
Declarations under Rule 41.7:
— as to applicant’s entitlement to apply for and be granted a patent (Rule 41.7(iii))
— as to the applicant’s entitlement to claim the priority of the earlier application (Rule 41.7(iii))

Published:
— with international search report (Art. 21(3))

(54) Title: TREATMENT FOR WOLFRAM SYNDROME AND OTHER ENDOPLASMIC RETICULUM STRESS DISORDERS

(57) Abstract: The present invention generally relates to a method of treating or preventing an endoplasmic reticulum stress disorder in subjects, such as a method of treating or preventing Wolfram syndrome.
TREATMENT FOR WOLFRAM SYNDROME AND OTHER ENDOPLASMIC RETICULUM STRESS DISORDERS

GOVERNMENT LICENSE RIGHTS

[0001] This invention was made with Government support under grant R01 DK067493 awarded by the U.S. National Institutes of Health. The Government has certain rights in the invention.

FIELD OF THE INVENTION

[0002] The present invention generally relates to a method of treating or preventing an endoplasmic reticulum stress disorder in subjects, including a method of treating or preventing Wolfram syndrome.

BACKGROUND OF THE INVENTION

[0003] Wolfram syndrome is a rare autosomal recessive disorder characterized by diabetes insipidus, diabetes mellitus, optic atrophy, and deafness (also known as DIDMOAD). Insulin dependent diabetes usually occurs as the initial manifestation during the first decade of life, while the diagnosis of Wolfram syndrome is invariably later with onset of the other features in the second and ensuing decades. Two causative genes for this genetic disorder have been identified and are named WFS1 and WFS2. It has been shown that multiple mutations in the WFS1 gene, as well as a specific mutation in the WFS2 gene, lead to β cell death and neurodegeneration through endoplasmic reticulum (ER) and mitochondrial dysfunction. WFS1 gene variants are also associated with a risk of type 2 diabetes. Moreover, a specific WFS1 variant can cause autosomal dominant diabetes.

[0004] Wolfram syndrome is caused by stress in the ER, a cell component involved in many vital functions of the eyes, brain, and pancreas. The ER is a membrane-bound organelle that is crucial for the folding and maturation of proteins, lipid biosynthesis, and homeostasis of intracellular Ca\(^{2+}\) and reduction-oxidation (redox) potential. Protein folding and modification in the ER is highly sensitive to disturbances of ER homeostasis, including altered glycosylation, ER Ca\(^{2+}\) depletion, increased mRNA translation, oxidative stress, energy deprivation, metabolic challenge, and inflammatory stimuli. The accumulation of unfolded and misfolded proteins in the ER lumen, termed ER stress, activates intracellular signaling pathways to resolve the protein folding defect. This unfolded protein response (UPR) increases the capacity of ER protein
folding and modification, reduces global protein synthesis, and activates ER-associated protein degradation (ERAD). If ER stress is too severe or chronic, or the UPR is compromised and not able to restore the protein folding homeostasis, numerous apoptotic signaling pathways are activated.

[0005] Despite the underlying importance of ER malfunction in ER stress disorders such as Wolfram syndrome and the identification of WFS1 and WFS2 genes, a molecular mechanism linking the ER to death of neurons and β cells has not been elucidated. Thus, to date there are no known treatments for Wolfram Syndrome and other ER stress disorders.

SUMMARY OF THE INVENTION

[0006] Briefly, various aspects of the present invention are directed to methods of treating or preventing an ER stress disorder in a subject in need thereof. The methods comprise administering to the subject a therapeutically effective amount of a ryanodine receptor (Ryr) inhibitor.

[0007] In various aspects, the present invention is also directed to methods of treating or preventing Wolfram Syndrome in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a ryanodine receptor inhibitor.

[0008] In further aspects, the present invention is directed to methods of treating or preventing clinical symptoms of an ER stress disorder, such as Wolfram Syndrome, in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a ryanodine receptor inhibitor.

[0009] Other objects and features will be in part apparent and in part pointed out hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figure 1 is a depiction of the pathogenesis of Wolfram syndrome.

[0011] Figure 2 is a scheme illustrating Wolfram syndrome as a prototype of human endoplasmic reticulum function.

[0012] Figure 3A is a depiction of a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of WFS2-associated proteins from HEK293T cell lysates transfected with glutathione S-transferase (GST) or GST-WFS2 expression plasmid.
[0013] Figure 3B is a depiction of a GST-tagged WFS2 pulled down of HEK293T cell lysates transfected with a GST-WFS2 expression plasmid, analyzed for CAPN2 by immunoblotting with anti-CAPN2 antibody.

[0014] Figure 3C is a depiction of a GST-tagged WFS2 pulled down of HEK293T cell lysates transfected with GST-WFS2 expression plasmid analyzed for CAPNS1 by immunoblotting with anti-CAPNS1 antibody.

[0015] Figure 3D is a depiction of an immunoblot of N-terminal FLAG-tagged WFS2 pull down of HEK293T cell lysates transfected with a WFS2 expression plasmid.

[0016] Figure 3E is a depiction of an immunoblot of C-terminal FLAG-tagged WFS2 pull down of HEK293T cell lysates transfected with a WFS2 expression plasmid.

[0017] Figure 3F is a depiction of an immunoblot of Neuro2a cell lysates immunoprecipitated with immunoglobulin G (IgG) or anti-calpain-2 antibodies.

[0018] Figure 3G is a depiction of an immunoblot of INS-1 832/13 cell lysates immunoprecipitated with IgG or anti-calpain-2 antibodies.

[0019] Figure 3H is a depiction of COS7 cells transfected with pDsRed2-ER vector and stained with calpain-2 antibodies.

[0020] Figure 3I is a depiction of an immunoblot of HEK293T cells transfected with WFS2 expression plasmids and fractioned into cytosolic and ER fractions.

[0021] Figure 3J is an immunoblot of HEK293T cells transfected with empty expression plasmid or a CAPN2 expression plasmid and a graph illustrating a change in expression levels of cleaved caspase 3.

[0022] Figure 4A is a depiction of an immunoblot of NSC34 cells transfected with control scramble siRNA or siRNA directed against WFS2 and a graph illustrating a change in expression levels of cleaved spectrin and caspase 3.

[0023] Figure 4B is a graph apoptosis of NSC34 cells transfected with scrambled siRNA or siRNA directed against WFS2 untreated or treated with thapsigargin.

[0024] Figure 4C is a depiction of an immunoblot of INS-1 832/13 cells transfected with scrambled siRNA or siRNA directed against WFS2 and a graph illustrating a change in expression levels of cleaved caspase 3.

[0025] Figure 4D is a depiction of an immunoblot of MIN6 cells transfected with scrambled siRNA or siRNA directed against WFS2 and a graph illustrating a change in expression levels of cleaved caspase 3.
Figure 4E is a depiction of an immunoblot of NSC34 cells transfected with empty expression plasmid, WFS2 expression plasmid, CAPN2 expression plasmid or co-transfected with WFS1 and CAPN2 expression plasmids and a graph illustrating a change in expression levels of cleaved caspase 3.

Figure 4F is a graph illustrating apoptosis in Neuro2a cells transfected with empty expression plasmid, WFS2 expression plasmid, CAPN2 expression plasmid or co-transfected with WFS1 and CAPN2 expression plasmids.

Figure 4G is a depiction of an immunoblot of NSC34 cells transfected with scrambled siRNA, siRNA directed against WFS2, siRNA directed against CAPN2 or co-transfected with siRNA directed against WFS2 and siRNA directed against CAPN2 and a graph illustrating a change in expression levels of cleaved caspase 3 and CAPN2.

Figure 5A is a depiction of an immunoblot of Neuro2a cells transfected with siRNA against WFS2 or a scrambled siRNA and a graph illustrating a change in expression levels of cleaved caspase 3.

Figure 5B is a depiction of an immunoblot of MIN6 cells transfected with scrambled with scrambled siRNA or siRNA directed against WFS2 untreated or treated with calpeptin.

Figure 5C is a graph of apoptosis in Neuro2a cells were transfected with scrambled siRNA or siRNA against WFS2 untreated or treated with calpeptin.

Figure 5D is a depiction of an immunoblot of INS-1 832/13 cells transfected with scrambled siRNA and siRNA directed against WFS2 and a graph illustrating a change in expression levels of cleaved caspase 3.

Figure 5E is a depiction of an immunoblot of MIN6 cells transfected with scrambled siRNA, siRNA directed against WFS2, or empty expression plasmid.

Figure 5F is of HEK293T cells transfected with empty expression plasmid, WFS2 expression plasmid, scramble siRNA, or siRNA directed against WFS2 and a graph illustrating a change in expression levels of CAPNS1.

Figure 5G is a depiction of an immunoblot of HEK293T cells transfected with empty or WFS2 expression plasmid untreated or treated with MG132 and a graph illustrating change in expression levels of CAPNS1.

Figure 5H is a depiction of an immunoblot of HEK293T cells transfected with empty or WFS2 expression plasmid, untreated or treated with cycloheximide and a graph illustrating a change in expression levels of CAPNS1 over time.
[0037] Figure 5I is a depiction of an immunoblot of NSC34 cell lysates transfected with an empty vector, a FLAG tagged ubiquitin plasmid or co-transfected with WFS2 expression plasmid and Ub-FLAG plasmid.

[0038] Figure 5J is a depiction of an immunoblot of brain lysates from control and WFS2 knockout mice and a graph illustrating a change in expression levels of cleaved spectrin and CAPNS1.

[0039] Figure 6A is a depiction of an immunoblot of protein was extracted from brain tissues of WFS1 brain specific knockout and control mice and a graph illustrating a change in expression levels of cleaved spectrin and CAPNS1.

