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(54) Title: IRON CHELATORS AND COMBINATION THEREOF FOR THE TREATMENT OF WOLFRAM SYNDROME 2

(57) Abstract: The present invention relates to pharmaceutical compositions and pharmaceutical combinations for the treatment of type 2 Wolfram syndrome, diabetes and diseases associated with focal siderosis. More particularly, the invention relates to iron chelators and combinations thereof with glutathione precursors and/or anti-diabetes drugs, e.g. glucagon-like peptide-1 receptor agonists, for use in treating type 2 Wolfram syndrome and other diseases.

IRON CHELATORS AND COMBINATION THEREOF FOR THE TREATMENT OF WOLFRAM SYNDROME 2

FIELD OF THE INVENTION

The present invention relates to pharmaceutical compositions and pharmaceutical combinations for the treatment of type 2 Wolfram syndrome, diabetes and diseases associated with focal siderosis. More particularly, the invention relates to iron chelators and combinations thereof with glutathione precursors and/or anti-diabetes drugs, e.g. glucagon-like peptide-1 receptor agonists, for use in treating type 2 Wolfram syndrome and other diseases.

BACKGROUND OF THE INVENTION

Wolfram syndrome (WFS), also known as DIDMOAD (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy and Deafness), is a multisystem neuronal and β-cell degenerative disorder of autosomal recessive inheritance. In WFS, β-cell degeneration results from cellular stress and apoptosis, and leads to severe insulin deficiency mimicking type 1 diabetes.

Type 1 WFS results from mutations in the *WFS1* gene, which encodes Wolframin, a transmembrane protein localized to the endoplasmic reticulum (ER). Cells lacking Wolframin exhibit ER stress and apoptosis. Type 2 WFS (T2-WFS; OMIM #604928) was originally described in a Jordanian descent and results from mutations in the *CISD2* (CDGSH iron-sulfur domain-containing protein 2) gene. A single missense mutation that truncates 75% of the protein was identified in affected patients. A homozygous intragenic deletion of CISD2 has been reported in an Italian girl presenting with diabetes mellitus, optic neuropathy, bleeding intestinal ulcers, and sensorineural hearing loss. T2-WFS is characterized by severe peptic ulcer disease and impairment in platelet aggregation that are uncommon in T1-WFS.

CISD2 encodes a small protein, called nutrient-deprivation autophagy factor-1 (NAF-1), that is localized to the mitochondrial outer membrane, the ER and the mitochondrial-ER interacting membranes (mitochondrial-associated membrane (MAM)). *Cisd2* knockout mice exhibit muscle weakness, along with distorted

mitochondrial morphology, growth retardation and premature aging with shortened life span.

NAF-1 belongs to a conserved family of 2Fe-2S proteins, called the NEET family. In humans, three NEET members were identified; CISD1 (mitoNEET, mNT), CISD2 (NAF-1), and CISD3 (Miner 2). These proteins contain a CDGSH iron-sulfur domain that coordinates 2Fe-2S cluster transfer to apo-acceptor proteins. NEET protein expression has recently been shown to affect the levels of cellular labile iron and those of reactive oxygen species (ROS). Suppression of NAF-1 in tumor cells resulted in uncontrolled mitochondrial accumulation of labile iron (i.e. focal siderosis), increased generation of ROS, mitochondrial dysfunction, and reduced tumor growth. Moreover, suppressed expression of NAF-1 was shown to lead to apoptosis.

WFS is often fatal by mid-adulthood due to complications from the many features of the condition, such as health problems related to diabetes mellitus or neurological complications. Currently available treatments of WFS are only symptomatic and supportive, and require a multidisciplinary effort to manage the various aspects of this condition, such as anti-diabetic drugs and hearing aids. However, there is no effective treatment to prevent β-cell and neuronal cell degeneration in WFS, thereby preventing or reducing the severity of WFS-associated complications and prolonging life span.

It is therefore an object of the invention to provide the use of a composition comprising at least one iron chelator for the treatment of T2-WFS.

Another object of the invention is to provide a combination treatment comprising at least one iron chelator and an anti-oxidant.

A further object of the invention is to provide a combination treatment comprising at least one iron chelator and an anti-diabetes drug.

An additional object of the invention is to provide a combination treatment comprising at least one iron chelator, a glutathione precursor, and an anti-diabetes drug.

It is still a further object of the invention to provide the use of these compositions and combinations for the treatment of T2-WFS, diabetes and other diseases demonstrating increased levels of focal labile iron.

These and other objects of the invention will become apparent as the description proceeds.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides an iron chelator for use in treating Wolfram syndrome 2 (T2-WFS).

In one embodiment, the iron chelator is a conservative iron chelator. In another embodiment the iron chelator is selected from N'-[5-(Acetyl-hydroxy-amino)pentyl]-N-[5-[3-(5-aminopentyl-hydroxy-carbamoyl)propanoylamino]pentyl]-N-hydroxy-butane diamide (desferrioxamine) and 3-hydroxy-1,2-dimethylpyridin-4(1H)-one (deferiprone).

In another aspect, the present invention provides a pharmaceutical combination comprising an iron chelator for use in treating T2-WFS.

In one embodiment, the present invention provides a pharmaceutical combination comprising an iron chelator and an anti-oxidant for use in treating T2-WFS. The anti-oxidant according to the invention is a glutathione-stimulating gene selected from a cell glutathione promoter, a glutathione precursor, a glutathione cleavable ester, and a glutathione coupled to membrane-crossing peptide. Specifically, the glutathione precursor is selected from N-acetyl cysteine (NAC) and L-2-oxothiazolidine-4-carboxylic acid.

In another embodiment, the present invention provides a pharmaceutical combination comprising an iron chelator and an anti-diabetic agent for use in treating T2-WFS. For example, the anti-diabetic agent according to the invention is selected from exenatide, pioglitazone and rosiglitazone.

In a further embodiment, the present invention provides a pharmaceutical combination comprising an iron chelator, an anti-oxidant and an anti-diabetic agent for use in treating Wolfram syndrome 2.

In a further aspect, the present invention provides a pharmaceutical combination comprising an iron chelator, an anti-oxidant and an anti-diabetic agent for use in treating Type I diabetes, Type II diabetes, or a disease demonstrating regional iron overload.

In a still further aspect, the present invention provides a kit comprising: (a) a container with an iron chelator; (b) a container with an anti-diabetic agent; and/or (c) a container with an anti-oxidant; and (d) label or package insert with instructions for treating T2-WFS.

In one specific embodiment of the invention, the active agents present in the containers comprised in the kit are provided as different dosage forms, each one in a suitable carrier.

In one specific embodiment, the iron chelator, the anti-diabetic agent and the anti-oxidant are administered simultaneously, sequentially or separately.

In another specific embodiment, the iron chelator is administered parenterally, and the anti-oxidant is administered orally.

In a further aspect, the present invention provides an iron chelator formulated for use in conjunction with an anti-oxidant and/or an anti-diabetic agent, for use in the treatment of Wolfram syndrome 2.

In a still further aspect, the present invention provides an anti-diabetic drug formulated for use in conjunction with an iron chelator, and optionally with an anti-oxidant, for use in the treatment of Wolfram syndrome 2.

In another aspect, the present invention provides a method for treating Wolfram syndrome 2 by administering an iron chelator to a subject. In one embodiment, the

method further comprises administering an anti-oxidant. In another embodiment, the method further comprises administering an anti-diabetic agent. In a further embodiment, the method further comprises administering an anti-oxidant and an anti-diabetic agent.

In another aspect, the present invention provides a method for treating Type I diabetes, Type II diabetes, or a disease demonstrating regional iron overload by administering an iron chelator, an anti-oxidant and an anti-diabetic agent to a subject.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1D show the effect of NAF-1 suppressed expression on INS-1E cells.

Fig. 1A shows total protein extractions from cell lines INS-1E NC (control) and INS-1E NAF-1(-) (transfected with NAF-1 shRNA). The extractions were chromatographed on PAGE and the gel was stained with Coomassie Blue.

Fig. 1B shows western blot analysis performed with protein extracts from INS-1E NC and INS-1E NAF-1(-) using anti-NAF-1 antibodies.

Fig. 1C shows quantitative analysis of the blots presented in Fig. 1B. The result is shown as % of NAF-1 expression relative to control.

*** $p<0.001$.

Fig. 1D shows the cell growth of the INS-1E NC and NAF-1(-) cell lines. The cell growth (activity) was determined by using the Alamar-blue cell viability probe.

Abbreviations: C (INS-1E NC, control); - (NAF-1(-)); CA (cell activity in arbitrary units).

Figs. 2A-2D shows the mitochondrial damages in INS-1E NAF-1(-) cells compared to the INS-1E NC cells, and the partially reversed effect of pre-treatment with DFP on mitochondrial damages.

Fig. 2A shows representative images of the mitochondrial membrane potential (MMP) of INS-1E NC and NAF-1(-) cells lines after incubation with tetramethylrhodamine ethyl ester (TMRE, in arbitrary units), with or without pre-treatment with the conservative iron chelator deferiprone (DFP, 50-100 μM for between 30 minutes up to overnight).

Fig. 2B shows a quantitative analysis of the images of Fig. 2A.

Images were analyzed with Image J software, ** $p<0.01$.

Fig. 2C shows images of mitochondrial labile iron accumulation in INS-1E NC and NAF-1(-) cells, after incubation with rhodamine B-[(1,10-phenanthrolin-5-yl) aminocarbonyl] benzyl ester (RPA, in arbitrary units), with or without pre-treatment with DFP (50-100 μM for 30-60 minutes).

Fig. 2D shows a quantitative analysis the images of Fig. 2C.

Images were analyzed with Image J software, *** $p<0.001$.

Abbreviations: C (INS-1E NC, control); - (NAF-1(-)).

Figs. 3A- 3C show the mitochondrial reactive oxygen species (ROS) formation in INS-1E NC and NAF-1(-) cells, and the partially reversed effect of pre-treatment with the iron chelator DFP on mitochondrial ROS formation.

Fig. 3A shows representative microscopic images of ROS accumulation at 0 and 60 minutes in INS-1E NC and NAF-1(-) cells, with or without pre-treatment with DFP.

Fig. 3B shows dihydroethidium (DHE) fluorescence (relative to time 0) in INS-1E NC and NAF-1(-) cells, with or without pre-treatment with DFP (50-100 μM), over time (0, 10, 20, 30, 40, 50 and 60 minutes).

Fig. 3C presents the quantitative analysis of DHE fluorescence (relative to untreated NC cells) in INS-1E NC and NAF-1(-) cells, with or without pre-treatment with DFP (100 μM), after 60 minutes.

Images were analyzed with Image J software. **$p<0.01$, *** $p<0.001$.

Abbreviations: C (INS-1E NC, control); - (NAF-1(-)); T (time, in minutes).

Figs. 4A-4B show MMP of INS-1E NC, NAF-1(-) and NAF-1(-)/(+) cell lines.

Fig. 4A shows representative microscopic images of the INS-1E cell lines after incubation with TMRE.

Fig. 4B shows the quantitative analysis of the images of Fig. 4A (relative to NC cells).

Images were analyzed by Image J software, ***$p<0.001$.

Abbreviations: C (INS-1E NC, control); - (NAF-1(-)); -/+ (NAF-1(-)/(+)).

Figs. 5A-5B show mitochondrial labile iron accumulation in INS-1E NC, NAF-1(-) and NAF-1(-)/(+) cell lines.

Fig. 5A shows representative microscopic images of the INS-1E cell lines after incubation with RPA.

Fig. 5B shows a quantitative analysis of the images of Fig. 5A (relative to NC cells).

Images were analyzed by Image J software, ***p<0.001.
Abbreviations: C (control); - (NAF-1(-)); -/+ (NAF-1(-)/(+)).

Figs. 6A-6C show NAF-1 protein levels in skin fibroblast from a T2-WFS patient compared to normal fibroblasts.
Fig. 6A shows coomassie blue protein staining after PAGE analysis of normal skin fibroblasts (control) and skin fibroblasts from a T2-WFS patient.
Fig. 6B shows western blots of control and T2-WFS fibroblasts using anti-NAF-1 antibodies.
Fig. 6C shows a quantitative analysis of western blots of fig 6B. The results are shown as % of NAF-1 expression relative to control.
***p<0.001.
Abbreviations: C (control fibroblasts); W (T2-WFS fibroblasts).

Figs. 7A-7B show mitochondrial damages in T2-WFS skin fibroblasts compared to normal fibroblasts.
Fig. 7A shows RPA fluorescence (relative to control cells), inversely correlated with mitochondrial labile iron accumulation, in control and T2-WFS fibroblasts.
***p<0.001.
Fig. 7B shows electron microscope images of the mitochondria in control and in T2-WFS fibroblasts.
Abbreviations: C (control fibroblasts); W (T2-WFS fibroblasts).

Figs. 8A-8B show the effect of pre-treatment with the iron chelator DFP, the cell GSH-precursor NAC (CGP), or combination thereof on mitochondrial labile iron accumulation in control and T2-WFS fibroblast cells (from a T2-WFS patient) and in INS-1E NC and NAF-1(-) cell lines and on ROS formation in control and T2-WFS fibroblast cells.
Fig. 8A shows the % in RPA fluorescence change in control and T2-WFS skin fibroblasts after pre-treatment with DFP (50-100 μM, for 30-60 minutes), CGP (75 μM for 2 hours), or the combination thereof. RPA was loaded for 15 minutes at 37 °C and the cells were intensively washed and analyzed by epifluorescence microscopy in conjunction with Image J analysis.

***p<0.001.

Fig. 8B shows RPA fluorescence (relative to untreated NC cells) in INS-1E NC and NAF-1(-) cells after pre-treatment with DFP (50-100 μM for 30-60 minutes), CGP (75 μM for 2 hours), or combination thereof. RPA was loaded for 15 minutes at 37 °C and the cells were intensively washed and analyzed by epifluorescence microscopy in conjunction with Image J analysis.

Fig. 8C shows ROS formation (in arbitrary units) in control and T2-WFS fibroblasts, after pre-treatment with DFP (50-100 μM for 30-60 minutes), CGP (75 μM for 2 hours), or combination thereof. Mito-Sox (10 μM) was loaded for 15 minutes at 37 °C and the cells were intensively washed with HBSS buffer and analyzed by epifluorescence microscopy in conjunction with Image J analysis.

***p<0.001.

Abbreviations: C (control fibroblasts); W (T2-WFS fibroblasts); NC (INS-1E NC); -(NAF-1(-)).

Figs. 9A-9C show the effects of DFP, CGP, or combination thereof, on mitochondrial labile iron accumulation, MMP and ROS accumulation in control and T2-WFS fibroblasts cells.

Fig. 9A shows RPA fluorescence (relative to untreated control fibroblasts) in control and T2-WFS fibroblast cells (from four T2-WFS patients) after pre-treatment with DFP (50-100 μM for 30-60 minutes), CGP (75-100 μM for 2 hours), or combination thereof. RPA was loaded for 15 minutes at 37 °C and the cells were intensively washed and analyzed by epifluorescence microscopy in conjunction with Image J analysis.

p<0.01;*p<0.001.

Fig. 9B shows a quantitative analysis of MMP as measured by TMRE fluorescence (relative to untreated control fibroblasts) in control and T2-WFS fibroblasts (from four T2-WFS patients) after pre-treatment with DFP (50-100 μM for 30-60 minutes), CGP (75-100 μM for 2 hours), or combination thereof. TMRE was loaded for 15 minutes at 37 °C and the cells were intensively washed and analyzed.

***p<0.001.

Fig. 9C shows the analysis of ROS formation (relative to untreated control fibroblasts) in control and T2-WFS fibroblasts (from four T2-WFS patients) after pre-

treatment with DFP (50-100 μM for 30-60 minutes), CGP (75-100 μM for 2 hours), or combination thereof. TMRE was loaded for 15 minutes at 37 °C and the cells were intensively washed and analyzed.

**p<0.01, *** p<0.001.

Abbreviations: C (control); P1-P4 (T2-WFS patients 1-4).

Figs. 10A-10G show the changes of glucose, insulin, glucagon and glucagon-like peptide-1 (GLP-1) levels in the serum of a T2-WFS patient in response to IVGTT and arginine/glucagon injections or a mixed meal, before and after treatment with the GLP-1-receptor agonist (GLP-1-RA) exenatide.

Fig. 10A shows glucose levels (in mM) in the serum of a T2-WFS patient in response to stimulation with glucose, arginine and glucagon, before and after treatment with exenatide. The patient was treated with exenatide for 9 weeks followed by a 4-day washout period; glucose levels were assessed by IV glucose injection (0.3 g/kg), followed by injection of arginine (5 g) and glucagon (0.5 mg) at 30 minutes, before (circle) and after (square) exenatide intervention.

Fig. 10B shows C-peptide levels (in pM) in the serum of a T2-WFS patient in response to glucose, arginine and glucagon, before and after 9-week treatment with exenatide.

Fig. 10C shows glucose levels (in mM) in the serum of a T2-WFS patient in response to a standard mixed meal, without (circle) or with injection of 10 μg exenatide 1 hour prior to meal ingestion (square).

Fig. 10D shows C-peptide levels (in pM) in the serum of a T2-WFS patient in response to a standard mixed meal, without (circle) or with injection of 10 μg exenatide 1 hour prior to meal ingestion (square).

Fig. 10E shows C-peptide/glucose ratio (in pmol/mmol) in the serum of a T2-WFS patient in response to a standard mixed meal, without (circle) or with injection of 10 μg exenatide 1 hour prior to meal ingestion (square).

Fig. 10F shows GLP-1 levels (in pM) in the serum of a T2-WFS patient in response to a standard mixed meal, without (circle) or with injection of 10 μg exenatide 1 hour prior to meal ingestion (square).

Fig. 10G shows glucagon levels (in pM) in the serum of a T2-WFS patient in response to a standard mixed meal, without (circle) or with injection of 10 µg exenatide 1 hour prior to meal ingestion (square).
Abbreviations: A (arginine); C-p (C-peptide); G (glucose); Gn (Glucagon); M (meal); T (time, in minutes).

Figs. 11A-11G show the levels of insulin secretion, cyclic adenosine monophosphate (cAMP) and thioredoxin-interacting protein (TXNIP) in INS-1E NAF-1(-) cells treated without or with 3-isobutyl-1-methylxanthine (IBMX), forskolin or exenatide.
Fig. 11A shows insulin secretion (in pmol/µg protein) from INS-1E NC and NAF-1(-) cells following static incubations with 1.7 mM glucose (basal) or after stimulation with 16.7 mM glucose (stimulated), without (Untreated) or with 0.5 mM IBMX, 5 µM forskolin, or 100 nM exenatide.
Results are means ± SEM of 3 independent experiments in triplicates; *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to INS-1E NC or NAF-1(-) cells at 16.7 mM glucose, or between the indicated groups.
Fig. 11B shows insulin secretion (in pmol/µg protein) from INS-1E NC and NAF-1(-) cells with 1.7 mM glucose (basal) and after stimulation with 60 mM KCl for 1 hour. Results are means ± SEM of 3 independent experiments in triplicates; *$p<0.05$.
Fig. 11C shows the data shown in Fig. 13A, expressed as fold of insulin secretion in INS-1E NC cells at 16.7 mM glucose.
Fig. 11D shows cAMP levels (in ng/µg protein) in INS-1E NC and NAF-1(-) cells incubated with 1.7 mM or 16.7 mM glucose, without or with 0.5 mM IBMX, 5 µM forskolin, or 100 nM exenatide for 5 minutes.
*$p<0.05$, **$p<0.01$, compared to INS-1E NC or NAF-1(-) cells at 16.7 mM glucose, or between the indicated groups.
Fig. 11E shows the effect of 30 µM 2',5',-dideoxyadenosine (ddA) on exenatide-stimulated insulin secretion (in pmol/µg protein) from INS-1E NC and NAF-1(-) cells. *$p<0.05$.
Fig. 11F is a representative blot of TXNIP expression in INS-1E NC and NAF-1(-) cells treated with or without exenatide for 48 hours. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control.
Fig. 11G shows the quantification of the blot shown in Fig. 11F, relative to untreated NC cells.

*p<0.05, **p<0.01.

Abbreviations: - (NAF-1(-)); B (basal); C (INS-1E NC, control); E (exenatide); F (forskolin); G (GAPDH) I (IBMX); S (stimulated); T (TXNIP); U (untreated).

Figs 12A-12D show the effects of exenatide, DFP, or combination thereof on mitochondrial iron accumulation, mitochondrial membrane potential and oxidative stress induced by NAF-1 supression in INS-1E cells.

Fig. 12A is a representative image of gastric sections of control patients undergoing sleeve gastrectomy and of a T2-WFS patient stained for iron with Prussian blue (magnification x400). Arrows indicate positive staining for iron.

Fig.12B shows the quantification of mitochondrial iron accumulation as indicated by RPA fluorescence (in arbitrary units) in INS-1E NC and NAF-1(-) cells treated for 48 hours with or without exenatide (100 nmol/l) and/or the iron chelator DFP (25 µmol/l for 48 hours).

*p<0.05, ***p<0.001.

Fig. 12C shows changes in MMP as indicated by TMRE fluorescence (in arbitrary units) in INS-1E NC and NAF-1(-) cells treated for 48 hours with or without exenatide (100 nmol/l) and/or the iron chelator DFP (25 µmol/l for 48 hours).