[0040] Figure 6B is a graph illustrating relative levels of WFS1 mRNA in different parts of the brain of WFS1 knockout and control mice.

[0041] Figure 6C is a 2-D fluorescence difference gel electrophoresis of proteins extracted from the cerebellum of WFS1 knockout and control mice.

[0042] Figure 6D is a protein expression profile illustrating difference between WFS1 knockout and control mice.

[0043] Figure 6E is a depiction of an immunoblot of proteins extracted from the cerebellum of WFS1 brain specific knockout and control mice and a graph illustrating a change in expression levels of cleaved MBP.

[0044] Figure 7A is an image of iPS cells derived from fibroblasts and a karyotype of Wolfram iPS cells.

[0045] Figure 7B is an image of alkaline phosphatase staining of the Wolfram iPS cells.

[0046] Figures 7C, 7D, 7E, and 7F are images of Wolfram syndrome iPS cells stained with pluripotent markers.

[0047] Figure 7G is a depiction of an immunoblot of cleaved spectrin and actin in neural progenitor cells derived from Wolfram syndrome subject iPS cells and a graph illustrating a change in expression levels of cleaved spectrin.

[0048] Figure 7H is a graph illustrating changes in cytosolic calcium levels in unaffected controls and Wolfram syndrome subjects measured by Fura-2.

[0049] Figure 7I is graph illustrating rates of ER calcium depletion in INS-1 832/13 cells transfected with scrambled siRNA or siRNA directed against WFS1.

[0050] Figure 7J is a graph illustrating rates of ER calcium depletion in HEK293 cells, transduced with lentivirus expressing scrambled shRNA or shRNA directed against WFS1, untreated or treated with thapsigargin.
[0051] Figure 7K is a graph illustrating cytosolic calcium levels in HEK293 cells transduced with lentivirus expressing expressing scramble shRNA or shRNA directed against WFS1.

[0052] Figure 8A is a graph illustrating apoptosis of INS-1 832/13 cells were pretreated with DMSO or various compounds that suppress thapsigargin mediated cell death.

[0053] Figure 8B is a graph illustrating changes in cytosolic calcium levels of INS-1832/13 cells untreated or treated with dantrolene.

[0054] Figure 8C is a graph illustrating changes in cytosolic calcium levels of NSC34 cells untreated or treated with dantrolene.

[0055] Figure 8D are graphs illustrating cytosolic calcium levels in control and WFS1 deficient INS-1 832/13 and NSC34 cells untreated or treated with dantrolene.

[0056] Figure 9A is a depiction of an immunoblot of INS-1 832/13 cells transfected with scrambled siRNA or siRNA against WFS1, untreated or treated with dantrolene and graphs illustrating a changes in expression levels of cleaved caspase 3.

[0057] Figure 9B is a depiction of an immunoblot of INS-1 832/13 cells transfected with scrambled siRNA or siRNA against WFS1, untreated or treated with dantrolene and a graph illustrating a change in expression levels of cleaved spectin.

[0058] Figure 9C is a depiction of an immunoblot of NSC34 cells transfected with scrambled siRNA or siRNA against WFS1 untreated or treated with dantrolene and graphs illustrating changes in expression levels of cleaved capase 3 and cleaved spectrin.

[0059] Figure 10A is a depiction of an immunoblot of INS-1 832/13 cells transfected with scrambled siRNA or siRNA directed towards WFS1 and untreated or treated with ryanodine and a graph illustrating a change in expression level of cleaved caspase 3.

[0060] Figure 10B is a depiction of an immunoblot of INS-1 832/13 cells transfected with scrambled siRNA or siRNA directed towards WFS1 and untreated or treated with dantrolene and a graph illustrating a change in expression level of cleaved caspase 3.

[0061] Figure 10C is a depiction of an immunoblot of INS-1 832/13 cells transfected with scrambled siRNA or siRNA directed towards WFS1 and untreated or treated with dantrolene and a graph illustrating a change in expression level of cleaved caspase 3.

[0062] Figure 10D is a graph of SERCA activity of HEK293T cells expressing a scrambled shRNA or a shRNA directed against WFS1.
[0063] Figure 10E is a depiction of an immunoblot of HEK293T cells stably expressing scrambled shRNA or a shRNA directed against WFS1 and a graph illustrating a change in expression levels of WFS1.

[0064] Figure 10F is a graph of FACS analysis of INS-1 832/13 cells expressing D1ER untreated or treated with digitonin, EGTA, or digitonin and CaCl₂.

[0065] Figure 10G is a graph of FACS analysis of INS-1 832/13 cells expressing D1ER treated with various concentrations of thapsigargin.

[0066] Figure 11A is a depiction of an immunoblot of neural progenitor cells pretreated with or without dantrolene, then treated with thapsigargin and a graph illustrating changes in expression level of cleaved caspase 3.

[0067] Figure 12A is a depiction of an immunoblot of brain lysates from WFS1 brain specific knockout mice untreated or treated with dantrolene and a graph illustrating a change in expression levels of cleaved spectrin.

[0068] Figure 13A is a graph illustrating a change in blood glucose level in Wolfram syndrome mice untreated or treated with dantrolene after injection of a dextrose solution at week 13.

[0069] Figure 13B is a graph illustrating a change in blood glucose level in Wolfram syndrome mice untreated or treated with dantrolene after injection of a dextrose solution at week 18.

[0070] Figure 13C is a graph illustrating a change in overall blood glucose level in Wolfram syndrome mice untreated or treated with dantrolene.

[0071] Figure 13D is a graph illustrating a change in body weight in Wolfram syndrome mice untreated or treated with dantrolene.

[0072] Figure 14A is a graph illustrating cell death in INS1E cells treated with dantrolene and challenged with thapsigargin.

[0073] Figure 14B is a graph illustrating cell death in INS1E cells treated with dantrolene and challenged with cytokines.

DETAILED DESCRIPTION

[0074] In general, the present invention is directed to the use of a ryanodine receptor inhibitor such as dantrolene to prevent or delay the progression of disorders associated with ER dysfunction. Surprisingly, it has been discovered that calpain-2 protease provides a link between the ER and death of neurons and β cells in Wolfram syndrome. This discovery of the
underlying molecular mechanisms provides strategies to treat various disorders driven by prolonged ER stress. Evidence indicates that ER dysfunction triggers a range of human chronic diseases, including Type 1 and Type 2 diabetes, atherosclerosis, inflammatory bowel disease, retinitis pigmentosa, and neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Parkinson's disease, and Alzheimer's disease. However, currently there is no effective therapy targeting the ER for such diseases due to the lack of clear understanding of the ER's contribution to the pathogenesis of such diseases.

[0075] Although Wolfram syndrome is a rare disease and is currently neglected from mainstay drug discovery efforts, the homogeneity of the subject population and disease mechanism has enabled identification of an important drug target, a calcium-dependent protease, calpain-2. Applicants have discovered that calpain-2 hyperactivation is seen in both cell models and mouse models of Wolfram syndrome. Without being bound by theory, Figure 1 presents a proposed explanation for the pathogenesis of Wolfram syndrome and the target a ryanodine receptor inhibitor such as dantrolene.

[0076] Despite its rarity, Wolfram syndrome represents an exceptional human disease model currently available to identify drugs and biomarkers associated with ER health. Because it is challenging to determine the exact effects of ER dysfunction on the fate of affected cells in common diseases with polygenic and multifactorial etiologies, applicants sought to define the role of ER dysfunction in mechanistically homogenous patient populations such as Wolfram syndrome. Accordingly, applicants have discovered that small molecule compounds capable of preventing calpain-2 hyperactivation or maintaining ER calcium homeostasis under ER stress conditions can be used to treat subjects (e.g., humans) with both Wolfram syndrome and common diseases in which the ER is involved. A depiction of human endoplasmic reticulum dysfunction, including Wolfram syndrome is shown in Figure 2.

[0077] Accordingly, various aspects of the present invention are directed to a method of treating or preventing ER stress disorders such as Wolfram syndrome in subjects (e.g., humans) in need thereof comprising administering to the subjects an effective amount of a compound that is a ryanodine receptor inhibitor in a pharmaceutically acceptable carrier. In further aspects, the present invention is directed to methods of treating or preventing clinical symptoms of an ER stress disorder such as β cell and neuronal cell death in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a ryanodine receptor inhibitor.

[0078] One ryanodine receptor inhibitor useful in the methods of the present invention includes dantrolene. Further in accordance with the present invention, it has been discovered
that dantrolene can prevent ER stress-mediated cell death associated with Wolfram syndrome. Thus, dantrolene and other drugs that can regulate ER calcium homeostasis can be used to delay the progression of Wolfram syndrome and other ER stress disorders including Type 1 and Type 2 diabetes, atherosclerosis, inflammatory bowel disease, retinitis pigmentosa, and neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Parkinson's disease, and Alzheimer’s disease.

[0079] "Dantrolene" as used herein refers to 1-[[5-(4-nitrophenyl)-2-furyl]methylideneamino]imidazolidine-2,4-dione or a pharmaceutically acceptable salt or hydrate thereof. One form of dantrolene is the monosodium salt of dantrolene or a hydrate thereof. Dantrolene sodium, a ryanodine receptor inhibitor, has been in clinical use since the 1980's for treating muscle dysfunction associated with malignant hyperthermia (MH). More recently, it has been used for the management of neuroleptic malignant syndrome, spasticity, heat stroke, and methamphetamine intoxication. Dantrolene is thought to depress excitation-contraction coupling in skeletal muscle by inhibiting the release of calcium (Ca\(^{2+}\)) from the sarcoplasmic reticulum, (smooth ER found in muscle cells). The molecular structure of dantrolene sodium is shown below.

![Molecular structure of dantrolene sodium]

[0080] Dantrolene is highly lipophilic and poorly soluble in water. A more water soluble analog of dantrolene called azumolene is under development for similar indications as dantrolene. Azumolene, has a bromine group instead of the p-nitro group found in dantrolene, and is thirty times more water soluble. "Azumolene" as used herein refers to 1-[[5-(4-bromophenyl)-2-furyl]methylideneamino]imidazolidine-2,4-dione or a pharmaceutically acceptable salt or hydrate thereof. Thus, another ryanodine receptor inhibitor useful in the methods of the present invention includes azumolene.

[0081] The mechanism by which a ryanodine receptor inhibitor such as dantrolene prevents cell death associated Wolfram syndrome is not entirely understood. However, without being bound by theory, applicants believe that loss of function of WFS1 increases cytoplasmic calcium levels leading to activation of the calcium-dependent cysteine protease calpain-2.
Calpains are involved in a variety of calcium regulated cellular processes, such as signal transduction, cell proliferation and differentiation, and apoptosis. Applicants also believe that WFS2, the other causative gene for Wolfram syndrome, is a suppressor of calpain-2 mediated cell death. Thus, loss of function of WSF2 results in increased apoptosis of β-cells in Wolfram syndrome subjects.

[0082] By exploiting induced pluripotent stem cells (iPSCs) derived from the skin cells of subjects with Wolfram syndrome, applicants have discovered drugs that block activation of this enzyme and cell death. In this approach, applicants have targeted the pathway to calpain activation instead of focusing on calpain specific inhibitors.

[0083] In accordance with the present invention, a pharmaceutical composition comprising a ryanodine receptor inhibitor such as dantrolene is administered to the subject in need thereof. The pharmaceutical composition can be administered to a subject to achieve a desired therapeutic effect, e.g., inhibiting calpain-2 protease activity. The pharmaceutical composition used in the practice of the present invention may suitably comprise, consist of, or consist essentially of one or more ryanodine receptor inhibitors and the pharmaceutically acceptable carrier.

[0084] The composition can be administered alone or in combination with at least one other active agent that modulates the transport of calcium (Ca\(^{2+}\)) ions to and/or from the endoplasmic reticulum in a cell. Intracellular calcium level is precisely regulated by cooperative action of a series of calcium permeable channels, calcium pumps and calcium exchangers in the plasma membrane and endoplasmatic reticulum. For instance, calcium is dynamically stored in the endoplasmic reticulum (ER), which is able to accumulate very high Ca\(^{2+}\) levels due to activity of sarco/endoplasmic reticulum calcium adenosine triphosphatase (SERCA) pumps. Release of Ca\(^{2+}\) from the endoplasmatic reticulum is controlled by two types of Ca\(^{2+}\) release channels, namely ryanodine receptors and inositol trisphosphate receptors (IP3Rs). The second active agent can be, for example, an inhibitor of an inositol 1,4,5-trisphosphate receptor (IP3R), such as rapamycin. Further, the second active agent can be a compound that enhances SERCA activation, such as pioglitazone.

[0085] The term “combination” designates a treatment wherein at least two or more drugs are co-administered to a subject to cause a biological effect. In a combined therapy according to this invention, the at least two drugs may be administered together or separately, at the same time or sequentially. Also, the at least two drugs may be administered through
different routes and protocols. As a result, although they may be formulated together, the drugs of a combination may also be formulated separately.

[0086] In addition to the active ingredients (e.g., the ryanodine receptor inhibitor), the pharmaceutical composition can contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active compounds into preparations which can be used pharmaceutically. The pharmaceutical composition can be administered by a routes including, but not limited to, oral, intravenous, intramuscular, intrarterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, parenteral, topical, sublingual, or rectal means. A pharmaceutical composition for oral administration can be formulated using pharmaceutically acceptable carriers known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the subject. In certain embodiments, the composition is formulated for parenteral administration. Further details on techniques for formulation and administration can be found in the latest edition of REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Publishing Co., Easton, Pa., which is incorporated herein by reference). After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. Such labeling would include amount, frequency, and method of administration.

[0087] As used herein, the term "pharmaceutically acceptable carrier" means a non-toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material, or formulation auxiliary of any type. Some examples of materials which can serve as pharmaceutically acceptable carriers are sugars such as lactose, glucose, and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil; and soybean oil; glycols such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; detergents such as TWEEN 80; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; artificial cerebral spinal fluid (CSF), and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring, and perfuming agents, preservatives and antioxidants can also be present in the
composition, according to the judgment of the formulator based on the desired route of administration.

[0088] The determination of a therapeutically effective dose for any one or more of the ryanodine receptor inhibitors described herein is within the capability of those skilled in the art. A therapeutically effective dose refers to that amount of active ingredient which provides the desired result. The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active ingredient or to maintain the desired effect. Factors which can be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions can be administered every 3 to 4 days, every week, or once every two weeks depending on the half-life and clearance rate of the particular formulation.

[0089] Typically, the normal dosage amount of the ryanodine receptor inhibitor can vary from about 0.05 to about 100 mg per kg body weight depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. It will generally be administered so that a daily oral dose in the range, for example, from about 0.1 mg to about 75 mg, from about 0.5 mg to about 50 mg, or from about 1 mg to about 25 mg per kg body weight is given. The active ingredient can be administered in a single dose per day, or alternatively, in divided doses (e.g., twice per day, three time a day, four times a day, etc.). In general, lower doses can be administered when a parenteral route is employed. Thus, for example, for intravenous administration, a dose in the range, for example, from about 0.05 mg to about 30 mg, from about 0.1 mg to about 25 mg, or from about 0.1 mg to about 20 mg per kg body weight can be used.

[0090] In one aspect of the invention, the ryanodine receptor inhibitor comprises dantrolene sodium. Dantrolene sodium for injection is commercially available as DANTRIUM INTRAVENOUS (JHP Pharmaceuticals, Parsippany, NJ). It is a sterile, non-pyrogenic, lyophilized formulation supplied in 70 mL vials containing 20 mg dantrolene sodium, 3000 mg mannitol, and sufficient sodium hydroxide to yield pH of approximately 9.5 when reconstituted with 60 mL sterile water for injection USP.

[0091] Dantrolene sodium capsules are commercially as DANTRIUM (JHP Pharmaceuticals, Parsippany, NJ). DANTRIUM is supplied in capsules of 25 mg, 50 mg, and 100 mg. Each capsule contains edible black ink, FD&C Yellow No. 6, gelatin, lactose,
magnesium stearate, starch, synthetic iron oxide red, synthetic iron oxide yellow, talc, and
titanium dioxide.

EXAMPLES

[0092] The following non-limiting examples are provided to further illustrate the present
invention.

Example 1: Materials and Methods

[0093] Thapsigargin, tunicamycin, calpeptin and cycloheximide were obtained from
Sigma-Aldrich (St. Louis, MO). Growth media RPMI-1640 and Dulbecco's Modified Eagle
Medium (DMEM) were obtained from Invitrogen (Carlsbad, CA). Neural induction media and
neural proliferation media were obtained from STEMCELL Technologies (Vancouver, B.C.,
CA). MitoProbe DilC1 (5) mitochondrial membrane potential assay kit, Annexin V Alexa
Flour488 conjugate, Fluo-4 and Fura-2 calcium indicators were obtained from INVITROGEN
(Carlsbad, CA). Caspase-glo 3/7 protease assay kit and calpain-glo protease assay kit were
obtained from Promega (Madison, WI). Mito Stress test kit was obtained from Seahorse
Bioscience (North Billerica, MA). Anti-WFS2 antibody and anti-WFS1 antibody were obtained
from Proteintech (Chicago, IL). Anti-caspase-3 and anti-CAPN2 antibodies were obtained from
Cell Signaling Technology, Inc. (Danvers, MA). Anti-CAPNS1 and anti-alpha II spectrin
antibody were obtained from EMD Millipore (Billerica, MA). Anti-actin antibody was obtained
from Sigma-Aldrich (St. Louis, MO). Anti-myelin basic protein antibody was obtained from
Santa Cruz Biotechnology (Santa Cruz, CA). Anti-calpain-2 antibody, which detects both
CAPN2 and CAPNS1, was raised in rabbits against bacterially expressed rat calpain-2.

Plasmids and siRNA

[0094] pCMV-SPORT6-WFS2 expression plasmid was purchased from Open
Biosystems (GE Healthcare, Pittsburgh, PA). pDsRed2-ER vector was purchased from
Clonetech Laboratories, Inc. (Mountain View, CA). FLAG tagged WFS2 plasmids were
constructed by inserting FLAG sequences into the N- and C-termini of the expression plasmid.
GST-WFS2 plasmid was generated by inserting WFS2 sequence into pEBG mammalian
expression plasmid. A CAPN2 expression plasmid was generated in plenty-cmv-pro plasmid
provided by E. Campeau (Campeau et al., 2009). LIPOFECTAMINE 2000 (INVITROGEN,
Carlsbad, CA) was used to transfect small interfering RNA (siRNA) directed against WFS2 and
CAPN2 into cells. siRNAs were designed and synthesized at QIAGEN (Valencia, CA) as
follows: mouse WFS2 with the sequence of SEQ ID NO: 1 (CAACAGAAGGAUAGCUUG),
human WFS2 with the sequence of SEQ ID NO: 2 (CGAAAGUGUGAAUGAAA), human
CAPN2 with the sequence of SEQ ID NO: 3 (CCGAGGAGGUUAGAAAGUA), and rat WFS1
with the sequence of SEQ ID NO: 4 (GUUUGACGCUACAAGUU). Cells were incubated
in media overnight after siRNA transfection, and then additional treatments were performed,
including ER stress induction.