**p<0.01.

Fig. 12D shows the quantification of DHE fluorescence (in arbitrary units) in INS-1E NC and NAF-1(-) cells treated for 48 hours with or without exenatide (100 nmol/l) and/or the iron chelator DFP (25 µmol/l for 48 hours)

**p<0.01, ***p<0.001.

Abbreviations: - (NAF-1(-)); C (INS-1E NC, control); CS (control gastric section); E (exenatide); WS (T2-WFS gastric section).

Fig. 13 shows the effects of exenatide and DFP on insulin content in INS-1E NC and NAF-1(-) cells. Insulin content of INS-1E NC and NAF-1(-) cells treated without or with exenatide (100 nM for 48 hours), the iron chelator DFP (25 µmol/l for 48 hours), or combination thereof. Data is shown for 3 independent experiments in 3 and 6 replicates. Results were normalized to protein content and are expressed as fold of control.

**p<0.01, ***p<0.001, compared to INS-1E NC or NAF-1(-) cells, or between the indicated groups.

Abbreviations: C (INS-1E NC, control); - (NAF-1(-)); IC (insulin content); E (exenatide).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of treating Wolfram syndrome-2 (T2-WFS), comprising administering to a subject in need of such treatment at least one iron chelator. The invention further relates to combinations comprising at least one iron chelator for treating T2-WFS.

The iron chelator according to the invention causes "conservative" iron chelation, meaning that the chelator does not deplete the subject from iron, but rather redeploys it or re-distributes it within the subject's body. Conserved iron chelators extract excess mitochondrial labile (i.e. redox active and chelatable) iron from cells and recycles it to other cells via the transferrin-transferrin receptor system. This mechanism of action results in iron detoxification concurrent with regional depletion via chelation of the labile iron from any available source, and its redeployment to different cells. Accordingly, the chelator according to the invention maintains the iron in the subject, contrary to chelators used for treating systemic iron overload. It should be noted, however, that iron chelators acceptable for reducing total iron levels in a subject are also applicable according to the present invention, provided they are administered in lower doses or less frequently than the recommended treatment regimen.

The term "iron chelator" as used herein refers to agents with specific binding affinity for complexing iron (II or III), thereby blocking iron's ability to catalyze redox reactions and engage in the generation of noxious reactive oxygen species (ROS). Iron (II or III) have six chemical coordination sites. Thus, a single chelator molecule that binds to all six coordination sites (referred to as "hexadentate"), can completely inactivate the "free" iron, as in the case of desferrioxamine (N'-[5-(Acetyl-hydroxy-amino)pentyl]-N-[5-[3-(5-aminopentyl-hydroxy-carbamoyl)propanoylamino]pentyl]-N-hydroxy-butane diamide). Other chelators, such as deferiprone (3-hydroxy-1,2-dimethylpyridin-4(1H)-one), are endowed with only two coordination binding sites (namely are bidentate), requiring three molecules to render the metal ion non-labile. Thus, a chemical excess of chelator over labile iron is required in order to attain formation of non-labile chelates (i.e. iron-chelator complexes). Deferiprone has

been shown experimentally applicable for treating Friedreich ataxia and Parkinson's disease.

The term "treating" in the contexts of the present invention refers to ameliorating, improving, or slowing down the deterioration of at least one symptom or undesired effect of the disease as compared to an untreated subject. Examples of conditions alleviated or showing reduced deterioration by the treatment with the iron chelator and combinations thereof according to the invention are sensorineural deafness, optic atrophy, diabetes insipidus, hypogonadism, neurogenic bladder, insulin-treated diabetes mellitus, and neurological or psychiatric disorders.

The term "subject" or "patient" refers to a mammal, and in one embodiment, the patient is a human. The administering of the drug combination of the invention to the patient includes both self-administration and administration to the patient by another person.

The term "combination" as used herein refers to either a fixed combination in one dosage unit form, or a number of therapeutic agents (also designated herein as "active ingredients") for the combined administration where the agents may be administered independently at the same time or separately within time intervals, at the same route of administration or via different routes of administration, especially where these time intervals allow the therapeutic agents of the combination to show an additive or synergistic effect.

The term "pharmaceutical combination" as used herein refers to a product that results from the mixing or combining of more than one active ingredient and includes both fixed and non-fixed combinations of the active ingredients. The term "fixed combination" means that the active ingredients are both administered to a patient simultaneously in the form of a single entity or dosage. The term "non-fixed combination" means that the active ingredients are both administered to a patient as separate entities either simultaneously, concurrently or sequentially with no specific time limits. The term "pharmaceutical combination" also applies the administration of three or more active ingredients.

The term "synergy" or "synergistic" as used herein refers to a therapeutic combination which is more effective than the additive effects of the two or more single active ingredients. Accordingly, synergic combination means that the therapeutic effect of the components of the combination is greater than the sum of the therapeutic effects of administration of any of these agents separately as a sole treatment. A synergistic effect may be attained when the active ingredients are: co-formulated and administered or delivered simultaneously in a combined, unit dosage formulation; or delivered by alternation or in parallel as separate formulations. When delivered in alternation therapy, a synergistic effect may be attained when the active ingredients are administered or delivered sequentially.

The term "optional" or "optionally" as used herein means that a subsequently described event or circumstance may, but need not, occur, and that the description includes instances where the event or circumstance occurs and instances in which it does not.

The iron chelator and combinations comprising thereof according to the present invention are directed to reducing and alleviating symptoms associated with T2-WFS, as well as other diabetic pathologies, i.e. Type I and/or Type II diabetes, and diseases and disorders showing regional iron overload (i.e. iron accumulation restricted to specific cells, tissue or organ).

Non-limiting examples of undesired manifestation that can be treated by the iron chelators and the combinations thereof according to the invention are selected from: high blood sugar levels resulting from a deficiency in the hormone insulin; progressive vision loss due to degeneration of the nerves (optic atrophy); pituitary gland dysfunction that results in the excretion of excessive amounts of urine (diabetes insipidus); hearing loss caused by changes in the inner ear (sensorineural deafness), urinary tract problems; reduced amounts of the sex hormone testosterone in males (hypogonadism), and neurological or psychiatric disorders. Specifically, the treatment according to the invention results in improvement (i.e., increase) or slowing down in the deterioration of insulin secretion in a subject.

The oral iron chelators encompassed by the present invention are efficient in treating regional iron overload (or focal siderosis) when used at moderate concentrations. All known iron chelators presently used in the clinic have been designed just for reducing chronic systemic iron overload, which is a condition caused either by hyper-absorption of iron, or by poly-transfusion of blood, resulting in iron accumulation in the subject's body. The major requirements from chelators to be approved for clinical application include safety and efficacy in removing excess iron from tissues and fluids without interfering with normal functions. Another important factor is the convenient mode of drug administration, thus the generally preferred mode is the oral route.

Regional iron overload is a condition of iron accumulating in specific regions within cells or tissues, irrespective of the levels of iron in the rest of the organism. The accumulation often results in depletion of iron from surrounding areas, resulting in a misdistribution of the metal and subsequently dual toxicity due to oxidative damage in areas of labile iron accumulation and metabolic deprivation resulting from iron depletion. The means to attain regional iron detoxification concurrent with regional depletion is by chelation of labile iron from any available source (including the toxic regional overload) and its redeployment to cells via the transferrin-transferin receptor system. A chelator that fulfills both requirements of chelation of labile iron and also redeployment to cells is deferiprone (DFP, in defined experimental conditions). Such mode of action of a chelator that does not deplete the organism from iron (but rather conserves it) is referred as “conservative chelation”, which is clinically applicable to conditions of normoferremia or even hypoferremia.

The iron chelator in accordance with the invention can be a small molecule or biologic (amino acid based molecule), a drug already on the market, a drug currently undergoing clinical trials, or a drug that will be developed in future. Importantly, the treatment according to the invention may include one or more iron chelator, optionally in combination with two or more active agents.

Accumulation of labile iron in mitochondria can induce cellular damage through hydroxyl radical-induced oxidation of proteins, lipids and DNA, intracellular organelle dysfunction, and eventually apoptosis, thus leading to β-cell degeneration. NAF-1-suppressed INS-1E β-cells as well as skin fibroblasts obtained from T2-WFS

patients exhibit decreased mitochondrial membrane potential (suggesting impaired mitochondrial function), increased mitochondrial labile iron accumulation, and increased accumulation of ROS (namely, increased oxidative stress).

The experimental results presented herein show, for the first time, that treatment of a model system of T2-WFS (NAF-1-suppressed INS-1E cells) and T2-WFS skin fibroblasts with either the iron chelator DFP alone, the cell GSH-precursor NAC alone, or the GLP-1-RA exenatide alone, partially prevented mitochondrial iron overload and its deleterious consequences on mitochondrial function and oxidative stress. Furthermore, the combination of DFP and CGP, or DFP and exenatide, showed a clear synergistic effect on mitochondrial defects, and completely restored the levels of the measured parameters to the levels of control INS-1E cells or skin fibroblasts from healthy subjects.

The surprising finding that treatment of NAF-1-deficient β-cells with iron chelation and GSH precursor or GLP-1-RA prevented mitochondrial labile iron accumulation and oxidative stress indicates that these combination therapies provide a means to attenuate the progression of diabetes and neuronal degeneration in patients with T2-WFS. Accordingly, the application of the conservative chelation can be extended beyond WFS-2 to other diabetic pathologies, e.g. type I and/or II diabetes.

Accordingly, the present invention shows, for the first time, the beneficial effects of deferiprone alone in a model system of T2-WFS (rat insulinoma cells), skin fibroblasts from WFS-2 patients, all demonstrating features of the WFS-2 phenotype.

Furthermore, the invention shows that additives that commonly synergize with the chelator in relieving cells from the effects of iron overload or internal misdistribution of iron are glutathione-stimulating genes, such as N-acetyl cysteine (NAC), and anti-diabetic agents, such as the GLP-1-RA exenatide.

The present invention therefore particularly relates to additive and synergistic combinations of an iron chelator with an anti-oxidant, an iron chelator with an anti-diabetic agent, or an iron chelator with an anti-oxidant and an anti-diabetic agent,

which are useful in treating a subject suffering from T2-WFS, Type I or Type II diabetes, or a disease demonstrating regional iron overload.

In another aspect the invention relates to a method for the treatment, amelioration or improvement of T2-WFS, or a disease associated with increased regional iron levels. The method of the invention comprises the step of administering to a subject in need thereof, a therapeutically effective amount of at least one iron chelator, specifically a conservative iron chelator, or any compositions comprising the same, or any combinations thereof with an additional therapeutic agent.

The iron chelators according to the invention are optionally a member of the families of hydroxypyridones or hydroxyquinoliones. Specific examples of iron chelators are desferrioxamine (N'-[5-(Acetyl-hydroxy-amino)pentyl]-N-[5-[3-(5-aminopentyl-hydroxy-carbamoyl)propanoylamino]pentyl]-N-hydroxy-butane diamide) and deferiprone (3-hydroxy-1,2-dimethylpyridin-4(1H)-one).

The additional therapeutic agent to be combined with the iron chelator according to the invention is an anti-oxidant, or an anti-diabetic agent, or both.

An anti-oxidant agent may be a glutathione-stimulating gene. Glutathione-stimulating genes suitable according to the invention are members of the groups of glutathione (GSH) promoters, glutathione precursors (such as NAC and L-2-oxothiazolidine-4-carboxylic acid), glutathione cleavable esters (such as glutathione diethyl ester), and glutathione coupled to membrane-crossing peptides. A non-limiting example for another anti-oxidant suitable according to the invention is tetrathionate.

Anti-diabetic agents useful according to the invention belong to the thiazolidinedione class of drugs, such as pioglitazone and rosiglitazone, or to the group of incretin mimetics, such as the GLP-1 agonist exenatide.

It should be noted that standard clinical protocols for iron chelation are intended to treat systemic iron overload and are designed to reduce body iron burden (BIB). The administration regime for the treatment of T2-WFS according to the present invention

is designed to carry out "conservative chelation", namely maintain the BIB, and only redistribute the iron within the subject's body.

The surprising findings of the present invention can be summarized as follows:

1. Biochemical Properties of Insulinoma cells (INS-1E) transfected with NAF-1 shRNA as model of T2-WFS2 phenotype (compared to mock transfected cells):
   A. Repression of NAF-1 protein expression by ~ 50%.
   B. Reduction in cell growth.
   C. Reduction in mitochondrial membrane potential (MMP).
   D. Increased mitochondrial labile iron (MLI) levels.
   E. Increased mitochondrial ROS production (MRP).
   F. Restoration of properties affected by NAF-1 suppression by overexpression of WT NAF-1.

2. Biochemical Properties of skin fibroblasts taken from T2-WFS patients (compared to skin fibroblasts from normal individuals):
   A. Undetectable NAF-1 protein.
   B. Reduction in mitochondrial membrane potential (MMP).
   C. Increased mitochondrial labile iron levels (MLI).
   D. Increased mitochondrial ROS production (MRP).

3. Attempts to pharmacologically restore functions affected by NAF-1 repression:
   A. Deferiprone: MMP fully recovered in the fibroblasts of T2-WFS patients but only partial recovery in INS-1E. MLI and MRP partially recovered in INS-1E cells and fibroblasts of T2-WFS patients.
   B. In the case of pancreas INS-1E cells, combinations of Deferiprone with an additive compound/drug like NAC and/or GLP-1-RA (exenatide) lead to full recovery, as follows:
      a. NAC: MMP fully recovered in the fibroblasts of T2-WFS patients but only partial recovery in INS-1E. MLI and MRP partially recovered in INS-1E cells and fibroblasts of T2-WFS patients.
      b. Deferiprone + NAC: full recovery of MMP, MLI and MRP in INS-1E cells and fibroblasts of T2-WFS patients.

c. Exenatide: partial recovery of MMP, MLI and MRP in INS-1E cells. Partial recovery of insulin content in INS-1E cells.
d. Deferiprone + Exenatide: full recovery of MMP, MLI, MRP and insulin content in INS-1E cells.

The treatment with the different active ingredients according to the invention may require the use of different doses or different time periods; these will be evident to the skilled medical practitioner.

It should be noted that for the method of treatment and prevention provided in the present invention, the therapeutic effective amount, or dosage, is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the combination therapy of the invention is administered in maintenance doses, once or more daily.

Therefore, the dosages to be used should be appropriate for each iron chelator, or combination of either iron chelators with other agents or treatments. As a non-limiting example, where the ion chelator used is deferiprone, the daily dose is between about 10 mg/kg body weight to 50 mg/kg, specifically, between about 20 to 40, more specifically, about 30 mg/kg per day. For example, a recommended dose can be 20 mg/kg/day, optionally administered in two separate doses of 10 mg/kg/day either daily or every other day.

Further to the combinations described above, the iron chelator, and the combinations thereof can be given together with standard Wolfram syndrome therapy, such as insulin replacement therapy, nasal or oral vasopressin, anticonvulsant medication and psychiatric and anti-depressants.

In one aspect of the invention, the active ingredients used, i.e., the iron chelator and any combinations thereof with anti-oxidants and/or anti-diabetic agents are

administered according to their marketed mode, or via any other mode of administration, for example, oral, intravenous, intramuscular, subcutaneous, intraperitoneal, parenteral, transdermal, intravaginal, intranasal, mucosal, sublingual, topical, rectal or subcutaneous administration, or any combination thereof. According to a specific embodiment, the iron chelator is administered orally.

Any active ingredient of the combination therapy according to the invention can be administered in various oral forms including, but not limited to, tablets, capsules, pills, powders, granules, elixirs, tinctures, suspensions, syrups, and emulsions. It is contemplated that the active ingredients can be delivered by any pharmaceutically acceptable route and in any pharmaceutically acceptable dosage form. These include, but are not limited to, the use of oral conventional rapid-release, time controlled-release, and delayed-release pharmaceutical dosage forms. The active ingredients can be administered in a mixture with suitable pharmaceutical diluents, excipients or carriers (collectively referred to herein as "carrier" materials) suitably selected with respect to the intended form of administration.

The combined active agents of the present invention are generally administered in the form of a pharmaceutical composition comprising the agents of this invention together with a pharmaceutically acceptable carrier or diluent. Thus, the active agents used by this invention can be administered either individually or together in a kit, in any conventional oral, parenteral or transdermal dosage form.

More particularly, the invention also relates as a further aspect, to combining separate pharmaceutical compositions in kit form, including at least two separate pharmaceutical compositions. In one embodiment, the kit comprises an iron chelator, and an anti-diabetic drug. In a second embodiment, the kit comprises an iron chelator, and an anti-oxidant. According to a third embodiment, the kit comprises an iron chelator, an anti-diabetic agent and an anti-oxidant. The active agents in the kit are provided in separate containers, such as a divided bottle or a divided foil packet. However, the separate compositions may also be contained within a single, undivided container.

The kit may further comprise a label or package insert, which provide instructions about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of the pharmaceutical compositions. The kit may further comprise directions for the simultaneous, sequential or separate administration, in any order, of the pharmaceutical compositions to a subject

The separate pharmaceutical compositions comprised in the kit are optionally administered in different dosage forms (e.g., oral and parenteral). For example, the ion chelator is administered parenterally, and the anti-oxidant is administered orally. Furthermore, the pharmaceutical compositions in the kit may be administered at different dosage intervals.

In one embodiment, the pharmaceutical compositions in the kit are adapted for oral administration, and may be packaged as different oral dosage forms (e.g., pills, capsules). According to another embodiment, pharmaceutical compositions of the kit are provided as different dosage forms. In such case, the kit may comprise separate vials of each of the active agents in a suitable carrier.

According to one embodiment, the kit of the invention is intended for treating Wolfram syndrome 2 (T2-WFS).

According to one embodiment, the kit of the invention is intended for achieving a therapeutic effect in a subject suffering from Type I or Type II diabetes, or a disease demonstrating regional iron overload. For example, the kit of the invention is beneficial for treating high blood sugar levels resulting from a deficiency in insulin; progressive vision loss due to degeneration of the nerves (optic atrophy); pituitary gland dysfunction that results in the excretion of excessive amounts of urine (diabetes insipidus); hearing loss caused by changes in the inner ear (sensorineural deafness), urinary tract problems; reduced amounts of the sex hormone testosterone in males (hypogonadism), and neurological or psychiatric disorders. Achieving a therapeutic effect is meant for example, improving or slowing down the deterioration of insulin secretion in a subject.

Still further, the invention provides a method of treatment of T2-WFS comprising the step of administering to a subject in need thereof a therapeutically effective amount of the dosage forms comprised in the kit according to the invention.

According to a specific embodiment, the iron chelator according to the invention is formulated (i.e. labeled, marketed or indicated) for use in conjunction with an approved anti-diabetic agent or an anti-oxidant, or both, as a combination treatment for treating T2-WFS.

According to another specific embodiment, the anti-diabetic drug encompassed by the present invention is formulated (i.e. labeled, marketed or indicated) for use in conjunction with an iron chelator, and optionally with an anti-oxidant, as a combination treatment for treating T2-WFS.

The invention will now be described with reference to specific examples and materials. The following examples are representative of techniques employed by the inventors in carrying out aspects of the present invention. It should be appreciated that while these techniques are exemplary of specific embodiments for the practice of the invention, those of skill in the art, in light of the present disclosure, will recognize that numerous modifications can be made without departing from the spirit and intended scope of the invention.

EXAMPLES

Materials and methods

Subjects

A 31 year-old woman of Palestinian origin was diagnosed with WFS based on hearing loss, optic atrophy, neurogenic bladder and insulin-treated diabetes mellitus. Her medical history included peptic ulcers with recurrent episodes of upper gastro-intestinal (GI) bleeding. Her diabetes, which debuted at age 24, required 45 U of insulin daily in multiple injections (0.6 U/kg/day). Sequencing of the *CISD2* gene identified the homozygous intervening sequence-1 (IVS1)+6G>C, p.E37Q mutation, previously described in T2-WFS. No mutations were found in the *WFS1* gene. The patient had fluctuations of blood glucose with recurrent episodes of hypoglycemia. The patient was then treated by subcutaneous injections of exenatide 5 µg bid (twice a

day) for 4 weeks and then 10 μg bid for additional 5 weeks. She was hospitalized before and after intervention and β-cell function was assessed by intravenous (IV) glucose tolerance test (IVGTT) together with glucagon and arginine injection and mixed meal tests with and without injection of exenatide prior to meal. The study protocol was approved by the Hadassah Helsinki Committee (Clinical Trial Registration numbers: HM-0438–10; NCT01302327) and appropriate informed consent was obtained.

Dynamic tests

A standard meal test was performed to assess insulin and glucagon secretion. At time 0, the patient received a 500 kcal mixed meal and blood was drawn at 0, 30, 60, 120 and 180 minutes for glucose, C-peptide and glucagon levels. The acute response to exenatide was studied by repeating the test the next day with subcutaneous injection of 5 μg exenatide at 60 minutes prior to meal. β-Cell function was assessed by IV glucose tolerance test (IVGTT) together with glucagon and arginine injection. At time 0, glucose (0.3 g/kg) was injected within 1–2 minutes and then 0.5 mg glucagon and 5 g arginine were added at 30 minutes. Blood samples were drawn for glucose and C-peptide levels at the following time points: 0, 1, 3, 5, 10, 20, 30, 45 and 60 minutes. The IVGTT/glucagon/arginine test was repeated after 9 weeks of exenatide treatment following a 4-day washout. The patient was treated with a continuous insulin infusion pump to achieve near-normoglycemia for 48 hours prior to testing, both before and after the intervention. Insulin infusion was discontinued 30 minutes prior to the test and renewed at the end of the test until the next day.