Cell Culture

[0095] Neuro2a, NSC34, HEK293T MEFs, and COS7 cells were cultured in DMEM
containing 10% FBS and penicillin and streptomycin (ThermoFisher Scientific, Waltham, MA).
MIN6 3 cells were grown in DMEM containing 15% FBS and penicillin and streptomycin.
Subject primary lymphoblasts and INS-1 832/13 cells were cultured in RPMI containing 10%
FBS before measurement. Neural progenitor cells were maintained in STEMDIFF Neural
Progenitor Medium from STEMCELL Technologies (Vancouver, BC, Canada).

iPS Cell and neural progenitor cell generation

[0096] To generate iPS cells, fibroblasts were obtained from non-affected controls and
subjects with Wolfram syndrome. Integration-free iPS cells were generated via Sendai viral
delivery of the four reprogramming factors, Oct4, Sox2, Klf4, and c-Myc using Life
Technologies’ (ThermoFisher Scientific, Waltham, MA) CYTOTUNE reagents and protocols.
All WFS- and control-iPSCs showed silencing of the four transgenes, exhibited characteristic
human embryonic stem cell morphology, expressed pluripotency markers including ALP,
NANOG, SOX2, SSEA4, TRA-1, and had a normal karyotype. To generate neural progenitor
cells, iPSCs were counted and plated ~50,000 per well in a 96-well plate to form uniform
embryoid bodies. After 5 days, embryoid bodies were suspended in neural induction media and
replated as adherent cultures. Fresh media was applied every day for 7 days. Neural rosettes
formed in these cultures were selected and plated. Plated rosettes were fed with neural induction
media every day for 4-7 days to obtain neural progenitor cells.

MALDI-TOF Mass Spectrometry

[0097] HEK293 cells were transfected with GST-WFS2 plasmid and empty GST
plasmid. Cell lysates were collected and immunoprecipitated with glutathione beads in lysis
buffer (150 mM NaCl, 0.5% TritonX-100, 50 mM HEPES, 1 mM EDTA, 1 mM DTT, pH7.5).
The precipitated proteins from both samples were resolved by SDS-PAGE and stained with
Coomassie blue staining. The distinct bands that only appear in GST-WFS2 lane but not GST
lane were analyzed by MALDI-TOF tandem mass spectrometry on a Shimadzu Axima TOF2
mass spectrometry at University of Massachusetts Medical School Proteomics and mass spectrometry facility.

**Immunostaining**

[0098] Cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature and then permeabilized with 0.1% TRITON X-100 for 2 minutes. The fixed cells were washed with phosphate buffered saline with TWEEN (PBS/TWEEN 20) 0.1%, blocked with Image-It FX signal enhancer (INVITROGEN, Carlsbad, CA) for 1 hour, and incubated in primary antibody overnight at 4 °C. The cells were washed three times in PBS/TWEEN 0.1% and incubated with secondary antibody for 1 hour at room temperature. Images were obtained with a Zeiss (Oberkochen, Germany) LSM 5 PASCALconfocal microscope with LSM Image software.

**FACS Analysis**

[0099] Flow cytometry analyses (FACS) were performed with LSR II Flow Cytometer (BD Biosciences, San Jose, CA) at the FACS core facility of Washington University School of Medicine Neural progenitor cells or NSC34 cells were plated in 24-well plates. After staining with Annexin V, cells were washed and resuspended in PBS. Cells were run using standard protocols, specifically Annexin V positive cell (i.e., apoptotic cells). The results were analyzed by FlowJo ver.7.6.3 (Ashland, OR).

**Quantitative Real-Time PCR (qrt-PCR)**

[0100] Total RNA was extracted by RNeasy kits (Qiagen, Waltham, MA). Reverse transcriptase PCR was performed using high capacity reverse transcription kit and quantitative PCR (q-PCR) was demonstrated with Applied Biosystems ViiA7 (ThermoFisher Scientific, Waltham, MA) using SYBR green dye.

**2-Dimensional gel electrophoresis**

[0101] Proteins were extracted from cerebellums from WFS1 knockout and control mice. Equal amount of protein extract from paired samples were labeled by CyDye DIGE fluorors, and the spectrally resolvable dyes enabled simultaneous co-separation and analysis of samples on a single multiplexed gel. These paired samples were simultaneously separated on a single 2D gel, using isoelectric focusing (IEF) in the first dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. After electrophoresis, the gel was scanned using a Typhoon image scanner (GE Healthcare Life Sciences, Pittsburgh, PA). Each scan revealed one of the CyDye signals (Cy3 and Cy5). ImageQuant software V. 8 (GE Healthcare Life Sciences, Pittsburg, PA) was used to generate the image presentation data including the single and overlay images. The comparative analysis
of all spots was done by the DeCyder analysis software (GE Healthcare Life Sciences, Pittsburg, PA). The protein expression ratios between WFS1 knockout and control mice were generated and differentially expressed spots were analyzed by MALDI-TOF mass spectrometry.

**Knockout Mice**

[W0102] WFS1 beta cell (brain-specific) knockout mice were generated by breeding Nestin-Cre transgenic mice (The Jackson Laboratory, Bar Harbor, ME) with WFS1 floxed mice. WFS2 whole body knockouts were purchased from MRC Harwell (Oxfordshire, UK).

**SERCA Assays**

[W0103] HEK293 cells were homogenized in hypotonic buffer containing 10 mM NaHCO_3, 250 mM sucrose, 5 mM NaN_3, and 0.1 mM PMSF. ER fractions were isolated using differential centrifugation. ER protein fractions (125 μg) were added to the assay mixture [100 mM KCl, 30 mM imidazole-histidine (pH 6.8), 5 mM MgCl_2, 5 mM ATP, 5 mM (CO)K_2, 5 mM NaN_3, and 50 μM CaCl_2 (10 μCi/μmol[^45]Ca); CaCl_2 American Radiolabeled Chemicals)] heated to 37°C for 15 minutes. The reaction was quenched by the addition of 250 mM KCl and 1 mM LaCl_3. The mix was then vacuum filtered through a 0.2 μm HT Tuffryn membrane (Sigma-Aldrich (St. Louis, MO)). SERCA-dependent calcium transport was measured by comparing calcium transport in the presence or absence of 10 μM thapsigargin, a SERCA inhibitor.

**ER Calcium Monitoring with D1ER**

[W0104] A β cell line was developed to monitor ER calcium levels under various conditions. The ER-localized calcium sensor D1ER was modified to contain two fluorescent proteins, CFP and YFP, which were inserted onto the N- and C-terminus, respectively. INS-1 832/13 cells were transfected with the modified D1ER calcium sensor. When Ca^{2+} binds to the calmodulin, an intramolecular rearrangement occurs and brings CFP and YFP into close proximity resulting in a fluorescence resonance energy transfer (FRET) from CFP to YFP. The FRET signal allows ratiometric measurements of free ER Ca^{2+} and changes in Ca^{2+} using FRET/CFP ratio.

**Caspase 3/7 Activity Assay**

[W0105] A Caspase 3/7 assay (Promega, Madison, WI) was utilized to measure caspase-3 and -7 activities in various cell lines. The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This caspase-3/7 substrate can be added directly to cell lysates. The substrate is cleaved to release a aminoluciferin, which is a substrate for luciferase used in the production of light. The signal is proportional to caspase-3/7
activity. The stabilized luciferase and proprietary buffer system improve assay performance across a wide range of assay conditions, and the assay is less likely to be affected by compound interference unlike fluorescent- or colorimetric-based assays.

**Statistical analysis**

[0106] Two-tailed t-tests were used to compare data. P values below 0.05 were considered significant. All values are shown as means ± s.d. if not stated.

**Example 2: WFS2 Interaction with CAPN2**

[0107] The causative genes for Wolfram syndrome, WFS1 and WFS2, encode transmembrane proteins localized to the ER. Mutations in the WFS1 or WFS2 have been shown to induce neuronal and β cell death. To determine the cell death pathways emanating from the ER, proteins associated with WFS1 and WFS2 were sought.

[0108] HEK293T cells were transfected with GST-WFS2 plasmid and empty GST plasmid. Cell lysates were collected and immunoprecipitated with glutathione beads in lysis buffer (150 mM NaCl, 0.5% TRITRON X-100, 50 mM HEPES, 1 mM EDTA, 1 mM DTT, pH 7.5). The precipitated proteins from both samples were resolved by SDS-PAGE and stained with coomassie blue staining. The distinct bands that only appear in GST-WFS2 lane but not GST lane were analyzed by MALDI-TOF. Analysis revealed 13 interacting proteins (Table 1).

[0109] One of the major WFS2-associated polypeptides was CAPN2, the catalytic subunit of calpain-2, a member of the calcium dependent cysteine proteases family whose members mediate diverse biological functions including cell death (Figure 3A).

Table 1. GST-WFS2 interacting proteins

<table>
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<tr>
<th>Order on gel</th>
<th>Gene Symbol</th>
<th>Full Name</th>
<th>MW (kDa)</th>
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<tr>
<td>1</td>
<td>PRKDC</td>
<td>DNA dependent protein kinase catalytic subunit</td>
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<tr>
<td>2</td>
<td>COPA</td>
<td>Coatamer subunit alpha</td>
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<td>3</td>
<td>IPO7, 4, 9</td>
<td>Importin 7, 4, and 9</td>
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<td>MMS nucleotide excision repair</td>
<td>110</td>
</tr>
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<td>6</td>
<td>CNX</td>
<td>Calnexin</td>
<td>67</td>
</tr>
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<td>7</td>
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<td>Calpain-2</td>
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</tr>
<tr>
<td>8</td>
<td>GRP78</td>
<td>GRP78</td>
<td>78</td>
</tr>
<tr>
<td>9</td>
<td>TUBA TUBB</td>
<td>Alpha and Beta Tubin</td>
<td>50</td>
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</table>
[0110] Calpain-2 is a heterodimer consisting of the CAPN2 catalytic subunit and the CAPNS1 (previously known as CAPN4) regulatory subunit. Interaction of WFS2 with calpain-2 was verified by showing that endogenous calpain-2 subunits CAPN2 (Figure 3B) and CAPNS1 (Figure 3C) each associated with GST-tagged WFS2 expressed in HEK293T cells. Endogenous calpain-2 was also found to be co-immunoprecipitated with N- or C-terminal FLAG-tagged WFS2 expressed in HEK293T cells (Figures 3D and 3E, respectively).

[0111] To further confirm these findings, a co-immunoprecipitation experiment using IgG or anti-calpain-2 antibodies in Neuro2a cells (a mouse neuroblastoma cell line) and INS-1 832/13 cells (a rat pancreatic β cell line) was performed. It was found that endogenous WFS2 interacted with endogenous CAPN2 (Figures 3F and 3G). Since WFS2 is a transmembrane protein localized to the ER, the possibility that calpain-2 might also localize to the ER was explored by transfecting COS7 cells with pDsRed2-ER vector to visualize ER localization (Figure 3H, center panel). Immunofluorescence staining of COS7 cells showed that endogenous calpain-2 was mainly localized to the cytosol, but also showed that a small portion colocalized with DsRed2-ER protein at the periphery of the ER (Figure 3H, left panel). Cells were fractionated into cytosolic and ER fractions followed by immunoblotting which further confirmed this observation (Figure 3I). Collectively, these results show that calpain-2 interacts with WFS2 at the periphery of the ER.

[0112] Calpain hyper-activation has been shown to contribute to cell loss in various diseases, raising the possibility that calpain-2 might be involved in the regulation of cell death. To address this issue, the catalytic subunit of calpain-2 (CAPN2) was overexpressed. HEK293T Cells were transfected with empty expression plasmid or a CAPN2 expression plasmid. Apoptosis was monitored by immunoblotting analysis of caspase-3 cleavage. Expression levels of CAPN2 and actin were measured by immunoblotting (Figure 3J, left panel). Quantification of the immunoblot is shown in Figure 3J, right panel (n=3, *P<0.05). Over expression of calpain-2 was found to increase the cleavage of caspase-3 in HEK293T cells indicating that hyper-activation of calpain-2 induces cell death (Figure 3J).

**Example 3: WFS2 Suppresses Cell Death Mediated by CAPN2**

[0113] To determine whether WFS2 plays a role in cell survival, WFS2 expression in NSC34 cells was suppressed using siRNA and cell viability was measured under normal and ER stress conditions. NSC34 cells were untreated (UT) or treated with 0.5 μM thapsigargin (TG) for 6 hours. WFS2 knockdown was associated with increased cleavage of caspase-3 in normal
or ER stressed conditions (Figure 4A and 4B). Next, calpain-2 activation levels were evaluated by measuring the cleavage of alpha II spectrin, a substrate for calpain-2. RNAi-mediated knockdown of WFS2 was found to induce calpain activation, especially under stress conditions (Figure 4A). Apoptosis was monitored by immunoblotting analysis of caspase 3 cleavage (Figure 4A) \((n=5, *P<0.05)\) and Annexin V staining (Figure 4B) \((n=3, *P<0.05)\). Additionally, proteins levels of cleaved spectrin, WFS2, and actin were measured by immunoblotting.

[0114] In subjects with Wolfram syndrome, destruction of β cells leads to juvenile-onset diabetes. However it is not known whether WFS2 is also involved in pancreatic β cell death. INS-1 832/13 (Figure 4C) and MIN6 (Figure 4D) cells were transfected with control scrambled siRNA or siRNA directed against WFS2 and then left untreated (UT) or treated with 0.5 μM thapsigargin (TG) for 6 hours. Expression levels of cleaved caspase-3, WFS2, and actin were measured by immunoblotting and are depicted in Figures 4C \((n=3, *P<0.05)\) and 4D \((n=3, *P<0.05)\).

[0115] As was seen in neuronal cells, knockdown of WFS2 in rodent β cell lines INS1 832/13 (Figure 4C) and MIN6 (Figure 4D), was also associated with increased caspase-3 cleavage under both normal and ER stress conditions.

[0116] NSC34 cells were transfected with empty expression plasmid (MOCK), WFS2 expression plasmid, CAPN2 expression plasmid or co-transfected with WFS1 and CAPN2 expression plasmids. After 24 hours, post transfection, cells were left untreated (UT) treated with 5 μg/mL tunicamycin (TM) for 16 hours. Apoptosis was monitored by immunoblotting analysis of the relative levels of cleaved caspase-3 (Figure 4E, left panel). Expression levels of CAPN2, WFS2, and actin were also monitored by immunoblotting. Quantification of cleaved caspase-3 levels under treated and untreated conditions are shown in Figure 4E, right panel \((n=5, *P<0.05)\).

[0117] The association of WFS2 with calpain-2 and their involvement in cell viability raised the possibility that calpain-2 activation might be the cause of cell death in WFS2 deficient cells. To test this hypothesis, WFS2 was expressed together with the calpain-2 catalytic subunit CAPN2 and apoptosis was measured. It was found that ectopic expression of WFS2 significantly suppressed calpain-2-associated apoptosis under normal and ER stress conditions (Figure 4E, lanes 4 and 8, Figure 4F). Next, the question of whether CPAN2 mediates cell death induced by WFS2 deficiency was tested.

[0118] Neuro2a cells were transfected with empty expression plasmid (MOCK), WFS2 expression plasmid, CAPN2 expression plasmid, or co-transfected with WFS1 and CAPN2
expression plasmids. The cells were examined for apoptosis by Annexin V staining followed by flow cytometry analysis (Figure 4F) (n=3, *P<0.05).

[0119] Additionally, NSC34 cells were transfected with scrambled siRNA (cont.), siRNA directed against WFS2, siRNA directed against CAPN2 or co-transfected with siRNA directed against WFS2 and siRNA directed against CAPN2. Apoptosis was detected by immunoblotting of cleaved caspase 3 (Figure 4G, left panel). Protein levels of CAPN2, WFS2, and actin are shown in Figure 4G (center and right panels) (n=3, *P<0.05).

[0120] When CAPN2 was silenced in WFS2 deficient cells, apoptosis was partially suppressed compared to untreated WFS2 deficient cells (Figure 4G). Taken together, these results show that WFS2 is a negative regulator of calpain-2 pro-apoptotic functions.

Example 4: WFS2 Regulates Calpain Activity through CAPNS1

[0121] To further confirm that loss of function of WFS2 leads to cell death mediated by calpain-2, it was tested whether calpeptin (a calpain inhibitor) could prevent cell death in WFS2 deficient cells. Neuro2a and MIN6 cells were transfected with siRNA against WFS2 or a control scrambled siRNA (Cont.). After 36 hours post-transfection, cells were untreated or treated with 100 μM calpeptin for 12 hours. Cleaved caspase 3 and actin levels were assessed by immunoblotting (Figure 5A, left panel). Quantified levels of cleaved caspase-3 are shown in Figure 5A, right panel (n=3, *P<0.05). Quantification of protein levels from MIN6 cells are shown in Figure 5B (n=3, *P<0.05). Additionally, early stage apoptosis was monitored by Annexin V staining followed by flow cytometry, Figure 5C. INS-1 832/13 cells were transfected with scrambled siRNA and WFS2 siRNA. After 24 hours post-transfection, cells were untreated or treated with 5 μM calpeptin for 24 hours. Cleaved caspase-3, WFS2, and actin levels were monitored by immunoblotting (Figure 5D, left panel) and quantified (Figure 5D, right panel) (n=3, *P<0.05).

[0122] In agreement with previous observations, calpeptin treatment prevented WFS2-knockdown mediated cell death in neuronal (Figure 5A and 5C) and β cell lines (Figures 5B and 5D). Collectively, these results indicate that WFS2 is a suppressor of calpain-2-mediated cell death.

[0123] CAPN2 is the catalytic subunit of calpain-2. CAPN2 forms a heterodimer with the regulatory subunit, CAPNS1, which is required for protease activity and stability. Though it has been shown that WFS1 regulates protein stability and mediates cell fate decision, the role of WFS2 in CAPN2 and CAPNS1 protein stability is not known.
[0124] CAPN2, WFS2, and actin levels were assessed by immunoblotting HEK293T cells transfected with empty expression plasmid (MOCK), WFS2 expression plasmid, scrambled siRNA (siCON), or siRNA directed against WFS2 (siWFS2) (Figure 5E). It was found that ectopic expression of RNAi-mediated knockdown of WFS2 did not correlate with changes in the steady-state expression of CAPN2 (Figure 5E).

[0125] CAPNS1, WFS1, WFS2, and actin levels were assessed by immunoblotting HEK293T cells transfected with empty expression plasmid (MOCK), WFS2 expression plasmid, scrambled siRNA (siCON) or siRNA directed against WFS2 (siWFS2) (Figure 5F, left panel). Protein levels of CAPNS1 were quantified and are shown in Figure 5F, right panel. By contrast, overexpression of WFS2 significantly reduced CAPNS1 protein expression and transient suppression of WFS2 slightly increased CAPNS1 protein expression (Figure 5F) (n=5, *P<0.05). This data shows that WFS2 might be involved in CAPNS1 protein turnover, which is supported by the data showing that GST-tagged WFS2 expressed in HEK293T cells associated with endogenous CAPNS1 (Figure 3C).