Hormone measurements

Serum samples were kept at –20 °C until analysis. Serum C-peptide was analyzed using the ADVIA Centaur ELISA assay (Siemens Healthcare Diagnostics Inc., Tarrytown, NY). The assay is standardized against World Health Organization IS84/510 and does not cross-react with proinsulin. The minimum detectable concentration was 17 pM. Glucagon and glucagon-like peptide-1 (GLP-1) measurements were performed in the Holst laboratory at the University of Copenhagen, Denmark. Frozen samples were shipped on dry ice and analyzed by glucagon radioimmunoassay (RIA) directed against the C-terminus of the molecule.

Cell culture

INS-1E β-cells were grown in RPMI 1640 with 11.1 mM D-glucose supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 µM β-mercaptoethanol. INS-1E with suppressed NAF-1 expression cell line (NAF-1(-)) were generated using shRNA transfections, as described previously, and maintained in the same medium as control cells. Puromycin (1 µg/ml) was added to the culture medium of NAF-1(-) and control (NC) cell lines to allow selective expansion of transfected cells. Cells were plated one day before experimentation onto 96-well plates in puromycin-free medium, or onto microscope slides glued to perforated 3 cm-diameter tissue culture plates, for cell viability or microscopic measurements.

Insulin secretion

Insulin secretion from INS-1E NC and NAF-1(-) was evaluated by static incubation. Cells were pre-incubated for 30 minutes in RPMI 1640 containing 1.7 mM glucose and then incubated at 1.7 or 16.7 mM glucose with or without 100 nM exenatide, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 5 µM forskolin (from Sigma-Aldrich) or 60 mM KCl for 1 hour at 37 °C in 1 ml modified Krebs-Ringer bicarbonate buffer containing 20 mM HEPES and 0.25% BSA (KRBH-BSA). Medium was collected at the end of the basal (1.7 mM glucose) or stimulatory (16.7 mM glucose) incubations, centrifuged, and supernatants were frozen at –20°C pending insulin assay. Cells were then extracted and subjected to repeated freeze-thaw cycles in 1.5-ml microfuge tubes containing 0.1% BSA in GB/NP-40 solution. Insulin immunoreactivity in the extracts and medium was determined using a rat insulin ELISA Kit (Mercodia, Uppsala, Sweden).

Cyclic adenosine monophosphate measurement

INS-1E cells (NC and NAF-1(-)) were pre-incubated with 1.7 mm glucose for 30 minutes, followed by incubation with 16.7 mm glucose, with or without 100 nM exenatide, 0.5 mM IBMX or 5 µM forskolin for 5 minutes. Cyclic adenosine monophosphate (cAMP) was measured in cell extracts, using the Cyclic AMP EIA Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions.

Mitochondrial membrane potential measurement

INS-1E control and NAF-1(-) cell lines as well as skin fibroblast from healthy subjects and T2-WFS patients were plated at a density of 600,000 cells per well in glass-bottomed Petri dishes. Cells were incubated with the agent of choice (exenatide, deferiprone (DFP, Apo Pharma), cell GSH-precursor NAC (CGP), or the indicated combination thereof), for the indicated time and then washed with DMEM-HEPES (pH 7.3). Cells were then incubated with 0.1 μM tetramethylrhodamine, ethyl ester (TMRE, Molecular Probes; excitation, 543 nm; emission, 633 nm) for 15 minutes at 37 °C, washed with DMEM-HEPES (pH 7.3) and analyzed by a Nikon TE 2000 microscope equipped with opti-grid device and a Hamamatsu Orca-Era CCD camera. Images were acquired and analyzed with Velocity or Image-J software programs for image processing.

Mitochondrial labile iron measurement

INS-1E control and NAF-1(-) cell lines as well as skin fibroblast from healthy subjects and T2-WFS patients were plated at a density of 600,000 cells per well in glass-bottomed Petri dishes. Cells were incubated with the agent of choice (exenatide, DFP, CGP, or the indicated combination thereof). The iron status in the mitochondria of these cells was evaluated using rhodamine B-[(1,10-phenanthrolin-5-yl)amino-carbonyl]benzyl ester (RPA, Squarix Biotechnology), which fluorescence intensity is inversely correlated with labile iron levels in the mitochondria. Cells were incubated with RPA (1 μM) for 15 minutes at 37 °C and then washed with DMEM-HEPES. Images were captured by epi-fluorescence microscopy (equipped with opti-grid device) and analyzed with image-J software program.

ROS formation assay

INS-1E NC and NAF-1(-) cell lines as well as skin fibroblast from healthy subjects and T2-WFS patients were plated at a density of 600,000 cells per well in glass-bottomed Petri dishes. Cells were incubated with the agent of choice (exenatide, DFP, CGP, or the indicated combination thereof). Reactive oxygen species (ROS) formation was determined by incubating cells with 10 μM MitoSOX™ or 10 μM dihydroethidium (DHE) for 30 min at 37°C, which display blue fluorescence in cell cytoplasm, while the oxidized form is obtained by reaction with ROS and turns the

fluorescence to red, which can be analyzed microscopically (excitation: 518 nm; emission: 605 nm).

Protein blots

Protein expression was studied by standard Western blotting, using antibodies against: NAF-1, mNT (mitochondrial NEET; CISD1) and thioredoxin-interacting protein (TXNIP) (MBL International Co, Woburn, MA), and GAPDH (Abcam, Cambridge, MA). Peroxidase-conjugated AffiniPure goat anti-rabbit and anti-mouse IgGs (Jackson ImmunoResearch Laboratories (West Grove, PA)) were used as secondary antibodies. Protein expression was calculated as percentage of controls from three different experiments. GAPDH was used as a loading control.

Statistical analysis

Data shown are means ± SE. Statistical significance of differences between groups was determined by one-way ANOVA followed by Newman-Keuls test using the Prism 6.01 statistical program from GraphPad Software Inc. (San Diego, CA). A paired-sample t test was used when the difference between a reference and a test was analyzed. $P<0.05$ was considered significant.

Example 1:

Suppression of NAF-1 expression in INS-1E cells

In order to generate an *in vitro* cellular model system for T2-WFS, the inventors used shRNA technology, by transfecting human insulinoma (insulinoma is a tumor of the pancreas that is derived from beta cells and secretes insulin) INS-1E cells with NAF-1 shRNA. The resulting stable cell line, denoted as "NAF-1(-)", demonstrating suppressed expression of NAF-1. The cell line was characterized for protein expression levels in comparison to normal wild type (WT) INS-1E cells (designated "NC"), which served as control. The data obtained indicate that while the total protein expression was not affected by the shRNA transfection (Fig. 1A), the NAF-1(-) cell line showed a 50% decrease in the expression of the NAF-1 protein, compared to the WT cells (Figs. 1B-1C). Furthermore, the growth rate of the NAF-1(-) cells was found to be slower compared to the control WT cells (Fig. 1D). Accordingly, the inventors succeeded in preparing an *in vitro* model for T2-WFS.

Example 2:

Recovery of Mitochondrial Damages in NAF-1(-) cells by iron chelator

In order to determine the effect of NAF-1 suppression on the mitochondria of the INS-1E NAF-1(-) cell line, the mitochondrial membrane potential (MMP) was assessed along with labile iron levels in mitochondria of INS-1E NAF-1(-) cells compared to WT INS-1E cells. Indeed, the MMP detected (with the fluorescent TMRE (Tetramethylrhodamine, Ethyl Ester) probe) in the NAF-1(-) cells was lower than the MMP of the WT cells (Figs. 2A – 2B).

Additionally, the mitochondrial labile iron accumulation level was considerably higher in the NAF-1(-) cells compared to the WT/NC cells (Figs. 2C-2D). The mitochondrial labile iron level was detected with the fluorescent RPA (rhodamine B-[(1,10-phenanthrolin-5-yl) aminocarbonyl] benzyl ester) probe, which fluorescence intensity is inversely correlated with labile iron levels in the mitochondria. Importantly, pre-treatment of the cell lines with the iron chelator DFP (deferiprone) resulted in partial recovery of the mitochondrial damages (i.e., MMP and labile iron accumulation) in the NAF-1(-) cell line. These results indicate that mitochondrial damages in cells exhibiting suppressed expression of NAF-1 can be reversed or corrected, at least partially.

Example 3:

Reduction in Mitochondrial ROS formation in NAF-1(-) cells by iron chelator

The labile iron accumulation in the NAF-1(-) cells induces substantial increase in ROS formation, as detected with the fluorescent DHE (dihydroethidium) probe. However, pre-treatment of the INS-1E cell lines with the iron chelator DFP significantly reduced the ROS accumulation in the NAF-1(-) cell line (Figs. 3A-3C). These results indicate that ROS formation in cells exhibiting suppressed expression of NAF-1 can be reversed or corrected, at least partially.

Example 4:

Restoration of NAF-1 expression reduces mitochondrial defects

To ascertain that the pathological phenotypes observed in the NAF-1(-) cells indeed resulted from the reduced expression of NAF-1, the inventors infected these cells with a second vector, which induces overexpression of NAF-1 (denoted "NAF-1(+)"). The

results indicate that restoring the NAF-1 levels in NAF-1(-) cells to normal by the second transfection and the appropriate antibiotic selection (puromycin for suppression shRNA vector and G-418 for overexpressing the NAF-1(+) vector), repaired the pathological phenotypes observed in the NAF-1(-). Accordingly, it can be concluded that all observed mitochondrial pathological signals resulted from the reduction in NAF-1 protein levels.

The newly created cell line, denoted INS-1E NAF-1(-)/(+), was characterized for its MMP using the TMRE probe. Figs. 4A-4B show that the reduced MMP found in INS-1E NAF-1(-) cells can be fully restored by over-expression of NAF-1 protein in these cells.

NAF-1(-)/(+) cell line was characterized for its mitochondrial labile iron using the RPA probe. -5B show that over-expression of NAF-1 in NAF-1(-) cells resulted in the restoration of mitochondrial labile iron levels to the levels of WT INS-1E cells.

The results presented in Figs. 4 and 5 clearly indicate that low levels of the NAF-1 protein lead to the mitochondrial pathologies demonstrated in Figs. 2 and 3. Furthermore, the mitochondrial defects in cells exhibiting low levels of NAF-1 (as NAF-1(-) cells) can be corrected by restoring NAF-1 expression to normal levels.

Example 5:

NAF-1 protein levels and mitochondrial damages in skin fibroblast from a T2-WFS patient

Skin fibroblasts obtained from a 14 years old patient (designated patient 2) were analyzed by western blot and found to have no detectable levels of the NAF-1 protein compared to normal WT fibroblasts which severed as control (Figs. 6B-6C). No differences in total protein expression were observed between the control and T2-WFS fibroblasts (Fig. 6A).

As NAF-1 levels in the skin fibroblasts from the T2-WFS patient were essentially undetectable, as expected, the inventors next analyzed mitochondrial parameters in those fibroblasts.

The Mitochondrial labile iron accumulation in the control and T2-WFS fibroblasts was determined by RPA probe. As shown in Fig. 7A, the T2-WFS fibroblasts exhibit increased labile iron accumulation in the mitochondria compared to normal fibroblasts. These results are consistent with those obtained in the INS-1E NAF-1-depleted cell model. Furthermore, electron microscope images of control and T2-WFS fibroblasts show that mitochondria in cells derived from a T2-WFS patient have a distorted morphology, i.e. the mitochondria are shorter and fragmented, compared with mitochondria in control cells (Fig. 7B).

Example 6:

Recovery of mitochondrial damages in T2-WFS fibroblasts by iron chelator and the cell GSH-precursor NAC.

The effects of treating control and T2-WFS fibroblasts with the iron chelator DFP alone, the cell GSH-precursor NAC (CGP) alone, and the combination of DFP with CGP were tested. As shown in Fig. 8A, treatment of T2-WFS fibroblasts with either DFP or CGP alone led to a decrease in mitochondrial labile iron accumulation. Moreover, the combination of DFP and CGP resulted in an augmented effect, suggesting that the combined treatment has at least an additive effect on decreasing labile iron accumulation in the mitochondria. Similar results were obtained in control and NAF-1(-) INS-1E cell lines treated with DFP, CGP, or the combination thereof (Fig. 8B).

ROS accumulation in the fibroblasts of the T2-WFS patient was analyzed using the Mito-SOX^{TM} probe (Fig. 8A). In these experiments we also investigated whether there is influence to treating the fibroblasts (control/WFS-2) with the iron chelator DFP alone and in combination with the cell GSH-precursor NAC (Fig. 8C).

A summary of the data relating to mitochondrial damages and their recovery by treatment of DFP and the cell GSH-precursor NAC in four different T2-WFS patients is shown in Figs. 9A-9C. As discussed above, while T2-WFS fibroblasts exhibit increased labile iron accumulation in the mitochondria compared to normal fibroblasts, treating T2-WFS fibroblasts with the iron chelator DFP alone, or CGP alone lead to a decrease in mitochondrial labile iron accumulation, and the combination of DFP and CGP augments this effect (Fig. 9A). In addition, treatment of

T2-WFS fibroblasts with DFP, CGP, or combination thereof, fully restored MMP in these cells (Fig. 9B). Moreover, ROS accumulation in T2-WFS fibroblasts, which were markedly increased compared to ROS levels in control cells, were significantly reduced by treatment of T2-WFS fibroblasts with DFP or CGP (Fig. 9C). As in the case of mitochondrial labile iron accumulation, treatment of T2-WFS skin fibroblasts with the combination of DFP and CGP had an augmented effect on reducing ROS levels in the mitochondria compares with the treatment of either DFP or CGP alone.

Example 7:

Improved β-cell function of a T2-WFS patient treated with exenatide.

A T2-WFS patient was treated with the GLP-1 receptor agonist (GLP-1-RA) exenatide for 9 weeks. This treatment enabled the gradual reduction of the insulin dose by ~70% with improved glycemic control, evident by fasting blood glucose in the range of 6.5–7.5 mmol/l, and a decrease in Hemoglobin-A1c (HbA1C) from 6.2% prior to intervention to 5.0% thereafter, along with lower incidence of hypoglycemia.

β-Cell function was evaluated by IVGTT at baseline and after 9-week exenatide treatment followed by drug washout. As shown in Fig. 10B, basal and glucose/glucagon/arginine stimulated C-peptide levels prior to intervention were very low prior to intervention of exenatide. Nine weeks after initiation of exenatide treatment, the IVGTT showed a higher basal C-peptide and markedly increased C-peptide secretion in response to glucagon and arginine stimulation, whereas the response to glucose remained minor (Fig. 10B). In the post-intervention period, the C-peptide area under the curve (AUC) and the maximal insulin response (glucose, glucagon and arginine together) were 6.2- and 7-fold higher, respectively (Fig. 10B). A standard mixed meal test preceded by exenatide injection showed reduction of postprandial blood glucose, while the C-peptide/glucose ratio was higher throughout the test (Figs. 10C-10E). As expected, GLP-1 and glucagon levels were increased in response to a meal, whereas pre-meal exenatide administration inhibited both endogenous GLP-1 and glucagon secretion (Figs. 10F and 10G).

Altogether, treatment of the T2-WFS patient with GLP-1-RA improved glycemia and markedly increased the insulin response to stimulation. Such an effect on β-cell function was unexpected, considering the long-standing insulin requirement and the

severe hypoinsulinemia prior to initiation of GLP-1-RA treatment. This suggests that treatment with GLP-1-RA may correct a fundamental defect that leads to β-cell dysfunction.

Example 8:

Suppression of NAF-1 decreases glucose- and KCl-stimulated insulin secretion, while preserving the response to cAMP

In order to better understand β-cell dysfunction in NAF-1-deficient cells, the inventors used INS-1E NAF-1(-) cells, which showed 50% decrease in NAF-1 protein expression (as shown in Figs. 1B and 1C), without affecting mNT (mitochondrial NEET; CISD1) protein level. As shown in Figs. 11A and 11B, the insulin response of NAF-1(-) cells to glucose and to plasma membrane depolarization by KCl was decreased by ~30%, indicating impairment of β-cell function. By contrast, IBMX-, forskolin- and exenatide-stimulated insulin secretion from NAF-1(-) cells was similar to that of control cells (Fig. 11A). The relative amplifying effect of cAMP on glucose-induced insulin secretion was therefore higher in NAF-1 deficient β-cells. Thus, in NAF-1(-) cells, exenatide, forskolin and IBMX increased insulin secretion by 1.9, 2.8 and 2.4 fold, respectively, compared to only 1.5, 2.2 and 1.9 fold in control cells (Fig. 11C). Transient transfections with NAF-1 siRNA oligos also sensitized the cells to secrete more insulin in response to IBMX and forskolin. Thus, NAF-1 may be implicated in the regulation of cAMP-stimulated insulin secretion. This effect was not via augmented cAMP generation because a similar increase in cAMP levels was observed in INS-1E NC and NAF-1(-) cells following treatment with exenatide and forskolin (which increase cAMP generation by adenylyl cyclase), or with IBMX (which inhibits cAMP degradation) (Fig. 11D). As shown in Fig. 11E, exenatide-stimulated insulin secretion was inhibited by the adenylyl cyclase inhibitor 2',5',-dideoxyadenosine (ddA), further indicating that exenatide stimulation of insulin secretion is mediated via cAMP. Collectively, NAF-1 deficiency impaired insulin secretion in response to glucose and plasma membrane depolarization, while the amplification by cAMP was preserved, thus partially mimicking the β-cell dysfunction in the T2-WFS patient. Prolonged treatment of the patient with exenatide augmented the maximal insulin secretion off-drug, suggesting that in addition to its acute amplification of insulin secretion, exenatide may also alleviate β-cell stress.

TXNIP is a key mediator of β-cell dysfunction in diabetes, and may lead to oxidative stress by inhibiting the antioxidant protein thioredoxin. Treatment with exenatide has been previously shown to inhibit TXNIP. As shown in Figs. 11F and 11G, TXNIP protein levels were indeed increased in NAF-1(-) cells, whereas treatment with exenatide decreased TXNIP in both INS-1E NC and NAF-1(-) cells.

Example 9:

Combination of exenatide and DFP completely reverses mitochondrial dysfunction in INS-1E NAF-1(-) cells

Dysregulation of intracellular iron transport has been associated with oxidative stress and dysfunction of β-cells in diabetes. NAF-1 has been implicated in the regulation of intracellular iron balance, hence the inventors hypothesized that iron dysregulation may play a role in the pathophysiology of T2-WFS. The patient had recurrent hospitalizations due to bleeding peptic ulcer, which eventually required performance of subtotal gastrectomy. Staining of non-ulcerative regions of the patient's stomach for iron with Perls' (Prussian blue) stain revealed the presence of hemosiderin deposits appearing as fine granular puncta in the cytoplasm of glandular epithelial cells in the fundus and the antrum (Fig. 12A). No iron deposits were found in stomachs of subjects undergoing sleeve gastrectomy. Iron deposition appeared in the deep gastric glands and not in the foveolar cells lining the gastric mucosa and there was no extracellular deposition. Based on these findings the inventors surmised that gastric siderosis in the T2-WFS patient might represent a form of iron dysregulation, which is not secondary to gastric ulceration and bleeding or blood transfusions.

The effects of NAF-1 deficiency and the combination treatment of exenatide and DFP on mitochondrial labile iron levels and oxidative stress were studied in INS-1E cells. As discussed in examples 2 and 3 above, NAF-1 suppression in INS-1E resulted in increased mitochondrial iron accumulation (Fig. 12B), decreased MMP (Fig. 12C), and increased mitochondrial ROS accumulation (Fig. 12D). Treatment with the iron chelator DFP reversed these NAF-1 deficiency-induced outcomes (Figs. 12B-12D), indicating that in NAF-1 deficient β-cells, mitochondrial dysfunction and increased ROS production were associated with aberrant cellular iron distribution. Strikingly, treatment with exenatide similarly reduced mitochondrial iron accumulation and the ensuing ROS production, as well as partially restored MMP (Figs. 12B-12D).

Moreover, treatment with exenatide and DFP together completely abolished mitochondrial dysfunction and ROS generation, indicating the treatment of NAF-1-deficient β-cells with the combination of DFP and exenatide has an augmented effect compared to treatment with either DFP or exenatide alone.

Example 10:

Combination of exenatide and DFP completely prevents reduction in insulin reserves in NAF-1(-)cells.

Chronic β-cell stress may lead to reduction in insulin reserves. In agreement, insulin content in INS-1E NAF-1(-) cells decreased by 33% compared to INS-1E NC cells (Fig. 13). Despite oxidative stress in NAF-1(-), apoptosis was not increased compared to INS-1E NC cells, indicating that the reduction of insulin content was not secondary to β-cell loss. A 48-hour treatment of NAF-1(-) cells with exenatide alone only partially increased the insulin content, whereas DFP had no effect. Importantly, combined treatment with exenatide and DFP increased β-cell insulin content in a synergistic manner and completely prevented the reduction in insulin reserves in NAF-1-deficient β-cells (Fig. 13).

CLAIMS

1. An iron chelator for use in treating Wolfram syndrome 2 (T2-WFS).

2. The iron chelator for use according to claim 1, wherein said iron chelator is a conservative iron chelator.

3. The iron chelator for use according to claim 1 or 2, wherein said iron chelator is selected from N'-[5-(Acetyl-hydroxy-amino)pentyl]-N-[5-[3-(5-aminopentyl-hydroxy-carbamoyl)propanoylamino]pentyl]-N-hydroxy-butane diamide (desferrioxamine) and 3-hydroxy-1,2-dimethylpyridin-4(1H)-one (deferiprone).

4. A pharmaceutical combination comprising an iron chelator for use in treating Wolfram syndrome 2 (T2-WFS).

5. The pharmaceutical combination according to claim 4, wherein said iron chelator is selected from N'-[5-(Acetyl-hydroxy-amino)pentyl]-N-[5-[3-(5-aminopentyl-hydroxy-carbamoyl)propanoylamino]pentyl]-N-hydroxy-butane diamide (desferrioxamine) and 3-hydroxy-1,2-dimethylpyridin-4(1H)-one (deferiprone).

6. The pharmaceutical combination according to claim 4 or 5, further comprising an anti-oxidant.

7. The pharmaceutical combination according to claim 6, wherein said anti-oxidant is a glutathione-stimulating gene selected from a cell glutathione promoter, a glutathione precursor, a glutathione cleavable ester, and a glutathione coupled to membrane-crossing peptide.

8. The pharmaceutical combination according to claim 7, wherein said glutathione precursor is selected from N-acetyl cysteine (NAC) and L-2-oxothiazolidine-4-carboxylic acid.

9. The pharmaceutical combination according to claim 4 or 5, further comprising an anti-diabetic agent.

10. The pharmaceutical combination according to claim 9, wherein said anti-diabetic agent is selected from exenatide, pioglitazone and rosiglitazone.

11. A pharmaceutical combination comprising an iron chelator, an anti-oxidant and an anti-diabetic agent for use in treating Wolfram syndrome 2.

12. A pharmaceutical combination comprising an iron chelator, an anti-oxidant and an anti-diabetic agent for use in treating Type I diabetes, Type II diabetes, or a disease demonstrating regional iron overload.

13. The pharmaceutical combination according to claim 11 or 12, wherein said iron chelator is desferrioxamine or deferiprone.

14. The pharmaceutical combination according to claim 11 or 12, wherein said anti-oxidant is a glutathione-stimulating gene selected from a cell glutathione promoter, a glutathione precursor, a glutathione cleavable ester, and a glutathione coupled to membrane-crossing peptide.

15. The pharmaceutical combination according to claim 11 or 12, wherein said anti-diabetic-agent is selected from exenatide, pioglitazone and rosiglitazone.

16. A kit comprising:
(a) a container with an iron chelator;
(b) a container with an anti-diabetic agent; and/or
(c) a container with an anti-oxidant; and
(d) label or package insert with instructions for treating T2-WFS,
wherein the agents present in the containers are provided as different dosage forms, each one in a suitable carrier.

17. The kit according to claim 15, wherein the iron chelator, the anti-diabetic agent and the anti-oxidant are administered simultaneously, sequentially or separately.

18. The kit according to claims 15 or 16, wherein the iron chelator is administered parenterally, and the anti-oxidant is administered orally.

19. An iron chelator formulated for use in conjunction with an anti-oxidant and/or an anti-diabetic agent for use in the treatment of Wolfram syndrome 2.

20. An anti-diabetic drug formulated for use in conjunction with an iron chelator, and optionally with an anti-oxidant, for use in the treatment of Wolfram syndrome 2.

21. A method for treating Wolfram syndrome 2 by administering an iron chelator to a subject.

22. The method of claim 16, further comprising administering an anti-oxidant.

23. The method claim 16, further comprising administering an anti-diabetic agent.

24. The method claim 16, further comprising administering an anti-oxidant and an anti-diabetic agent.

25. A method for treating Type I diabetes, Type II diabetes, or a disease demonstrating regional iron overload by administering an iron chelator, an anti-oxidant and an anti-diabetic agent to a subject.