[0126] To find out whether WFS2 regulates CAPNS1 stability through the ubiquitin-proteasome pathway, HEK293T cells ectopically expressing WFS2 were treated with a proteasome inhibitor, MG132 (2 μM), and then the CAPNS1 protein level was measured. MG132 treatment stabilized CAPNS1 protein in HEK293T cells ectopically expressing WFS2 (Figure 5G) (n=4, *P<0.05).

[0127] To further confirm that WFS2 is involved in the degradation of CAPNS1, cycloheximide chase experiments were performed using HEK293T cells ectopically expressing WFS2 and control cells. HEK293T cells were treated with 100 μM cycloheximide for various lengths of times, e.g., 0, 2, 4, and 8 hours. It was found that ectopic expression of WFS2 was associated with significantly accelerated CAPNS1 protein loss, indicating that WFS2 contributes to post-translational regulation of CAPNS1 (Figure 5H) (n=3, *P<0.05).

[0128] To assess whether WFS2 is involved in the ubiquitination of CAPNS1, the levels of CAPNS1 ubiquitination in cells ectopically expressing WFS2 were measured. NSC34 cells were transfected with mock empty vector, FLAG tagged ubiquitin (Ub-FLAG) plasmid or co-transfected with WFS2 expression plasmid and Ub-FLAG plasmid. Cell lysates were immunoprecipitated with FLAG affinity beads and analyzed for ubiquitin conjugated proteins by immunoblotting. Levels of CAPNS1 and Ub-FLAG proteins were measured in the precipitates. WFS2, CAPNS1, and actin expression was monitored in the input samples. It was found that CAPNS1 ubiquitination was increased by ectopic expression of WFS2 (Figure 5I).
[0129] To further investigate the role of WFS2 in calpain-2 regulation, brain lysates from WFS2 knockout mice were collected, immunoblotted, and levels of cleaved spectrin were determined (spectrin is a well-characterized substrate for calpain). Notably, protein expression levels of cleaved spectrin, as well as CAPNS1, were significantly increased in WFS2 knockout mice as compared to control mice (Figure 5J) (each group, n=3, *P<0.05). Collectively, these results indicate that WFS2 inhibits calpain-2 activation by regulating CAPNS1 degradation mediated by ubiquitin-proteasome system.

Example 5: Calpain-2 Activation in a Mouse Model of Wolfram Syndrome

[0130] Calpain-2 is a calcium-dependent protease. WFS1, the other causative gene for Wolfram syndrome, has been shown to be involved in calcium homeostasis, raising the possibility that the loss of function of WFS1 is also involved in calpain activation. To test this idea, calpain activation levels in brain tissues from WFS1 knockout and control mice were measured. It was found that increased levels of a spectrin cleavage product, reflecting calpain activation, were observed in WFS1 knockout mice as compared to control mice (Figure 6A). Cleaved alpha II spectrin and actin levels were determined by immunoblot analysis (Figure 6A, left panel). Quantification of cleaved spectrin is shown in Figure 6A, right panel (each group, n=10, *P<0.05). WFS1 mRNA levels in different parts of the brain in WFS1(-/-) and WFS1(+/-) mice were measured by qRT-PCR. The suppression levels of WFS1 in different parts of brain are shown in Figure 6B.

[0131] To further confirm that calpain is activated by the loss of function of WFS1, other substrates for calpain in brain tissues from WFS1 knockout mice were identified by proteomics. 2-D fluorescence gel electrophoresis identified 12 proteins differentially expressed between cerebellums of WFS1 knockout mice and those of control mice (Figure 6C, circled). Protein expression ratios between WFS1 knockout and control mice were generated and differentially expressed spots were analyzed by MALD-TOF mass spectrometry. Quantitative diagrams of spots #2 and #3, identified as myelin basic protein, show lower levels of expression in WFS1 knockout mice as compared to control mice (Figure 6D, the positive numbers indicate an increase in expression level whereas a negative number indicates a decrease in expression level). Among these, myelin basic protein (MBP) is a known substrate for calpain in the brain. To confirm that myelin basic protein cleavage was enhanced in WFS1 knockout mice, myelin basic protein levels in brain lysates, extracted from the cerebellum, from WFS1 knockout and control mice were measured. It was found that the cleavage and degradation of myelin basic protein
was increased in WFS1 knockout mice as compared to control mice (Figure 6E) (each group, n=3, *P<0.05).

**Example 6: High Cytosolic Calcium Levels and Hyper-Activation of Calpain in Neural Progenitor Cells**

[0132] To further confirm these observations, evidence was sought for increased calpain activity in neural progenitor cells derived from induced pluripotent stem cells (iPSCs) of Wolfram syndrome subjects with mutations in WFS1. Fibroblasts from four nonaffected controls and five subjects with Wolfram syndrome were transduced with four reprogramming genes (Sox2, Oct4, c-Myc, and Klf4) (28) (Table 2). At least ten clones from each control- and Wolfram-iPSCs were produced. All control- and Wolfram-iPSCs, exhibited characteristic human embryonic stem cell morphology, expressed pluripotency markers including ALP, NANOG, SOX2, SSEA4, TRA-1-81, and had a normal karyotype (Figure 7A, 7B, 7C, 7D, 7E, and 7F). To create neural progenitor cells, neural aggregates from iPSCs were formed. Neural aggregates were harvested at day 5 and re-plated onto new plates to give rise to colonies containing neural rosette structures. At day 12, neural rosette clusters were collected, re-plated, and used as neural progenitor cells. It was found that spectrin cleavage was increased in neural progenitor cells derived from Wolfram-iPSCs as compared to ones from control iPSCs, which indicates increased calpain activity (Figure 7G). The relative levels of the spectrin cleavage product are indicated (Figure 7G, left panel) and quantified (Figure 7G, right panel) (n=4, *P<0.05).
Table 2. Information on genotypes and phenotypes of Wolfram syndrome and control subjects

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<tr>
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<th>Source</th>
<th>Clinical Diagnosis</th>
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<th>Age at biopsy</th>
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<th>Age of Deafness</th>
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<td>WFS</td>
<td>H313Y</td>
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</table>

*DM: diabetes mellitus; *OA: optic atrophy; *DF: diabetes insipidus; *WUWC: Washington University Wolfram Clinic; *WFS: Wolfram syndrome; *CRI: Correll Research Institute; *ATCC:

The finding that calpain appeared to be hyperactivated in neural progenitor cells derived from Wolfram-iPSCs raised the possibility that cytoplasmic calcium levels might be increased in these cells. To test this idea, cytoplasmic calcium levels of neural progenitor cells derived from control- were compared with Wolfram-iPSCs cells using Fura-2, a fluorescent calcium indicator allowing accurate measurements of cytoplasmic calcium concentrations. Figure 7H (left panel) shows that cytoplasmic calcium levels were higher in Wolfram-iPSCs derived cells than in control cells. This was confirmed by staining these cells with another fluorescent calcium indicator, Fluo-4 (Figure 7H, right panel) (n=4, *P<0.05).

Additionally, INS-1 832/13 cells transfected with scrambled siRNA or siRNA directed against WFS1 and then untreated or treated with 10 mM thapsigargin for 24 hours or 25 mM high glucose for 48 hours. The percent rates of ER calcium depletion in INS-823/13 cells stained with Fluro-4 are shown in Figure 7I. RNAi-mediated knockdown of WFS1 in INS-1 832/13 cells increased the rate of ER-calcium depleted cells under ER stress or high glucose conditions.
[0135] HEK293 cells were transfected with lentivirus expressing scrambled shRNA or shRNA directed against WFS1 and then untreated or treated with 10 nM thapsigargin for 24 hours. shRNA-mediated knockdown of WFS1 in HEK293 cells, stained with Fluo-4, also increased the rate of ER calcium-depleted cells under normal and ER stress conditions (Figure 7J) and increased the cytosolic calcium concentrations (Figure 7K). Collectively, these results indicate that loss of function of WFS1 increases cytoplasmic calcium levels, leading to calpain activation.

Example 7: Prevention of Cell Death and Restoration of Calcium Homeostasis

[0136] The results shown above demonstrate that the pathway leading to calpain-2 activation provides potential therapeutic targets for Wolfram syndrome, such as leakage of ER calcium to the cytosol. To test this concept, a small-scale screen was performed to identify chemical compounds that could prevent cell death mediated by thapsigargin, a known inhibitor for ER calcium ATPase. INS-1 823/13 cells were pretreated with DMSO or drugs for 24 hours the incubated in media containing 2 nM of thapsigargin (TG) overnight. Apoptosis was detected by caspase 3/7-Glo luminescence. Among 73 well-characterized chemical compounds that we tested (Table 3), eight compounds could significantly suppress thapsigargin mediated cell death. These eight compounds were PARP Inhibitor, dantrolene sodium, NS398, rapamycin, pioglitazone, calpain inhibitor III, docosaheaxenoic acid (DHA), and GLP-1 (Figure 8A).

Table 3. Chemical compounds used for a screen targeting the ER calcium homeostasis.

<table>
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<th>No.</th>
<th>Drug</th>
<th>Treatment Concentration</th>
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<td>Nicotinamide (Vitamin B3)</td>
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<td>(-)-Riboflavin (Vitamin B2,Vitamin G)</td>
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[0137] GLP-1, pioglitazone, and rapamycin are FDA approved drugs, and have been shown to confer protection against ER stress-mediated cell death. Dantrolene is another FDA approved drug clinically utilized for muscle spasticity and malignant hyperthermia. Though previous studies have shown that dantrolene is an inhibitor for the ER-localized ryanodine receptors and suppresses leakage of calcium from the ER to the cytosol, it was unknown whether dantrolene could be used to confer protection against cell death in Wolfram syndrome.