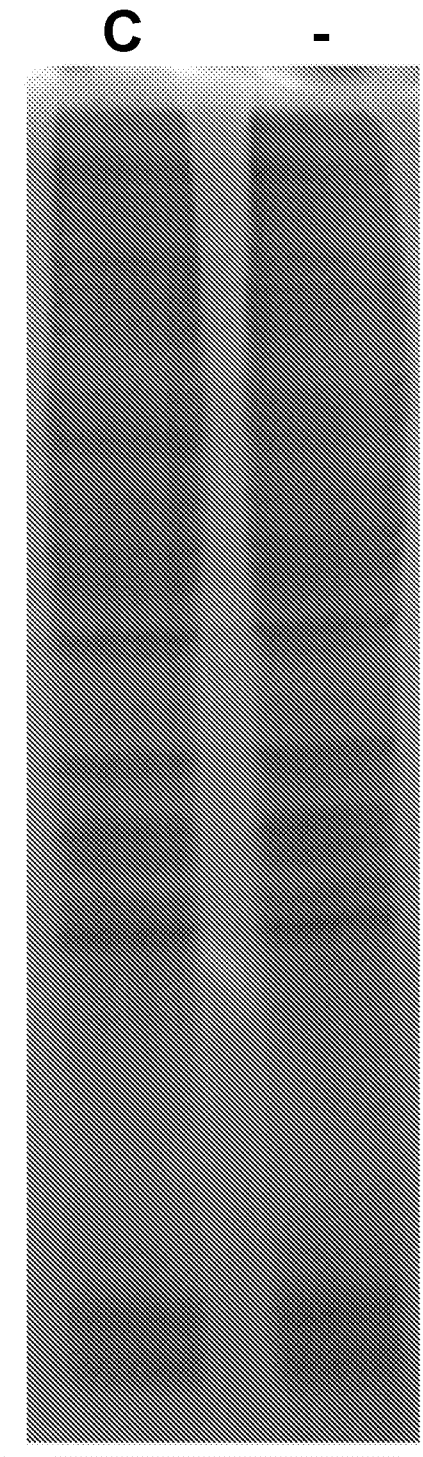


Fig. 1A

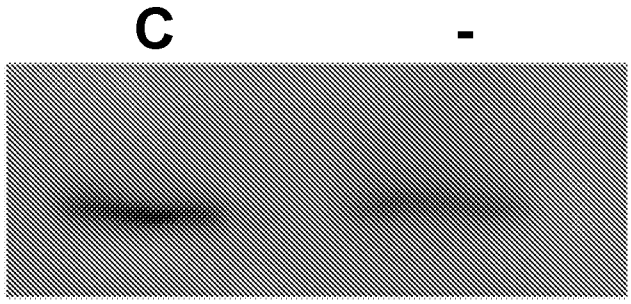


Fig. 1B

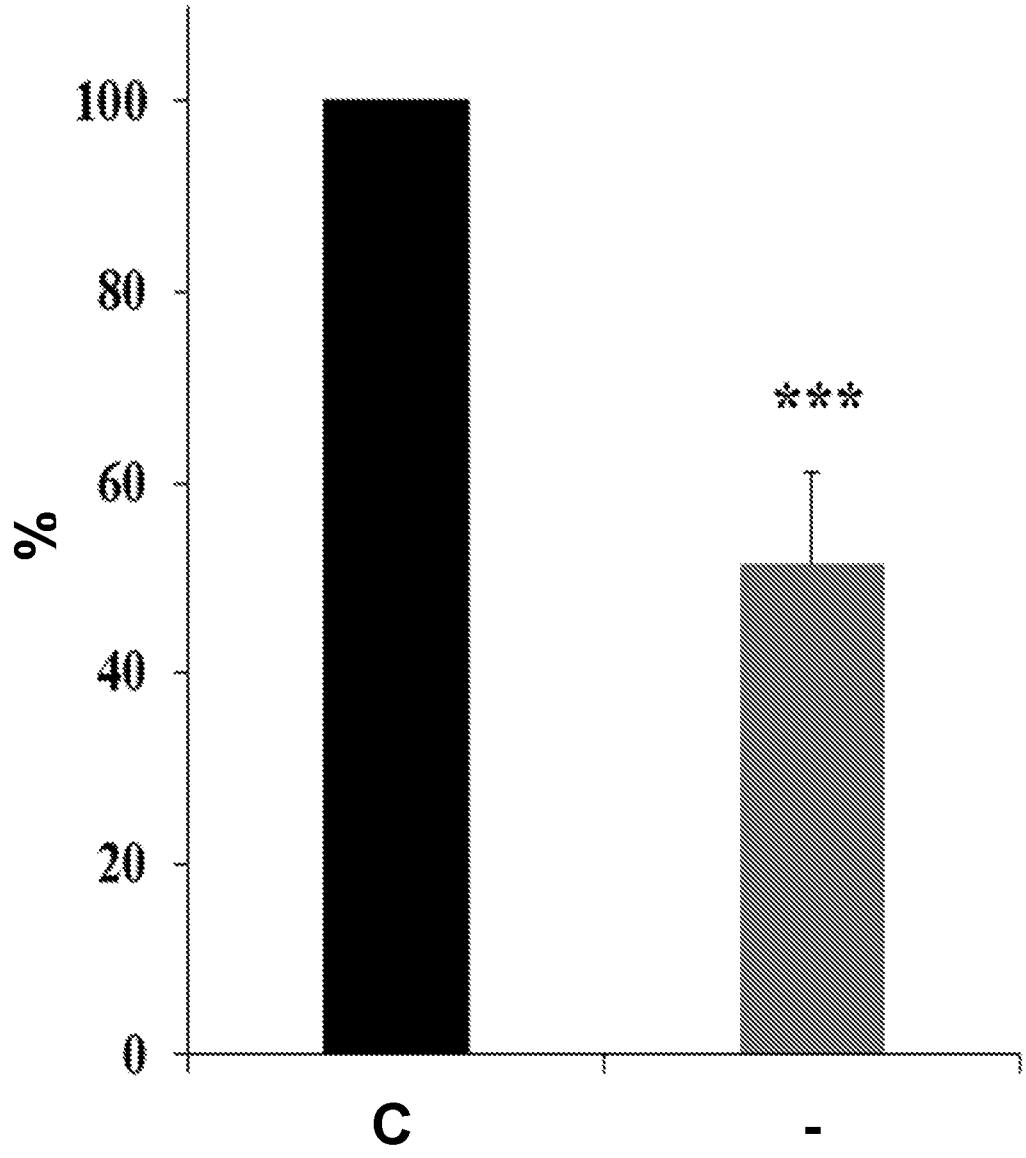


Fig. 1C

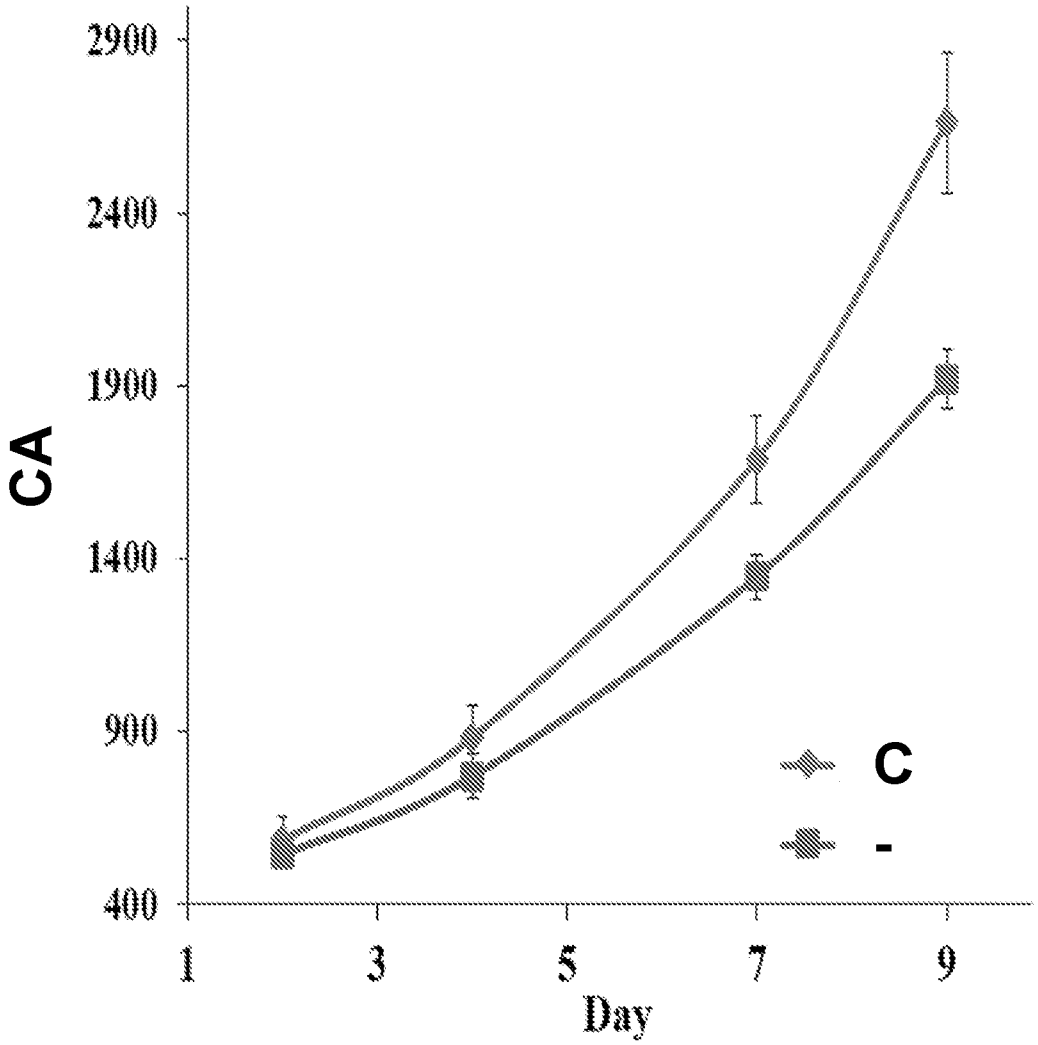


Fig. 1D

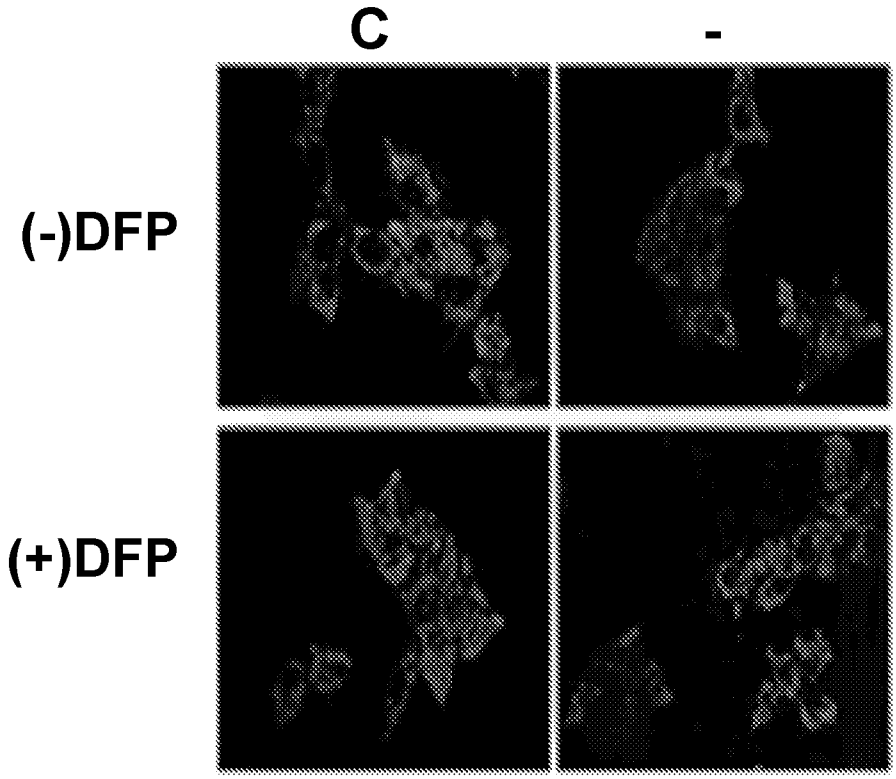


Fig. 2A

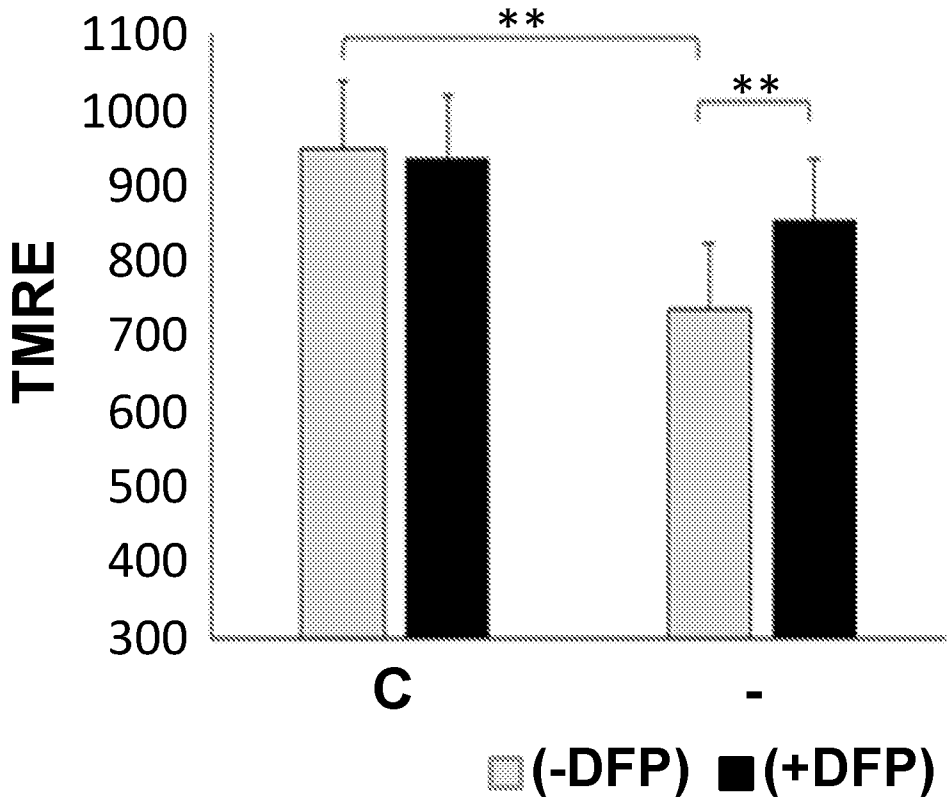


Fig. 2B

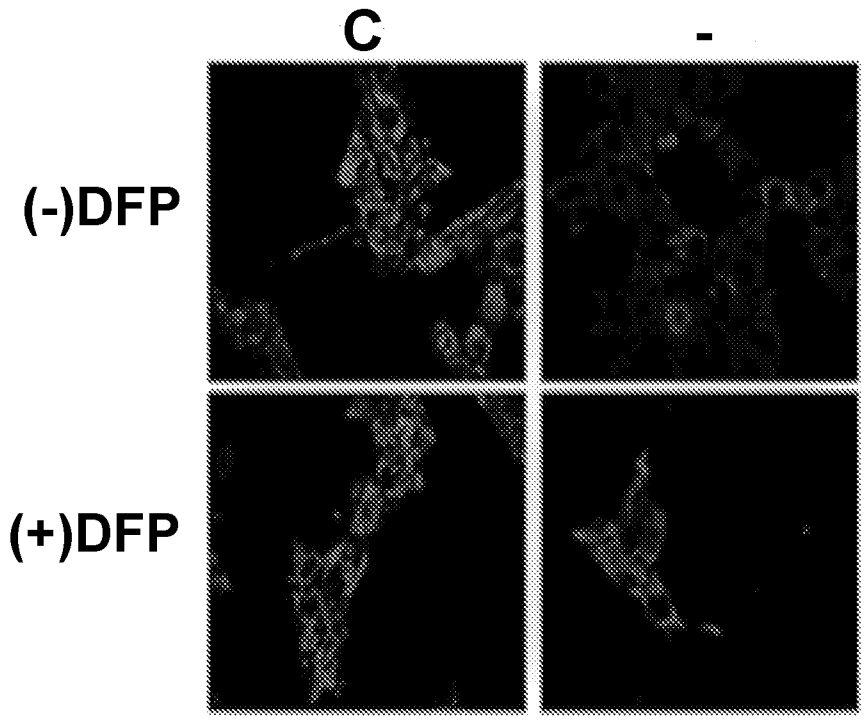


Fig. 2C

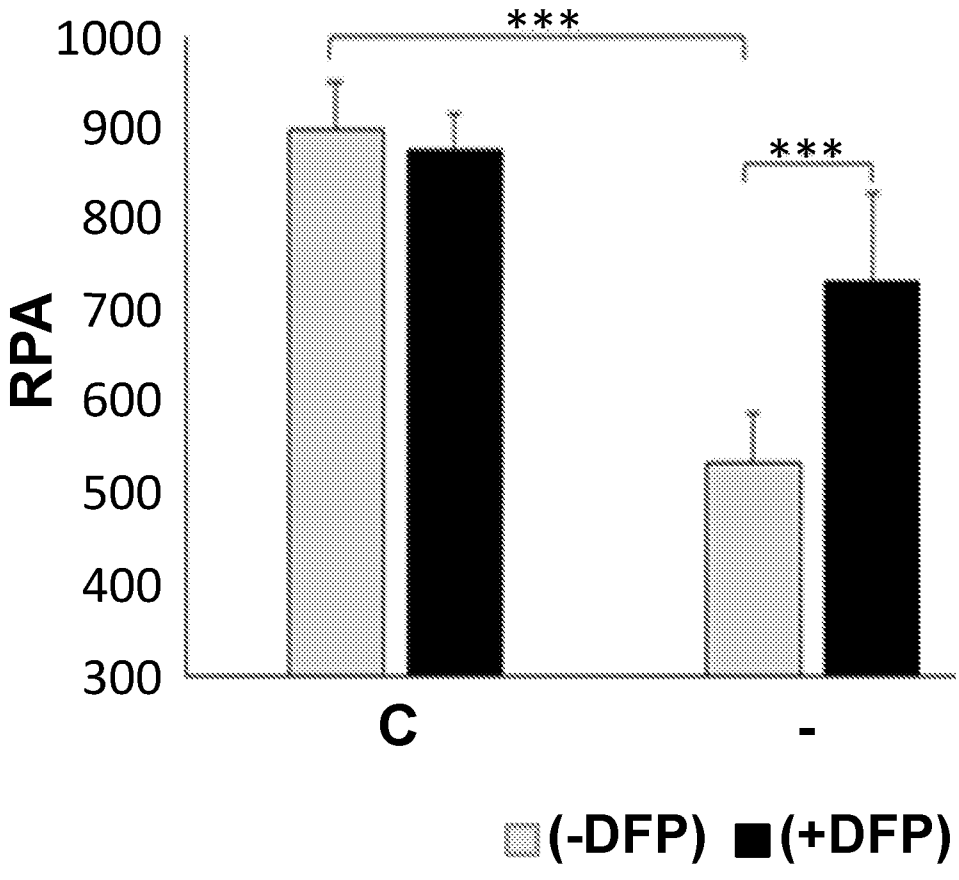


Fig. 2D

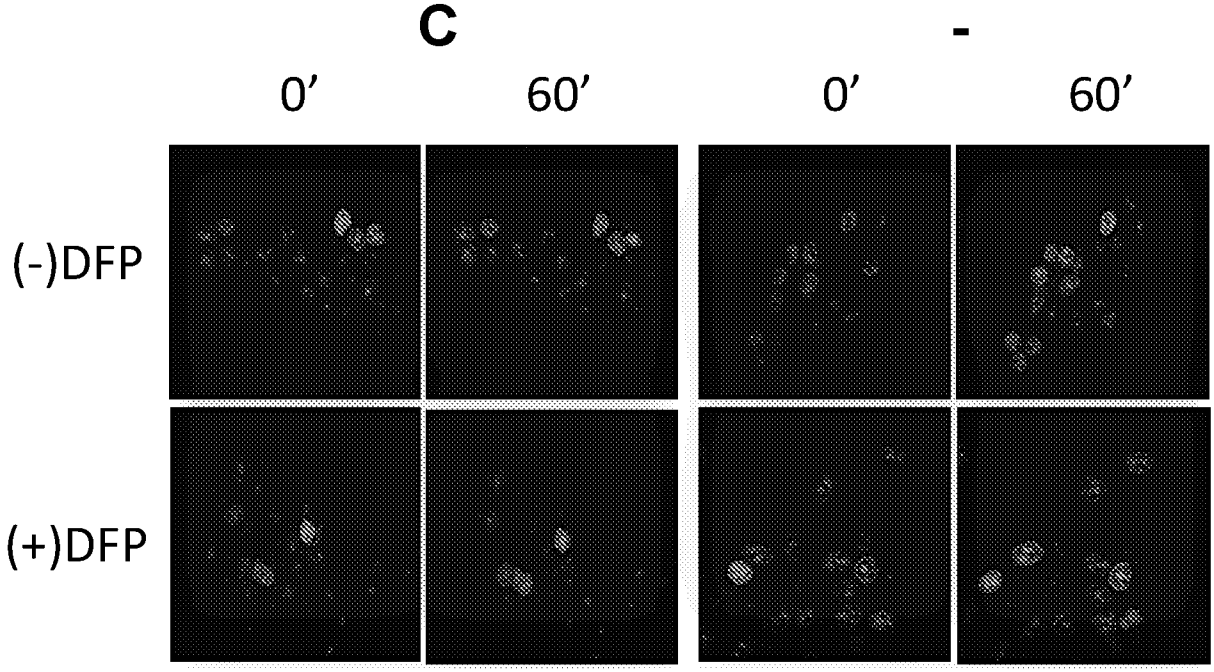

C
-
0'
60'
0'
60'
(-)DFP
(+)DFP

Fig. 3A

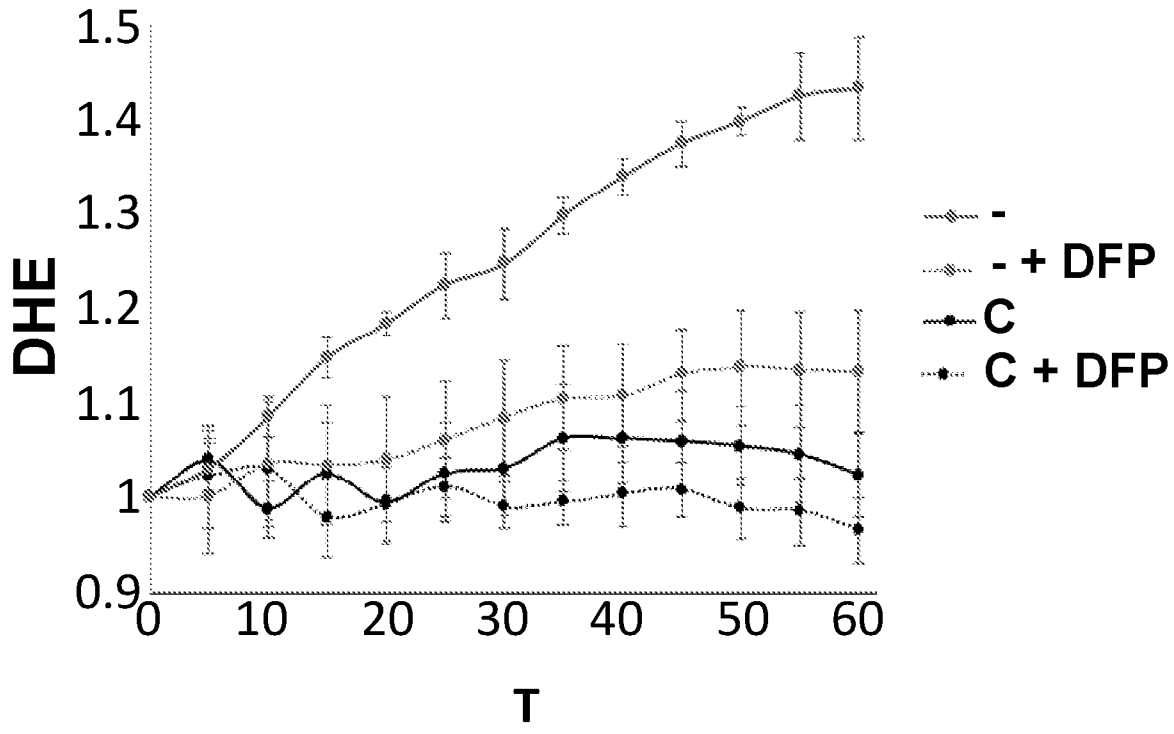

DHE
1.5
1.4
1.3
1.2
1.1
1
0.9
0
10
20
30
40
50
60
T
-
- + DFP
C
C + DFP

Fig. 3B

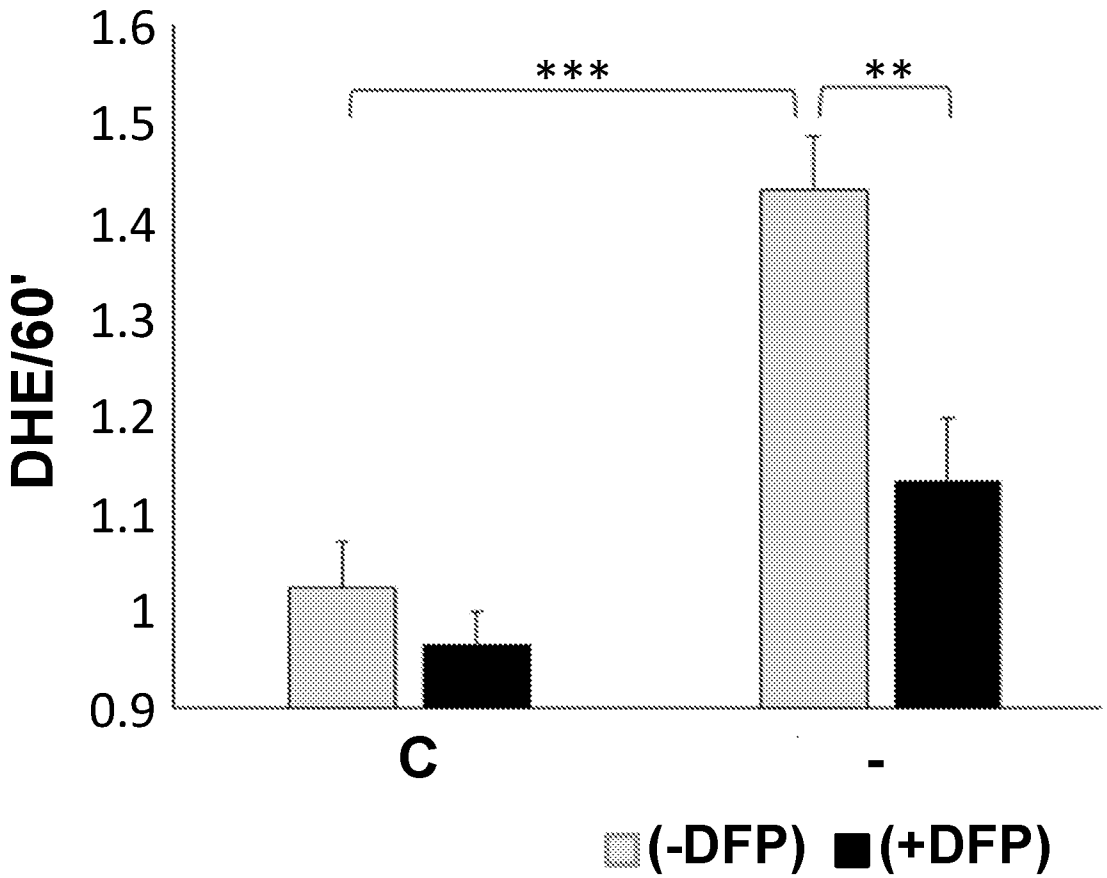


Fig. 3C

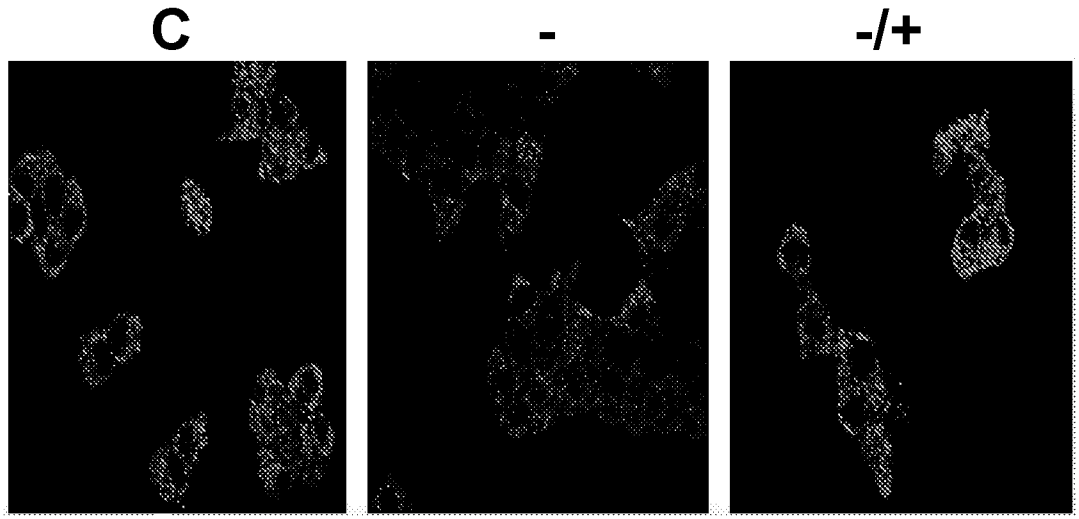


Fig. 4A

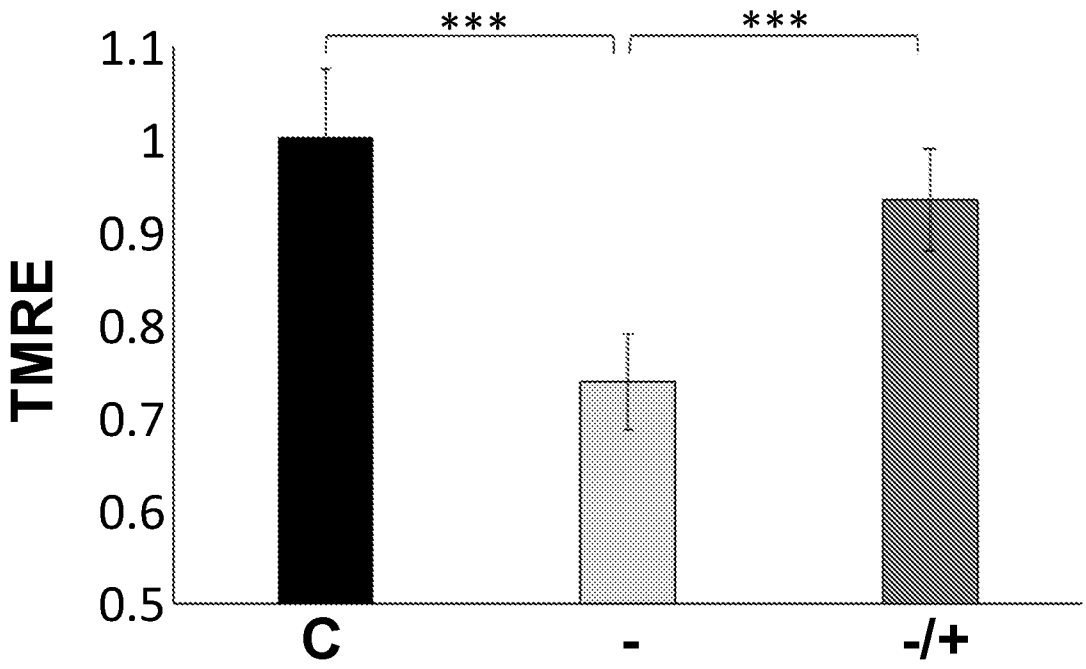


Fig. 4B

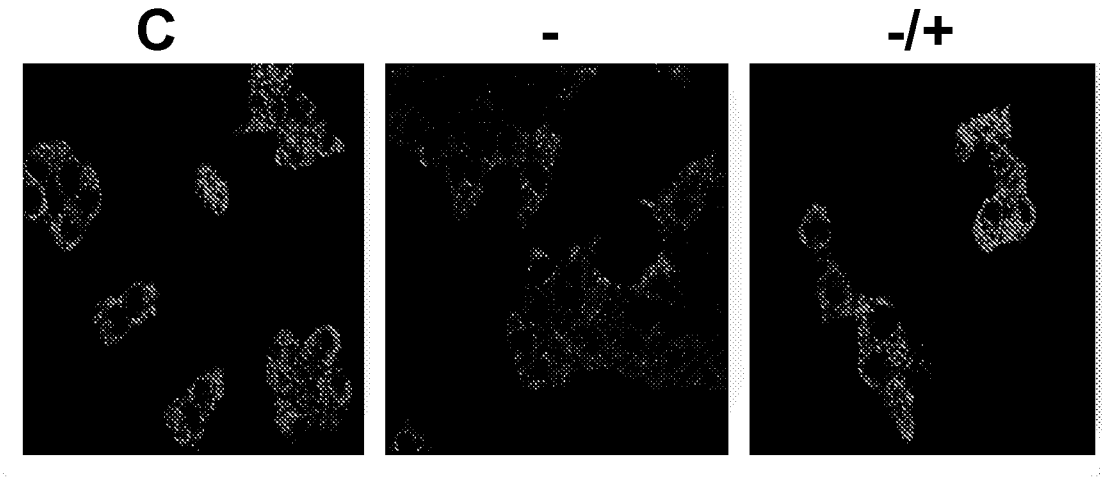


Fig. 5A

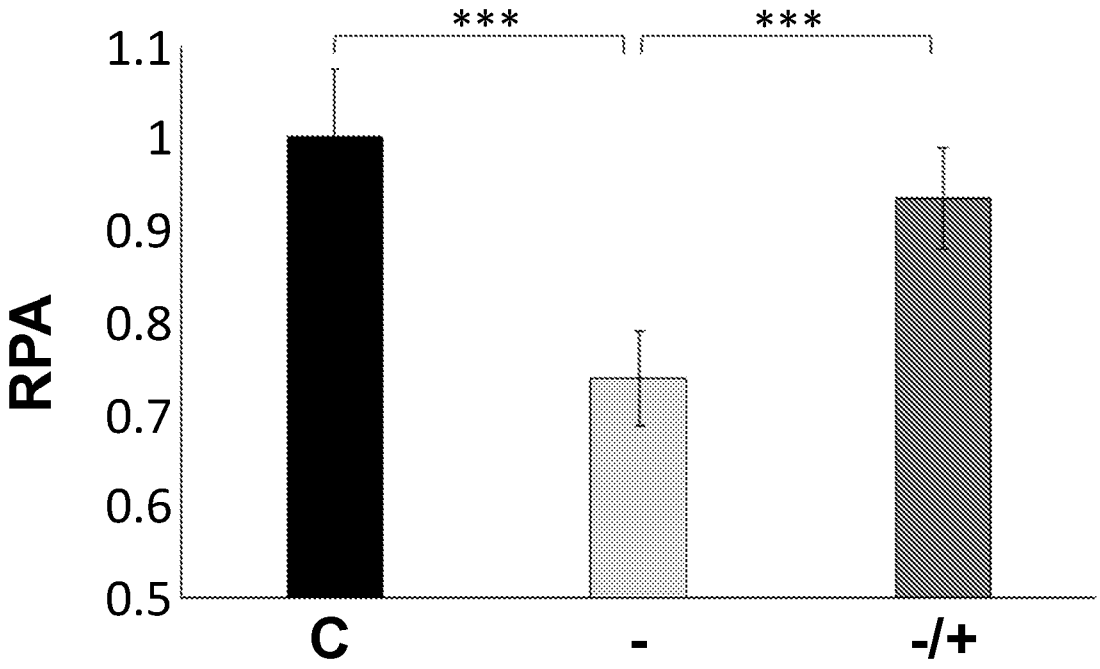


Fig. 5B

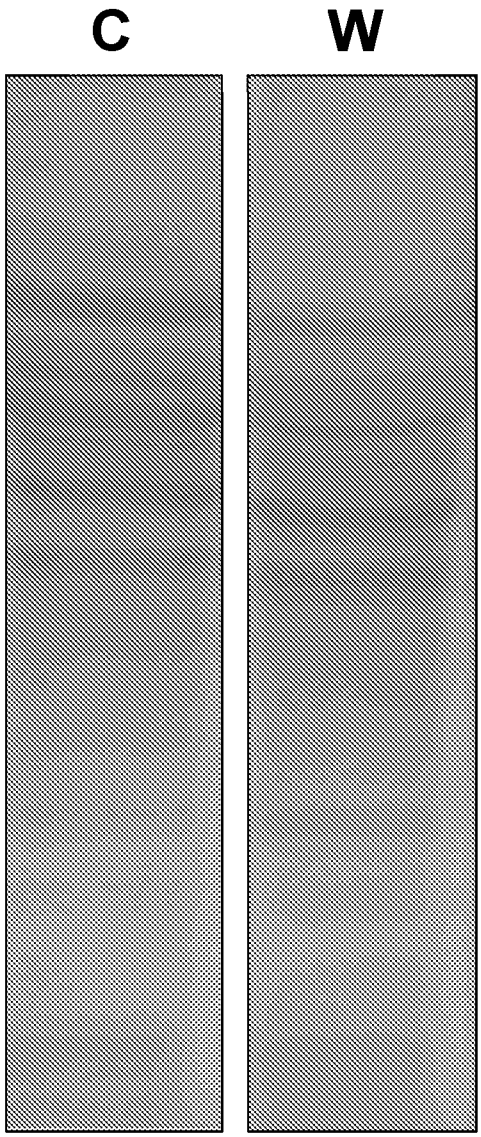


Fig. 6A

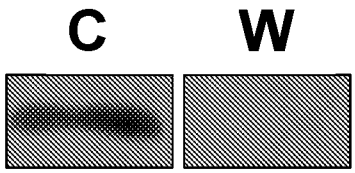


Fig. 6B

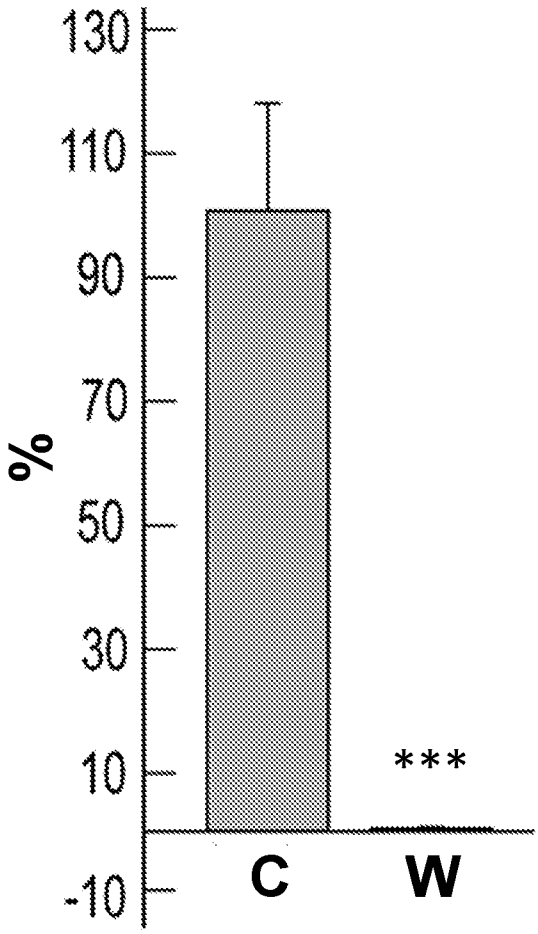


Fig. 6C

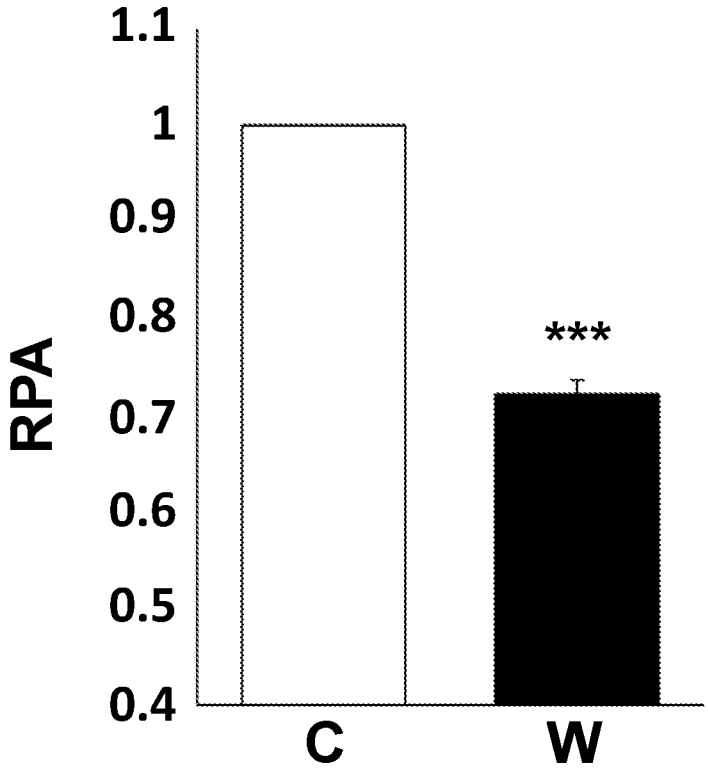


Fig. 7A

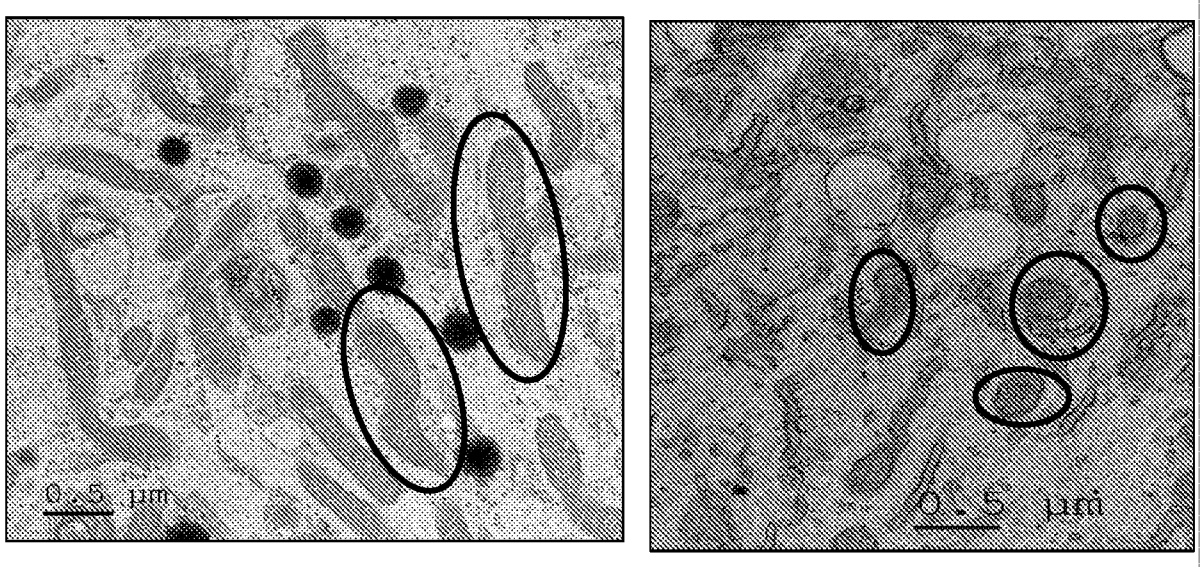


Fig. 7B

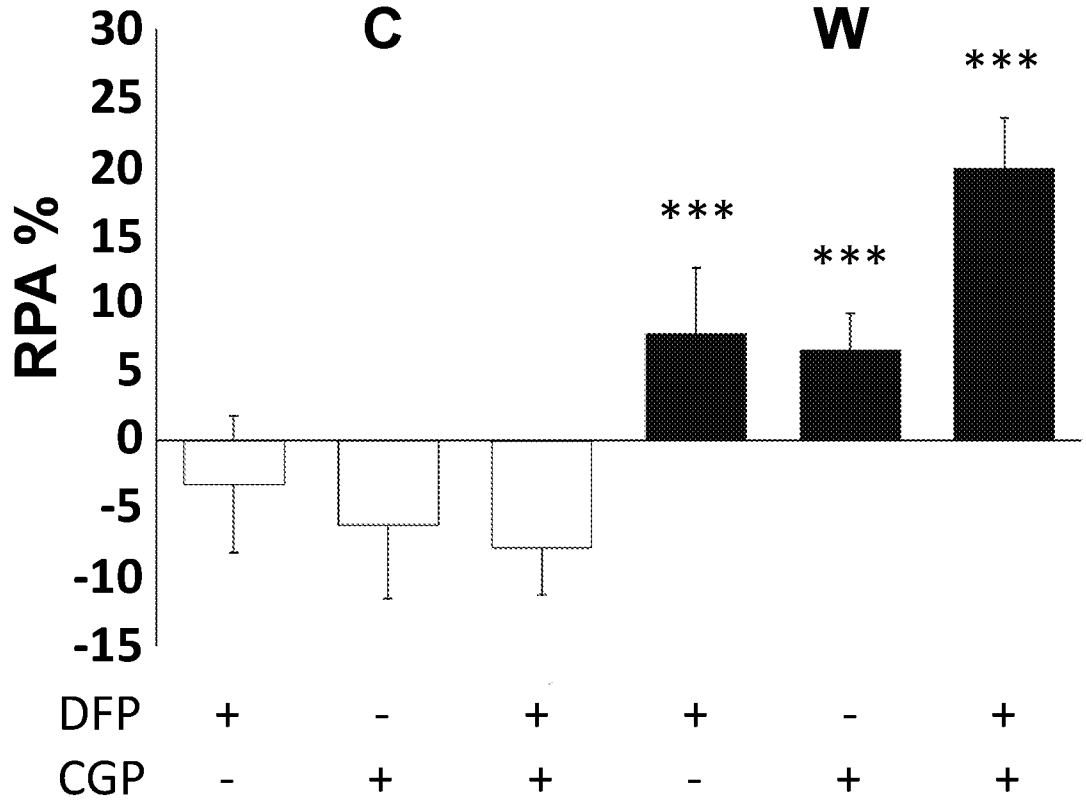

C
W
RPA %
30
25
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-10
-15
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DFP + - + + - +
CGP - + + - + +

Fig. 8A

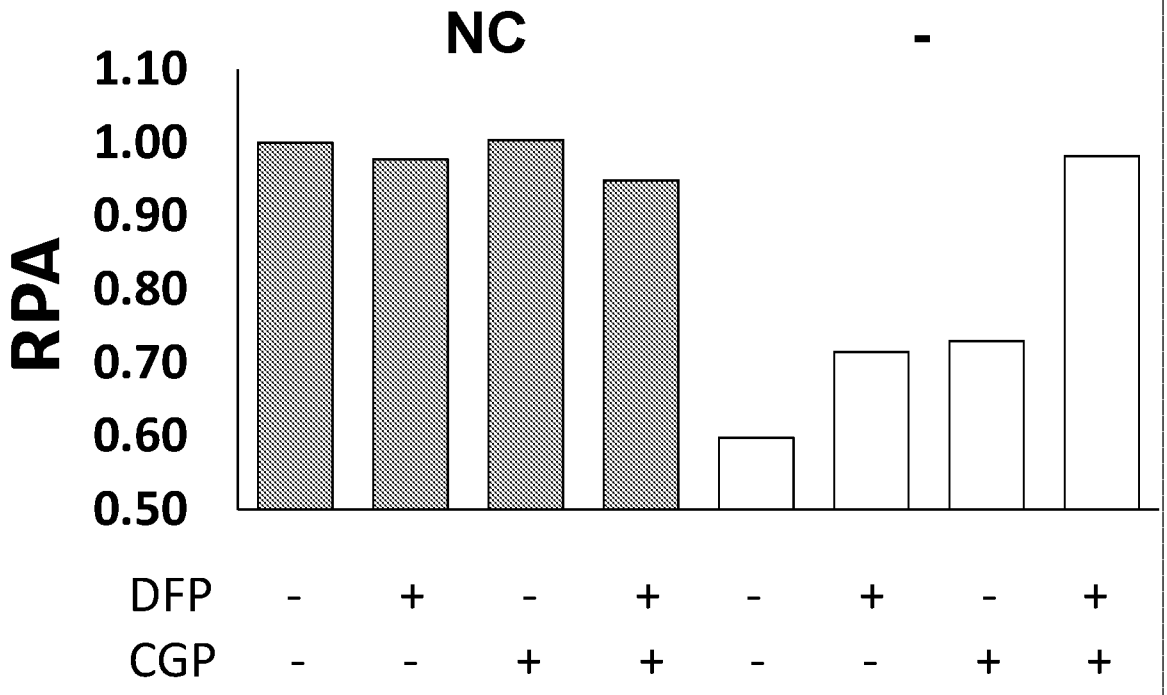

NC
-
RPA
1.10
1.00
0.90
0.80
0.70
0.60
0.50
DFP - + - + - + - +
CGP - - + + - - + +

Fig. 8B

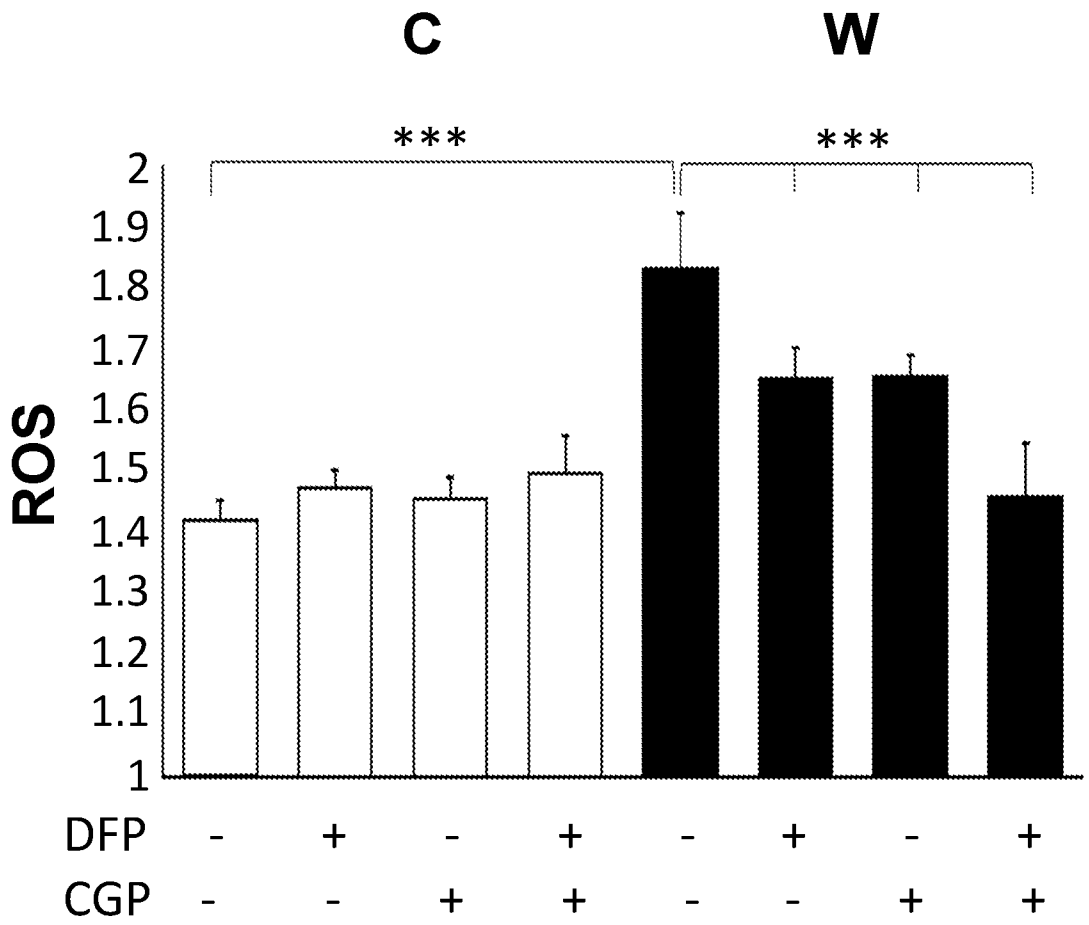


Fig. 8C

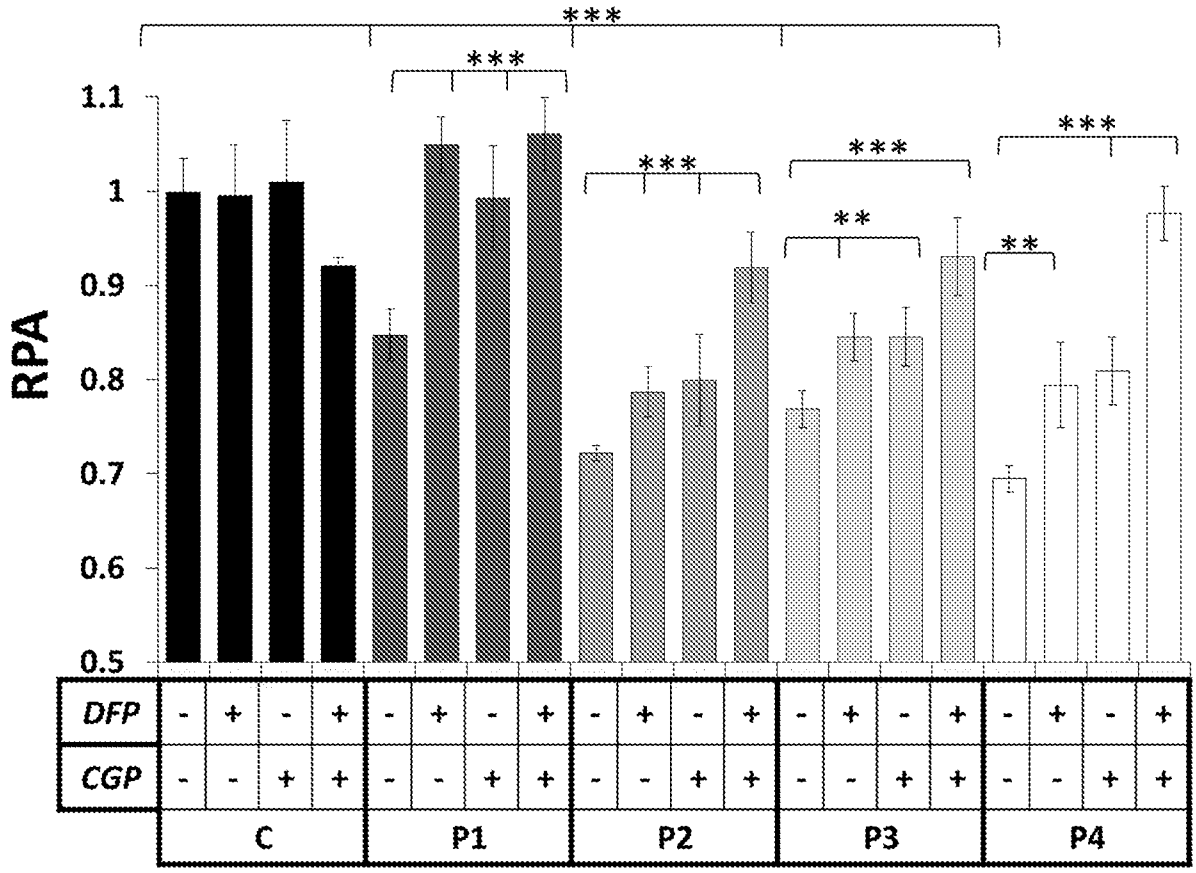

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RPA
1.1
1
0.9
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0.5
DFP
CGP
C
P1
P2
P3
P4

Fig. 9A

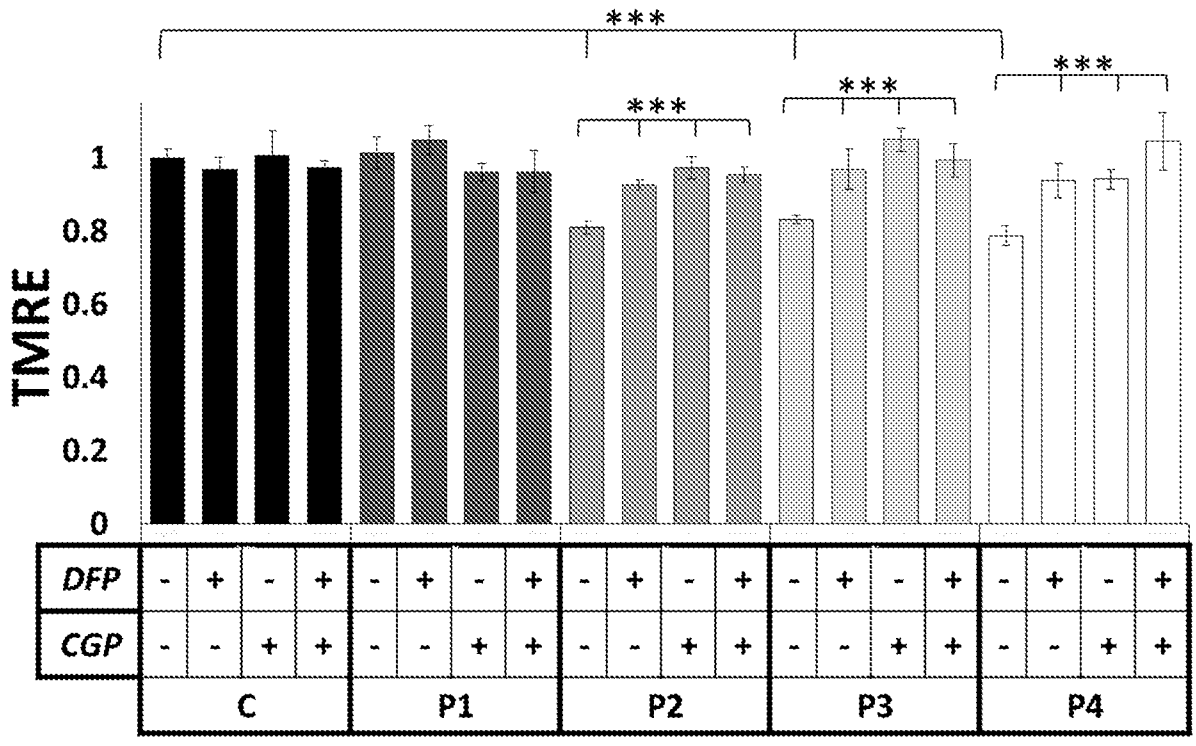

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TMRE
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0.2
0
DFP
CGP
C
P1
P2
P3
P4