[0138] First, the question of whether dantrolene could decrease cytoplasmic calcium levels was tested. INS-1 832/13 and NSC34 cells were untreated or treated with 10 μM dantrolene for 24 hours. Cytoplasmic calcium levels were measured by Fura-2 calcium indicator over a period of 5 minutes. Thapsigargin (1μM) was added at time 0. It was found that dantrolene treatment decreased cytosolic calcium levels in INS-1 832/13 and NSC34 cells (Figures 8B and 8C, respectively).

[0139] Next, it was necessary to determine if dantrolene could restore cytosolic calcium levels in WFS1-deficient cells. RNAi-mediated WFS1 knockdown increased cytosolic calcium levels relative to control cells, and dantrolene treatment (10 μM for 24 hours) restored cytosolic calcium levels in WFS1-knockdown INS-1 832/13 cells (Figure 8D, left panel) as well as WFS1-knockdown NSC34 cells (Figure 8D, right panel) (n=6, *P<0.05).

**Example 8: Protection of WFS1 Deficient Cells**

[0140] In order to determine whether dantrolene conferred protection in WFS1 deficient cells and WFS1 silenced INS-1 832/13 cells were treated with dantrolene. INS-1 832/13 cells
were transfected with scrambled siRNA or siRNA directed against WFS1. Cells were untreated or treated with 10 μM dantrolene for 48 hours, then incubated in media containing 0.5 μM thapsigargin for 6 hours. Expression levels of cleaved caspase-3 under untreated and thapsigargin treated conditions (Figure 9A, left panel) were quantified and shown in Figure 9A, center and right panels (n=3, *P<0.05). Protein levels of cleaved spectrin, WFS1, and GAPDH were analyzed by immunoblotting (Figure 9B, left panel) and quantified (Figure 9B, right panel) (n=3, *P<0.05). Dantrolene treated cells resulted in suppression of apoptosis and calpain activity.

[0141] NSC34 cells were transfected with scrambled siRNA or siRNA directed against WFS1, then untreated or treated with 10 μM dantrolene for 24 hours. Protein levels of cleaved spectrin, cleaved caspase-3, WFS2, and GAPDH were determined by immunoblotting (Figure 8G, left panel) and quantified (Figure 9C, right panel) (n=3, *P<0.05). Dantrolene treatment also prevented calpain activation and cell death in WFS1-knockdown NSC34 cells (Figure 9C).

Example 9: ER Calcium Homeostasis in β cells

[0142] In to determine if dantrolene affects ER calcium homeostasis, cells were treated with ryanodine or dantrolene. INS-1 832/13 cells were transfected with scrambled siRNA or WRFS1 siRNA. After 24 hours post-transfection, cells were untreated or treated with 2 μM ryanodine for 24 hours. Cleaved caspase-3, WFS1, and GAPDH levels were monitored by immunoblotting (Figure 10A, left panel) and quantified (Figure 8H, right panel). INS-1 832/13 cells (Figure 10B) and NSC34 cells (Figure 10C) were transfected with scrambled siRNA or WRFS2 siRNA. After 24 hours post transfection, cells were untreated or treated with 10 μM dantrolene for 24 hours. Cleaved caspase-3, WFS2, and GAPDH levels were monitored by immunoblotting. Quantification of cleaved caspase 3 protein levels are shown in Figures 10D and 10E, right panels (n=3, *P < 0.05).

[0143] Sarco/endoplasmic reticulum calcium transport ATPase (SERCA) activity was measured in HEK293 cells stably expressing scrambled sequence (cont.) or shRNA directed against WFS1. SERCA activity for control and shWFS1 cells is shown in Figure 10D (wild-type, n=6, WFS1 knockout, n=7, *P<0.05). Protein levels of WFS1 and actin were monitored in HEK293 cells stably expressing scrambled shRNA (cont.) or shRNA directed against WFS1 (Figure 10E, left panel) and were quantified (Figure 10E, right panel) (n=3, *P<0.05).
[0144] RNAi-mediated WFS1 knockdown HEK293 cells significantly reduced the activation levels of SERCA, indicating that WFS1 may play a role in the modulation of SERCA activation and ER calcium levels.

[0145] INS-1 832/13 cells that stably express D1ER were treated with digitonin and ethylene glycol tetraacetic acid (EGTA) which causes the minimum value of FRET/CFP ratio, Figure 10F. Additionally, cells treated with digitonin and CaCl₂ led to the maximum value of FRET/CFP ratio, Figure 10F.

[0146] FRET/CFP ratio was decreased in INS-1 832/13 cells treated with thapsigargin in a dose-dependent manner (Figure 10G). Additionally, the rates of ER calcium-depleted cells increased in cells treated with thapsigargin in a dose-dependent manner (Figure 10G).

Example 10: Human Brain Tissue

[0147] To confirm these observations in patient cells, neural progenitor cells derived from iPS cells of a Wolfram syndrome subject and an unaffected parent were pretreated with dantrolene, and then treated with thapsigargin (TG). Wolfram patient neural progenitor cells were pretreated with or without 10 µM dantrolene for 48 hours. Then cells were treated with 0.125 µM thapsigargin for 20 hours. Apoptosis was monitored by immunoblotting (Figure 11A, left panel). Quantification of cleaved caspase-3 protein levels are indicated in Figure 11A, right panel. Thapsigargin-induced cell death was increased in neural progenitor cells derived from the Wolfram syndrome subject as compared to those derived from the unaffected parent, and dantrolene could prevent cell death in the subject iPSC-derived neural progenitor cells.

Example 11: Wolfram Syndrome Mouse Model

[0148] In addition, brain-specific WSF1 knockout mice were treated with dantrolene and evidence of suppressed calpain activation in brain lysates from these mice was observed. Control and WFS1 brain specific knockout mice were treated with water or dantrolene (20 mg/kg) for 4 weeks. Brain lysates of these mice were examined by immunoblotting. Protein levels of cleaved spectrin and GAPDH were monitored (Figure 12A, left panel) and quantified (Figure 12A, right panel) (n>3, *P<0.05). These results indicate that dantrolene can prevent cell death in Wolfram syndrome by suppressing calpain activation. Additionally, dantrolene treatment did not block cell death mediated by WFS2 knockdown, showing that WFS2 does not directly alter the ER calcium homeostasis.
[0149] β-cell specific knockout mice were used to determine if dantrolene could improve glucose tolerance. WSF1 beta cell-specific knockout mice were divided into two test groups consisting of 10 mice, 8 weeks old. Subjects in test group 1 were injected (intraperitoneal) with 6 mg/kg dantrolene for 5 days per week for up to 18 weeks. Subjects in test group 2 were injected (intraperitoneal) with 0.9% saline. Additionally, 10 Littermate control mice of similar age were injected (intraperitoneal) with 0.9% saline.

[0150] Weekly non-fasting blood glucose levels and body weight were measured throughout the course of the study. Intraperitoneal glucose tolerance test (IPGTT) was performed on week 0, 13, and 18. Blood glucose levels were obtained over a period of time following injection of a 2g/kg dextrose solution at week 13 (Figure 13A) and at week 18 (Figure 13B).

[0151] An average blood glucose level for the three groups throughout the course of the study is shown in Figure 13C. Body weight of all three groups throughout the course of the study is shown in Figure 13D.

Example 12: Dantrolene prevents ER stress- and cytokine-induced β cell death

[0152] To determine whether dantrolene can prevent ER calcium depletion and confer protection against ER stress-mediated cell death in a cell model of type 1 diabetes. INS1E cells were untreated or treated with dantrolene (100 nM), challenged with thapsigargin (10 nM for 24 hours) or an inflammatory cytokine (50 ng/mL), and then the cells were monitoring for cell death via a caspase 3/7 activity assay. Treatment with dantrolene prevented thapsigargin-induced (Figure 14A) and cytokine induced (Figure 14B) β cell death.

[0153] The following publications are hereby incorporated herein by reference for all relevant purposes.


[0154] When introducing elements of the present invention or the preferred embodiments(s) thereof, the articles "a", "an", "the" and "said" are intended to mean that there are one or more of the elements. The terms "comprising", "including" and "having" are intended to be inclusive and mean that there may be additional elements other than the listed elements.

[0155] In view of the above, it will be seen that the several objects of the invention are achieved and other advantageous results attained.

[0156] Having described the invention in detail, it will be apparent that modifications and variations are possible without departing from the scope of the invention defined in the appended claims.
WHAT IS CLAIMED IS:

1. A method of treating or preventing an endoplasmic reticulum (ER) stress disorder in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a ryanodine receptor inhibitor.

2. The method of claim 1, wherein the ER stress disorder is Wolfram Syndrome.

3. The method of claim 1 or 2, wherein the ER stress disorder is Type 1 or Type 2 diabetes.

4. The method of any one of claims 1 to 3, wherein the ER stress disorder comprises atherosclerosis, inflammatory bowel disease, retinitis pigmentosa, and neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Parkinson’s disease, and/or Alzheimer’s disease.

5. The method of any one of claims 1 to 4, wherein the ER stress disorder is a result of calpain-2 hyperactivation.

6. The method of any one of claims 1 to 5, wherein the ER stress disorder is associated with the deregulation of intracellular Ca2+ homeostasis in a subject.

7. The method of any one of claims 1 to 6, wherein the ryanodine receptor inhibitor is administered in a composition further comprising a pharmaceutically acceptable carrier.

8. The method of claim 7, wherein the composition further comprises at least one second active agent, wherein the second active agent modulates the transport of calcium (Ca2+) ions to and/or from the endoplasmic reticulum in a cell.

9. The method of any one of claims 1 to 8, wherein the ryanodine receptor inhibitor comprises dantrolene or a pharmaceutically acceptable salt or hydrate thereof.

10. The method of any one of claims 1 to 9, wherein the ryanodine receptor inhibitor comprises dantrolene sodium.
11. The method of any one of claims 1 to 10, wherein the ryanodine receptor inhibitor is administered intravenously in an amount of from about 0.05 mg to about 30 mg, from about 0.1 mg to about 25 mg, or from about 0.1 mg to about 20 mg per kg of body weight.

12. The method of claim any one of claims 1 to 10, wherein the ryanodine receptor inhibitor is administered orally in an amount of from about 0.1 mg to about 75 mg from about 0.5 mg to about 50 mg, or from about 1 mg to about 25 mg per kg of bodyweight.

13. The method of claim any one of claims 1 to 12, wherein the subject is a human.

14. A method of treating Wolfram Syndrome in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a ryanodine receptor inhibitor.

15. The method of claim 14, wherein the ryanodine receptor inhibitor is administered in a composition further comprising a pharmaceutically acceptable carrier.

16. The method of claim 14 or 15, wherein the composition further comprises at least a second active agent, wherein the second active agent modulates the transport of calcium (Ca2+) ions to and/or from the endoplasmic reticulum in a cell.

17. The method of any one of claims 14 to 16, wherein the ryanodine receptor inhibitor comprises dantrolene or a pharmaceutically acceptable salt or hydrate thereof.

18. The method of any one of claims 14 to 17, wherein the ryanodine receptor inhibitor comprises dantrolene sodium.

19. The method of any one of claims 14 to 18, wherein the ryanodine receptor inhibitor is administered intravenously in an amount of from about 0.05 mg to about 30 mg, from about 0.1 mg to about 25 mg, or from about 0.1 mg to about 20 mg per kg of body weight.
20. The method of any one of claims 14 to 18, wherein the ryanodine receptor inhibitor is administered orally in an amount of from about 0.1 mg to about 75 mg from about 0.5 mg to about 50 mg, or from about 1 mg to about 25 mg per kg of bodyweight.

21. The method of any one of claims 14 to 20, wherein the subject is a human.

22. A method of treating or preventing clinical symptoms of an endoplasmic reticulum (ER) stress disorder in a subject in need thereof comprising administering to the subject a therapeutically effective amount of a ryanodine receptor inhibitor.

23. The method of claim 22, wherein the clinical symptoms comprise β cell and neuronal cell death.

24. The method of claim 22 or 23, wherein the ER stress disorder comprises Wolfram Syndrome.

25. The method of any one of claims 22 to 24, wherein the ryanodine receptor inhibitor comprises dantrolene or a pharmaceutically acceptable salt or hydrate thereof.

26. The method of any one of claims 22 to 25, wherein the ryanodine receptor inhibitor comprises dantrolene sodium.

27. The method of any one of claims 22 to 26, wherein the ryanodine receptor inhibitor is administered intravenously in an amount of from about 0.05 mg to about 30 mg, from about 0.1 mg to about 25 mg, or from about 0.1 mg to about 20 mg per kg of body weight.

28. The method of any one of claims 22 to 27, wherein the ryanodine receptor inhibitor is administered orally in an amount of from about 0.1 mg to about 75 mg from about 0.5 mg to about 50 mg, or from about 1 mg to about 25 mg per kg of bodyweight.

29. The method of any one of claims 22 to 28, wherein the subject is a human.
30. The method of any one of claims 22 to 29, wherein the ER stress disorder comprises atherosclerosis, inflammatory bowel disease, retinitis pigmentosa, and neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Parkinson's disease, and/or Alzheimer's disease.
Figure 1

Wolfram syndrome

Absence of a single gene $\rightarrow$ ER dysfunction $\rightarrow$ Beta cell death
Neurodegeneration

Common diseases

Multiple Genetic Factors $\rightarrow$ ER dysfunction
Environmental Factors $\rightarrow$ Oxidative stress
$\quad$ Impaired cell signaling
$\quad$ Multiple other causes $\rightarrow$ Beta cell death
$\quad$ Neurodegeneration
Figure 3C

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Figure 3D

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Figure 3H

Calpain 2  ER  Merge
Figure 4A
- sRNA
- Spectrin 150
- Cleaved Caspase 3
- WFS2
- Actin

Figure 4B
- UT
- TG
- Apoptotic cells
- Annexin V positive cells (%)

The graphs show the relative protein level of cleaved caspase 3 and cleaved spectrin under different conditions, with statistical significance indicated by p-values.
Figure 4C

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Figure 4D

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Cleaved caspase 3

- **p = 0.0015**
- **p = 0.0007**
- **p = 0.018**
- **p = 0.026**
Figure 4G

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Cont</th>
<th>WFS2</th>
<th>CAPN2</th>
<th>WFS2+CAPN2</th>
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<tr>
<td>Cleaved Caspase 3</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>1.0</td>
<td>1.7</td>
<td>0.5</td>
<td>0.8</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WFS2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cleaved caspase 3

- Relative protein level
- p=0.014

CAPN2

- Relative protein level
- p=0.0015
Figure 5E

<table>
<thead>
<tr>
<th></th>
<th>siCON</th>
<th>siWFS2</th>
<th>MOCK</th>
<th>WFS2</th>
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<tbody>
<tr>
<td>CAPN2</td>
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<td></td>
<td></td>
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<tr>
<td>WFS2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5H

- WFS2
- CAPNS1
- Actin

- CX
- Mock

Time Points:
- 0h
- 2h
- 4h
- 8h
Figure 51

IB: CAPNS1

IP: Flag

Input

WFS2
CAPNS1
Actin

Mock vector
Ub-Flag
Ub-Flag+WFS2

Ub-CAPNS1
Figure 6B

Relative mRNA level

- WFS1+/-
- WFS1-/-

Brain stem, Cerebellum, Hippocampus, Cortex
1. Calbindin (-1.31) or SNAP-25 increased/decreased

<table>
<thead>
<tr>
<th>WFS1+/+</th>
<th>WFS1+-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Myelin basic protein (-2.49)</td>
<td></td>
</tr>
</tbody>
</table>

2. Myelin basic protein (-2.51)

4. Cytochrome c oxidase subunit 6B1 (>1.5)
5. GFAP (1.22)
6. FKBP4 (1.32)
7. Syntaxin-binding protein 1 (1.38)
8. Stathmin (-2.51)
9. Cytochrome C1 (-1.48)
10. Phosphatidylethanolamine-binding protein 1 (1.7)
11. Purkinje cell protein 4 (1.33)
12. Hemoglobin subunit alpha (-1.81)
Figure 7B

Figure 7C
Figure 8C

![Graph showing the ratio of A340/380 over time (min) for DMSO and Dantrolene.](image)

Figure 8D

**Cytosolic Calcium**

- p = 0.0006
- p = 0.038

![Bar charts comparing cytosolic calcium levels between UT and Dantrolene.](image)
Figure 9C

- **UT**
- **Dactclole**

**siRNA**
- Cleaved spectrin
- Cleaved caspase 3
- WFS1
- GAPDH

**Control**
- WFS1
- Cont.

**P-value**
- 0.018
- 0.001
Figure 14A

**ER Stress Induced Apoptosis**

![Bar chart showing ER stress induced apoptosis](chart1)

Figure 14B

**Cytokine Induced Apoptosis**

![Bar chart showing cytokine induced apoptosis](chart2)
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/00, G01N 33/567 (2016.01)
CPC - A61K 38/00
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)
IPC(8): A61K 38/00, G01N 33/567 (2016.01)
CPC: A61K 38/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 514/17.4, 514/7.21

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWest, PatBase, Google Scholar, Google Patents: Ryanodine receptor, inhibitor, antagonist, wolfram syndrome, endoplasmic reticulum, diabetes, Type 1, Type 2, neuronal cell death, ER stress

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>LUCIANI et al., Roles of IP3R and RyR Ca2 Channels in Endoplasmic Reticulum Stress and Beta-Cell Death, Diabetes, February 2009, Vol. 56, pages 422-432; Abstract, page 425, col 1, para 1; Page 426, col 2, para 1; Page 427, col 1, para 1; page 430, col 1, para 3- col 2, para 1</td>
<td>1-3, 14, 16/14, 22, 24/22</td>
</tr>
<tr>
<td>Y</td>
<td>US 2009/0203605 A1 (SEGATORI et al.) 13 August 2009 (13.08.2009); para [0019], [0171]</td>
<td>15, 16/15</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C.

* 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
7 January 2016 (07.01.2016)

Date of mailing of the international search report
02 FEB 2016

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer:
Lee W. Young
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)
INTERNATIONAL SEARCH REPORT

Box No. 1  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 13ter.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 13ter.1(a)).
      ☐ on paper or in the form of an image file (Rule 13ter.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:

Form PCT/ISA/210 (continuation of first sheet (1)) (January 2015)
INTERNATIONAL SEARCH REPORT

<table>
<thead>
<tr>
<th>Box No. II</th>
<th>Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)</th>
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<td></td>
<td>This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:</td>
</tr>
<tr>
<td>1. ✔️</td>
<td>Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:</td>
</tr>
<tr>
<td>2. ❌</td>
<td>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:</td>
</tr>
<tr>
<td>3. ☒️</td>
<td>Claims Nos.: 4-13, 17-21, 25-30 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).</td>
</tr>
</tbody>
</table>

<table>
<thead>
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<th>Box No. III</th>
<th>Observations where unity of invention is lacking (Continuation of item 3 of first sheet)</th>
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<tbody>
<tr>
<td></td>
<td>This International Searching Authority found multiple inventions in this international application, as follows:</td>
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<tr>
<td>1. ❌</td>
<td>As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.</td>
</tr>
<tr>
<td>2. ❌</td>
<td>As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.</td>
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<tr>
<td>3. ❌</td>
<td>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.:</td>
</tr>
<tr>
<td>4. ❌</td>
<td>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.:</td>
</tr>
</tbody>
</table>

**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.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)