Fig. 9B

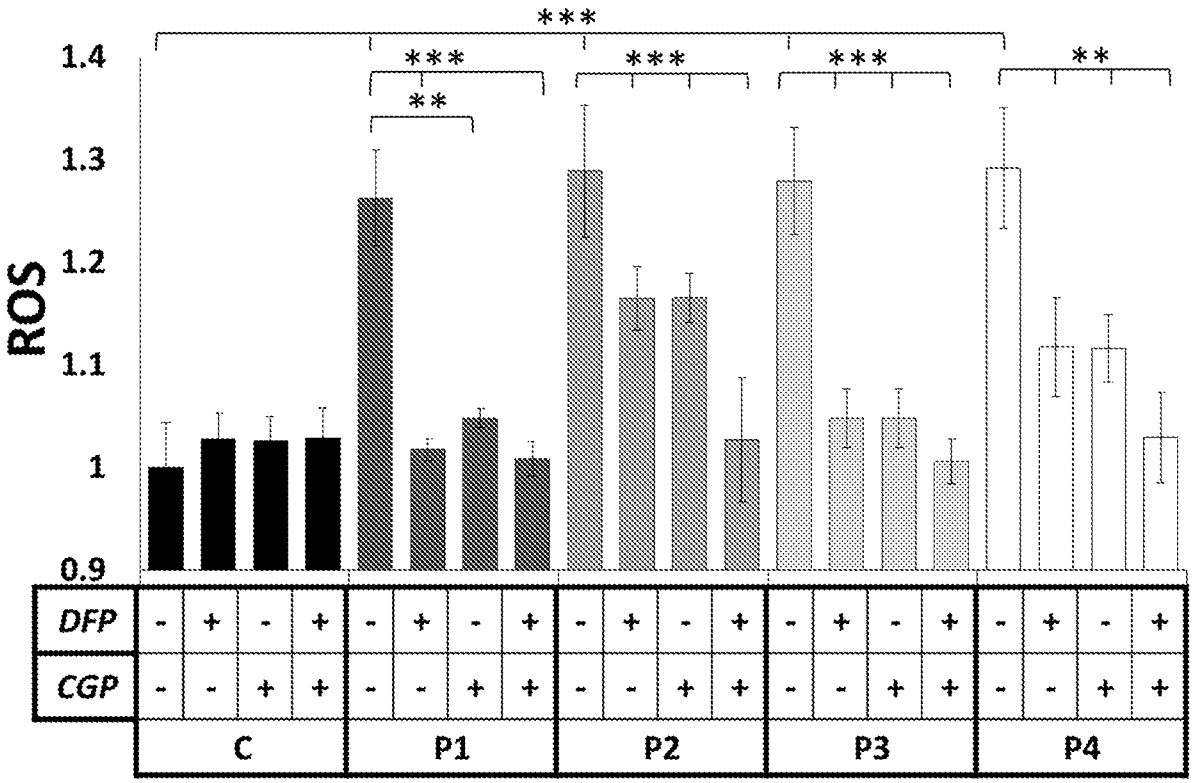


Fig. 9C

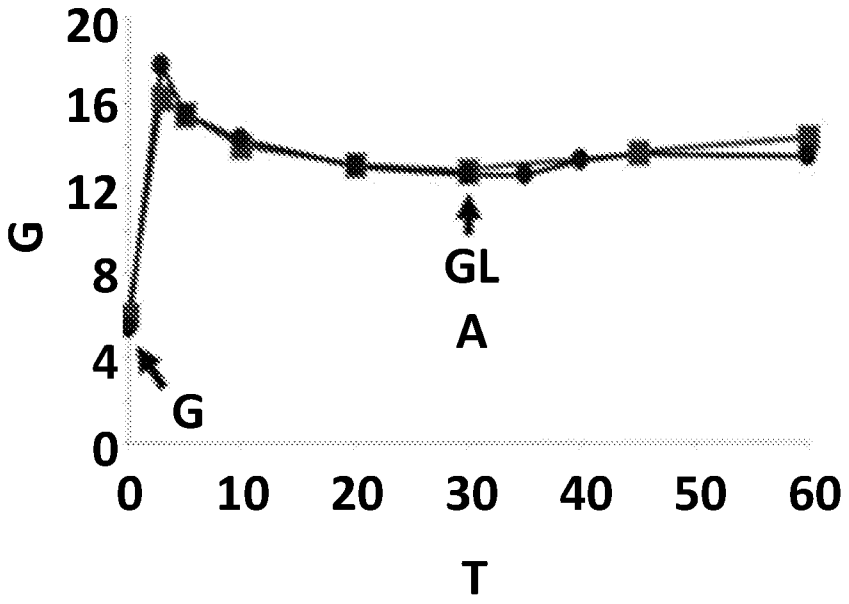


Fig. 10A

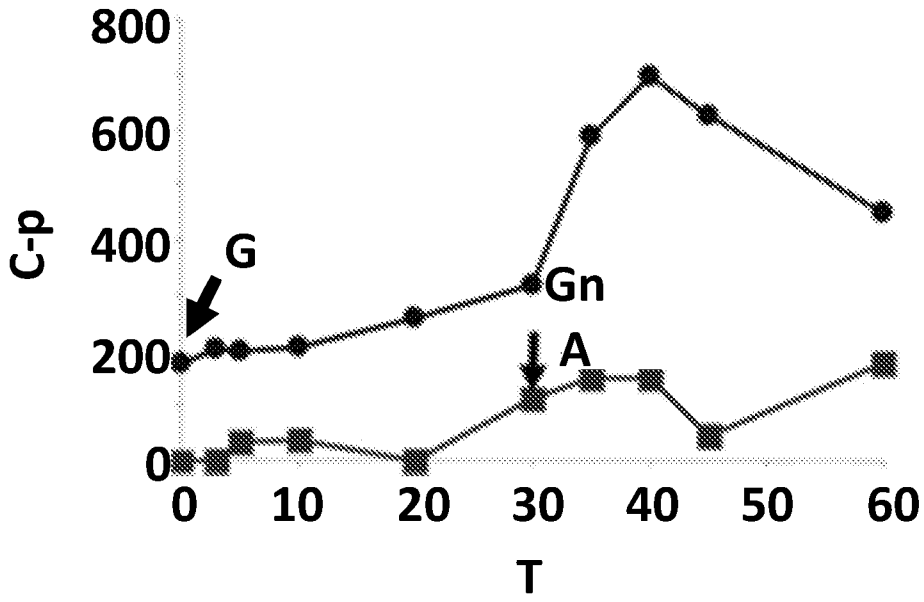


Fig. 10B

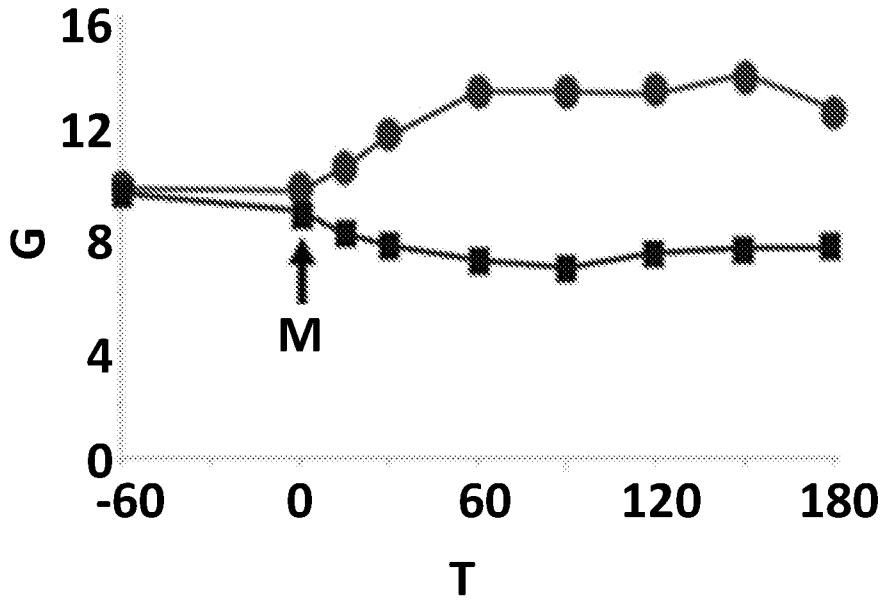


Fig. 10C

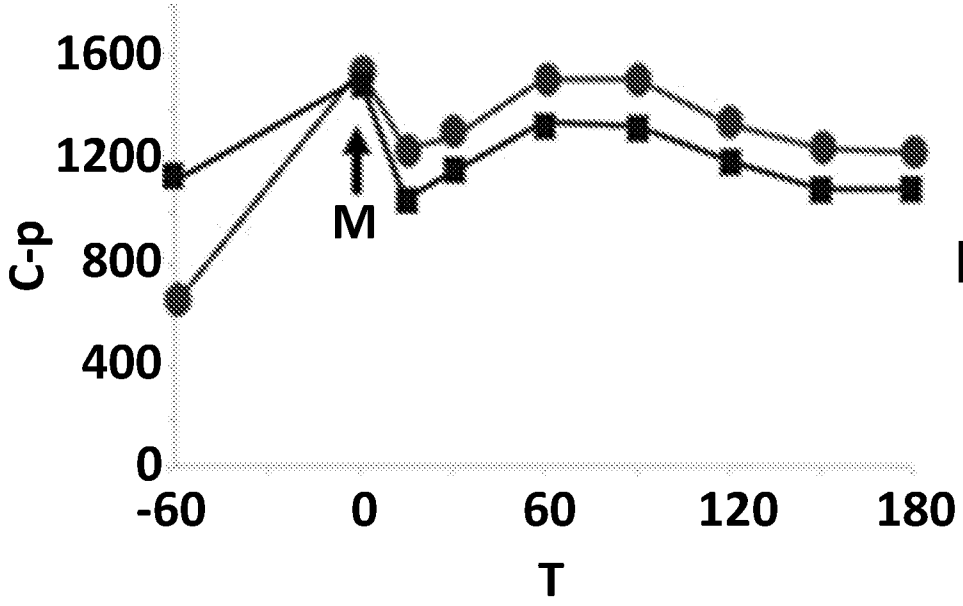


Fig. 10D

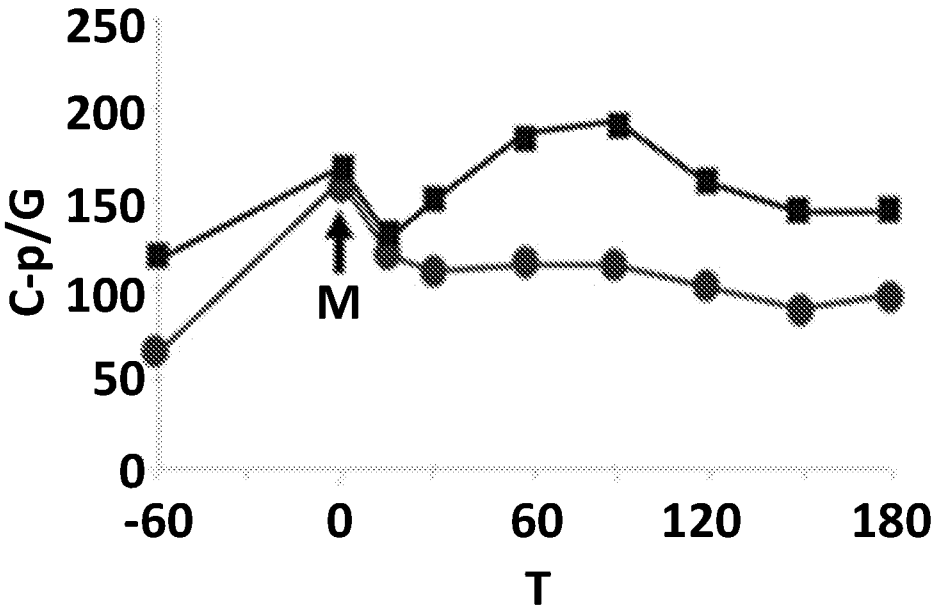


Fig. 10E

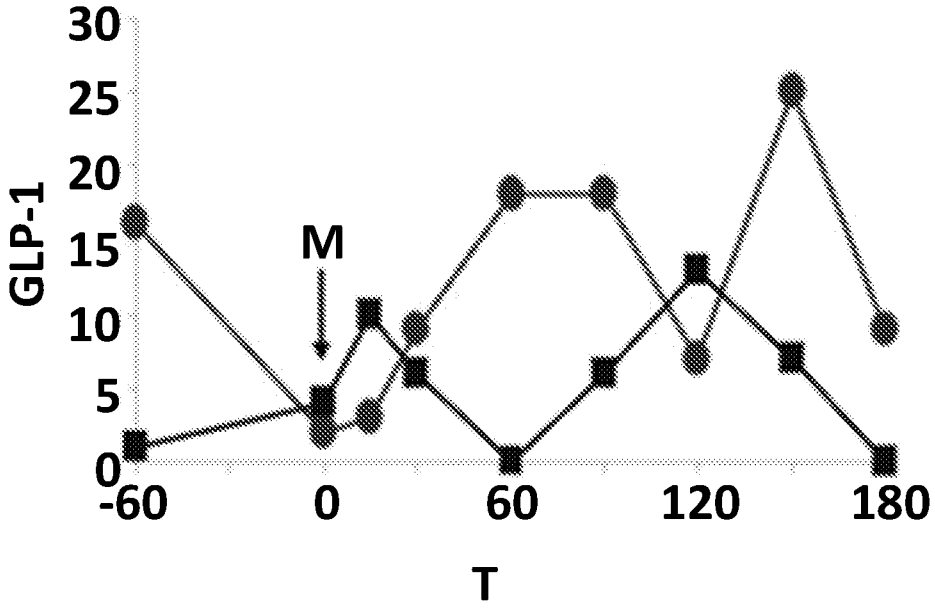


Fig. 10F

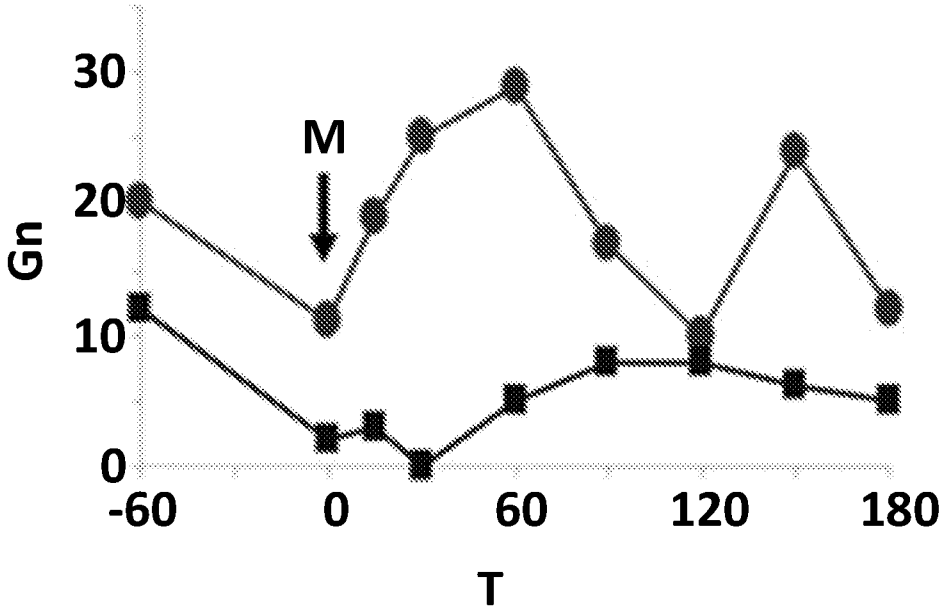


Fig. 10G

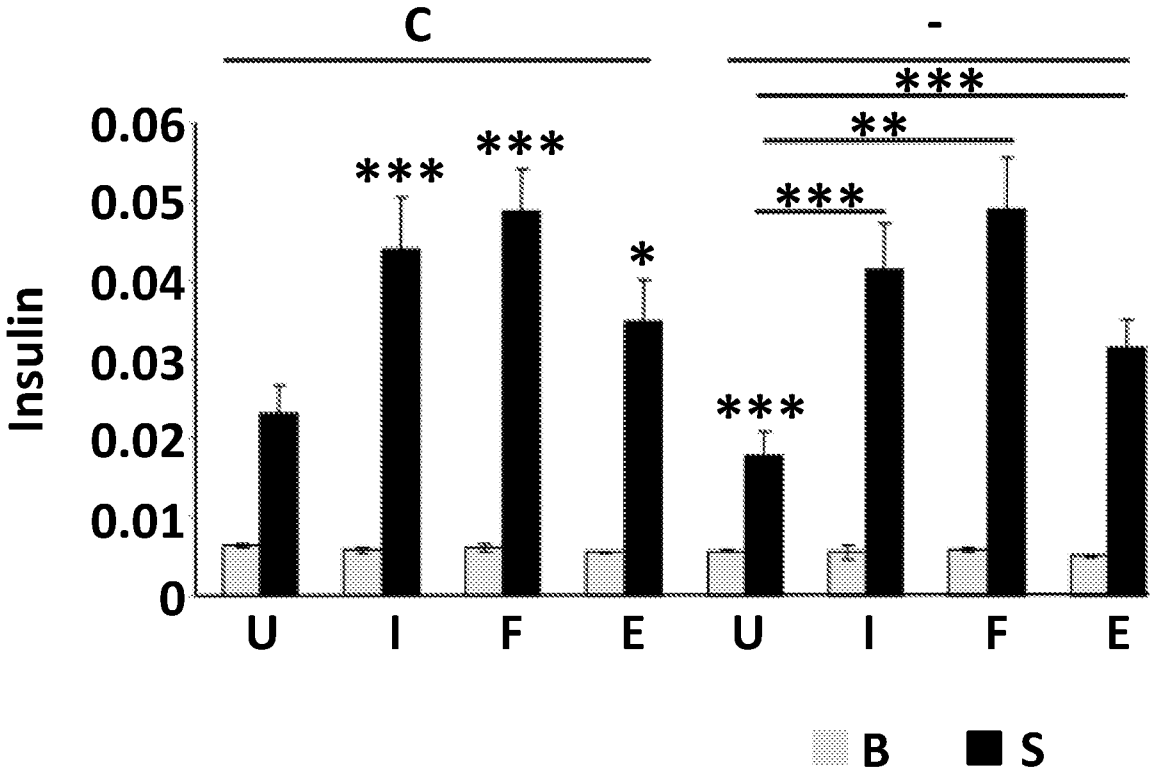


Fig. 11A

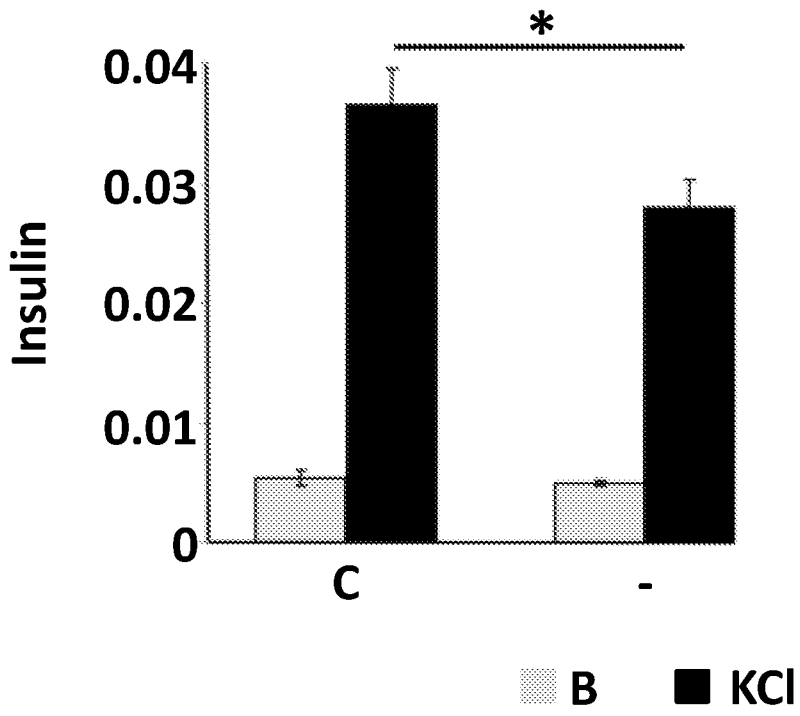


Fig. 11B

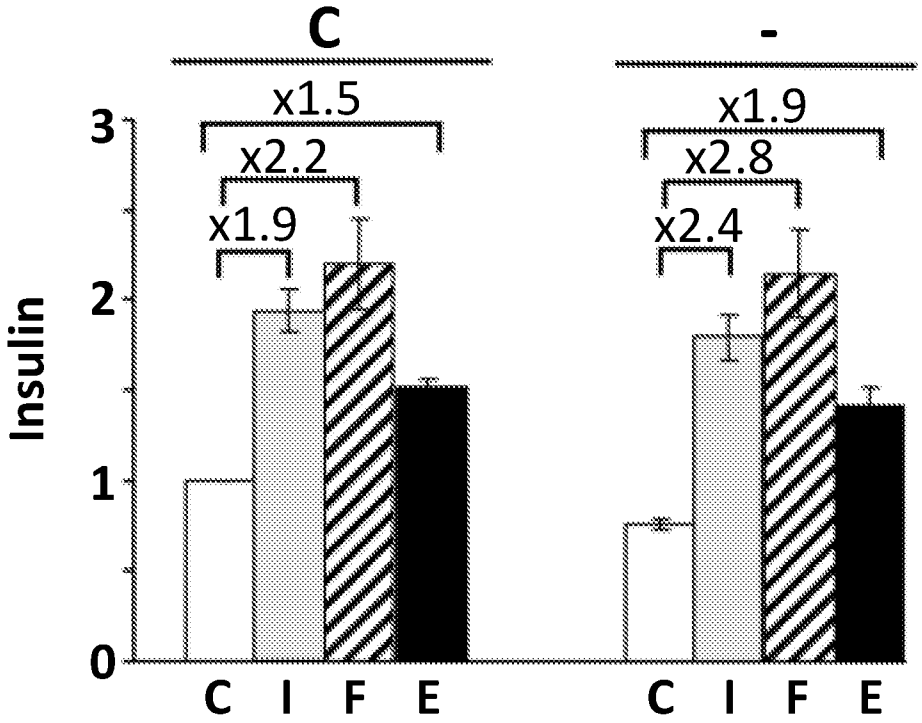


Fig. 11C

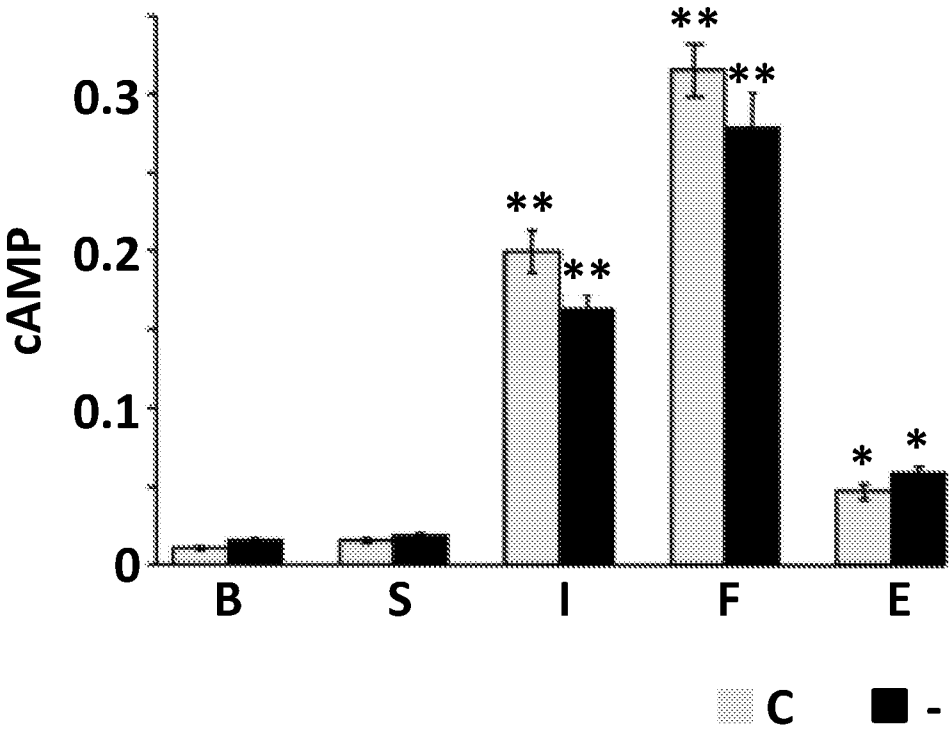


Fig. 11D

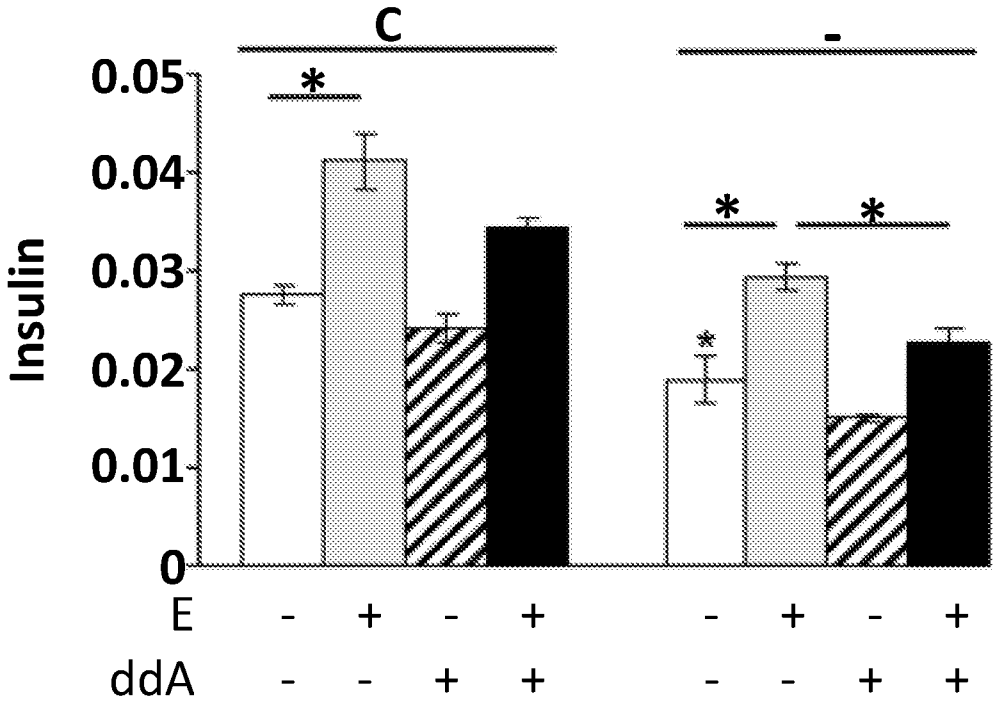


Fig. 11E

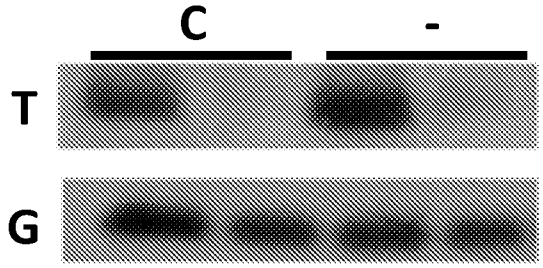


Fig. 11F

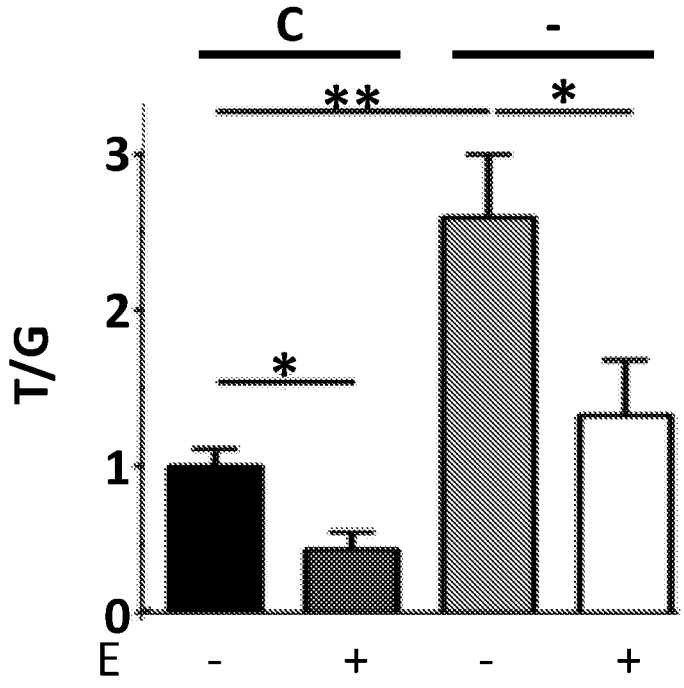


Fig. 11G

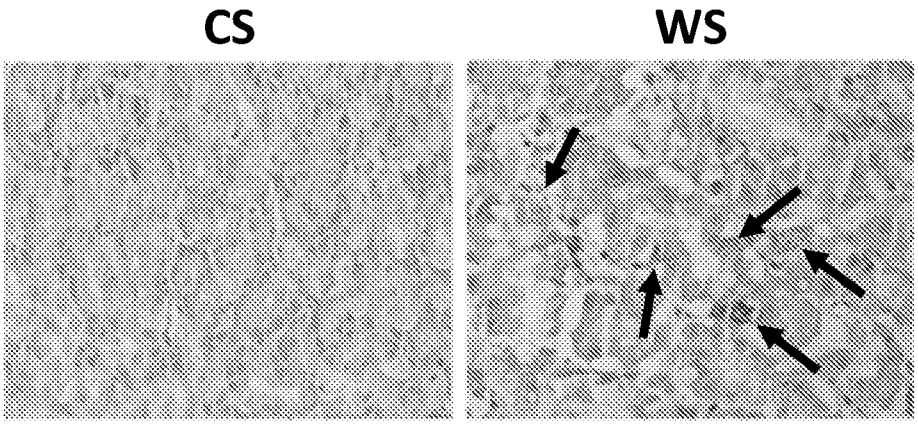


Fig. 12A

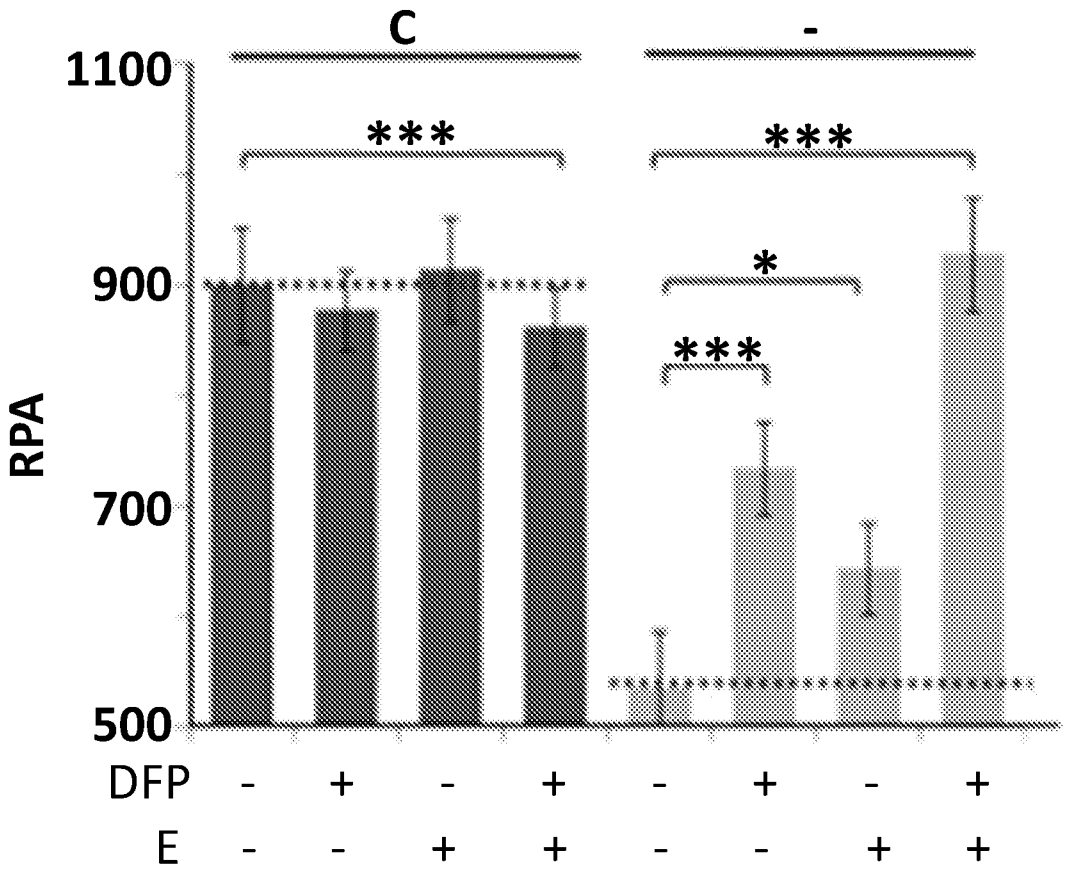


Fig. 12B

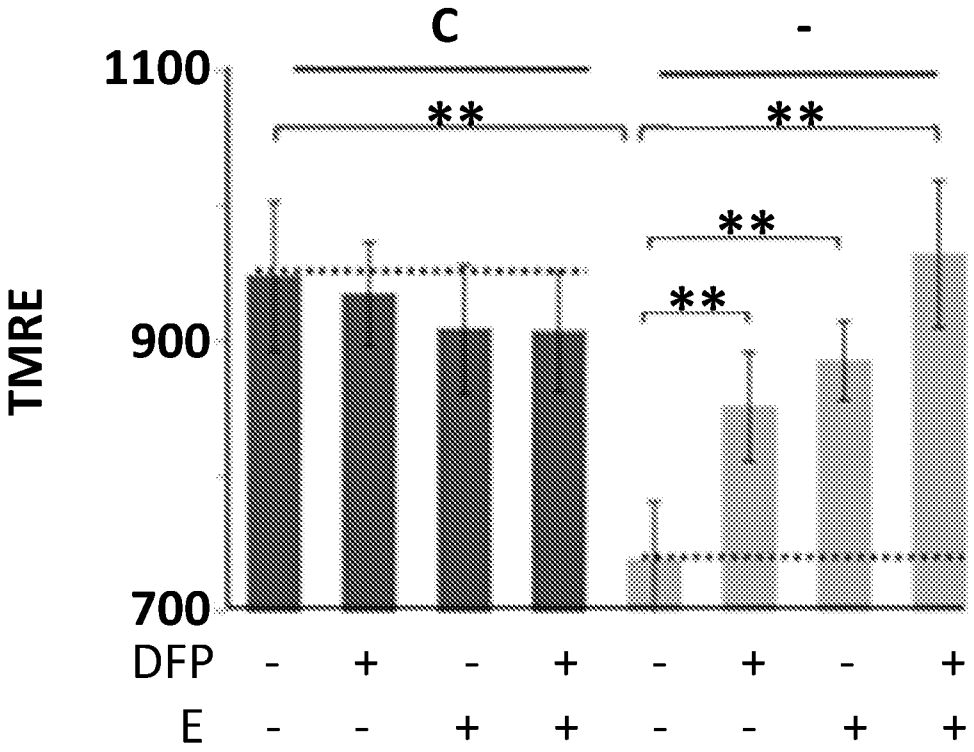


Fig. 12C

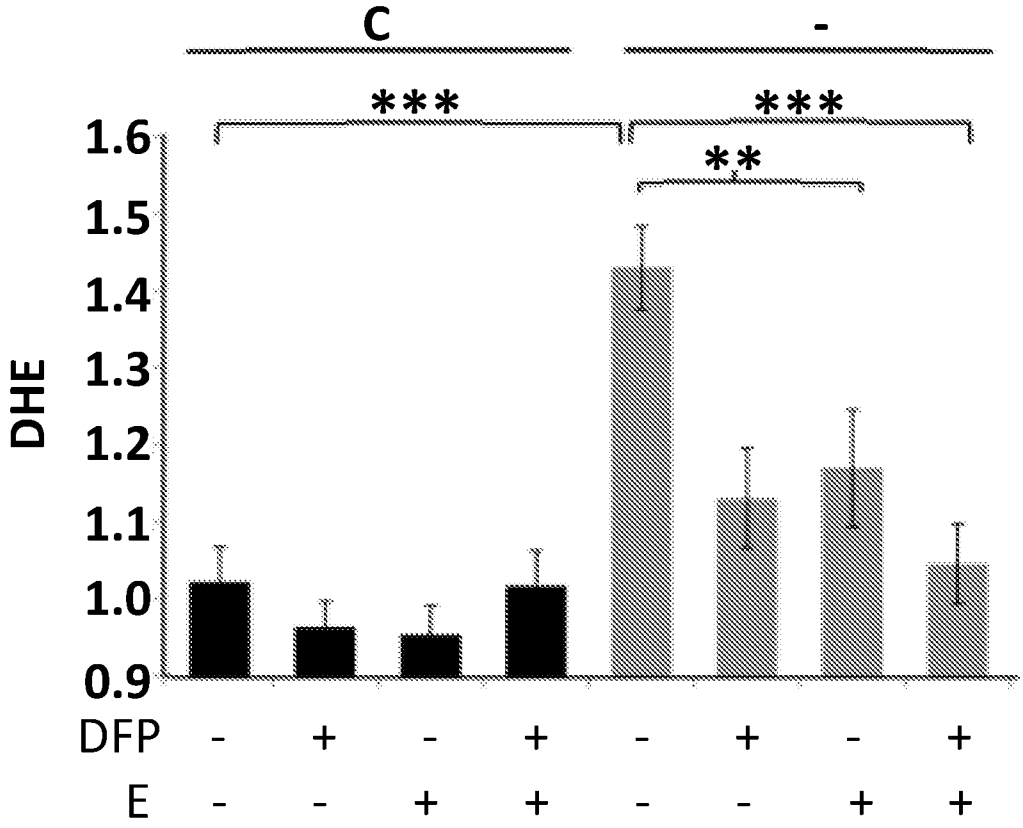


Fig. 12D

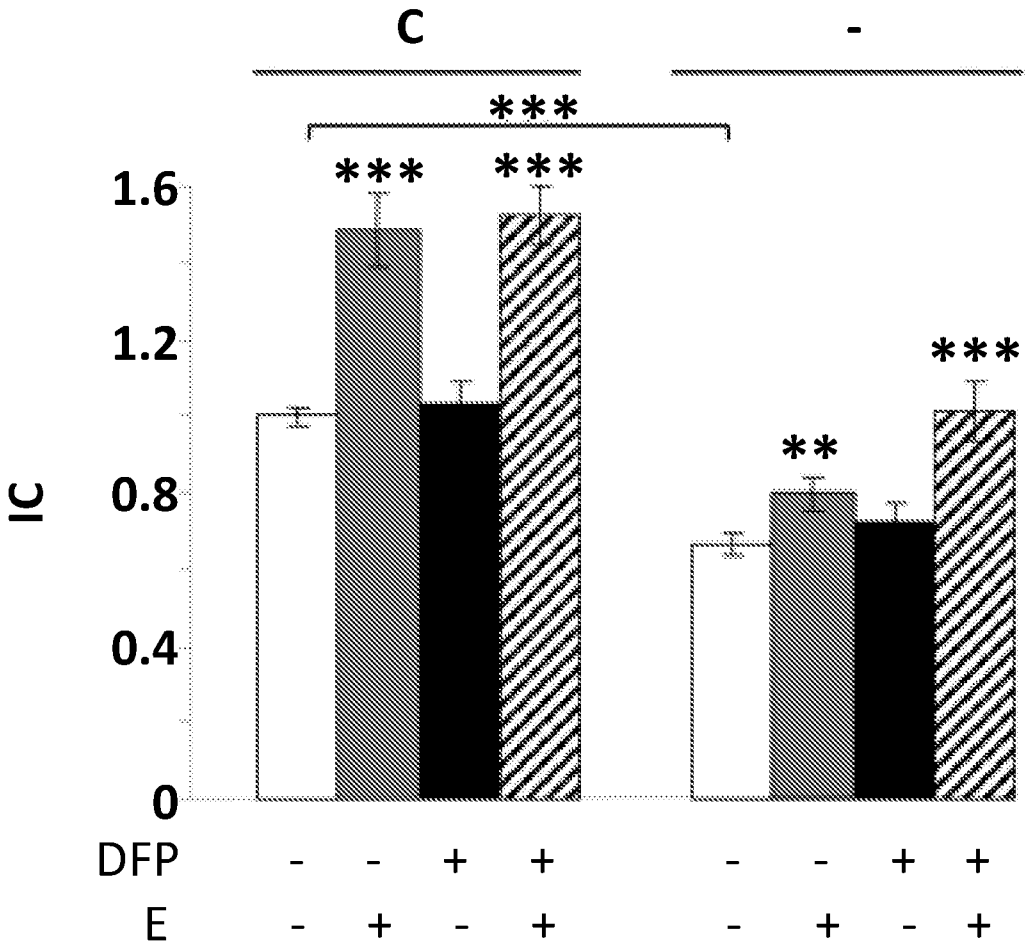


Fig. 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2017/050377

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K45/06 A61P43/00 A61P3/10 A61K38/22 A61K31/16
A61K31/198 A61K31/427 A61K31/4412 A61K31/4439
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/075910 A1 (PROTEMIX CORP LTD [NZ]; COOPER GARTH JAMES SMITH [NZ]; BAKER RICHARD J) 18 September 2003 (2003-09-18) claims 1,4,6,8,10-12, 41,43,64 figures 6,7 example 1 -----	1-11, 13-15, 19-24
X,P	DANIELPUR LIRON ET AL: "GLP-1-RA Corrects Mitochondrial Labile Iron Accumulation and Improves beta-Cell Function in Type 2 Wolfram Syndrome", JOURNAL OF CLINICAL ENDOCRINOLOGY & METABOLISM, ENDOCRINE SOCIETY, US, vol. 101, no. 10, 1 October 2016 (2016-10-01), pages 3592-3599, XP008185057, ISSN: 0021-972X, DOI: 10.1210/JC.2016-2240 [retrieved on 2016-07-26] abstract -----	1-11, 13-15, 19-24

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 July 2017	11/09/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Strack, Eberhard

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2017/050377

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

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

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

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

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

1-11, 19-24(completely); 13-15(partially)

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-11, 19-24(completely); 13-15(partially)

   An iron chelator for use in treating Wolfram syndrome 2 (T2-WFS); the combination of claim 4, the method of claim 21.

   ---

2. claims: 12, 16-18, 25(completely); 13-15(partially)

   A pharmaceutical combination comprising an iron chelator, an anti-oxidant and an anti-diabetic agent for use in treating Type I diabetes, Type II diabetes, or a disease demonstrating regional iron overload; the kit of claim 16, the method of claim 25.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IL2017/050377

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03075910 A1	18-09-2003	AU 2003222513 A1	22-09-2003
		WO 03075910 A1	18-09-2003