

MITOCHONDRIA-TARGETED ANTIOXIDANTS FOR USE IN THE TREATMENT OF
CELL BALLOONING-ASSOCIATED DISEASES, SUCH AS NON-ALCOHOLIC
FATTY LIVER DISEASE (NAFLD) AND NON-ALCOHOLIC STEATOHEPATITIS (NASH)

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BACKGROUND

Ballooning is a component and marker of severe cell injury under many pathological conditions (*Clinical Hepatology: Principles and Practice of Hepatobiliary Diseases*, Vol.1, Dancygier, H. (ed.), Springer, 2010, P. 207, *The Washington Manual of Surgical Pathology: Department of Pathology and Immunology*, by P. A. Humphrey, L.P. Dehner, J.D. Pfeifer (eds), Washington University School of Medicine, Lippincott Williams & Wilkins, 2008, P. 209; *Robbins & Cotran Pathologic Basis of Disease*, 9th Edition, V. Kumar, A. Abbas, J. Aster, Elsevier, 2014). Ballooned cells can undergo apoptosis and necrosis, and ballooning may cause organ disfunction, inflammation, and fibrosis, especially in the liver (Guy, C.D., et al. *Hepatology*, 2012, 55, 1711-172; Kleiner, D. E., et al. *Hepatology*, 2005. 41, 1313-1321; Lackner C, et al. *J Hepatol.*, 2008, 48(5), 821-828). Ballooned hepatocytes in steatohepatitis, both alcoholic and nonalcoholic in origin, as well as in other liver diseases including conditions of chronic cholestasis, increased copper storage, or ischemia–reperfusion injury of the liver allograft, demonstrate pronounced diminution or even loss of cytoplasmic expression of K8/18.

20 Ballooned or enlarged hepatocytes are also a feature found in a variety of other liver diseases, including acute hepatitis, neonatal giant cell hepatitis, autoimmune hepatitis and chronic hepatitis B. Ballooned cells frequently contain Mallory–Denk bodies (MDB), which are composed of K8/18 and the oxidative stress-induced 1/p62 sequestosome (p62), among other proteins (Lackner C, et al. *J. Hepatol.* 2008, 48, 821–828; Zatloukal K, et al. *J. Pathol.* 2004, 204, 367–376; Zatloukal K, et al. *Exp. Cell Res.* 2007, 313, 2033–2049). Recent studies demonstrate that apoptosis of hepatocytes associated with MDB can lead to an increase of CK-18 in blood plasma of patients suffering from liver diseases (Feldstain et al., *Am J Gastroenterol.* 2013 108:1526-31).

Diminution or loss of cytoplasmic K8/18 staining can be regarded as a marker for a distinguished form of hepatocellular damage that results in ballooning degeneration and eventually MDB formation, which preferentially occurs in liver diseases such as nonalcoholic steatohepatitis (NASH), alcoholic steatohepatitis, chronic cholestasis, increased copper storage, or ischemia–reperfusion injury

Many diseases such as renal failure, neurologic and intestinal infection or other injuries including toxic cardiac injuries, are conditions with ballooning formation (in kidneys, brain, intestine, heart respectively) (*Clinical Hepatology: Principles and Practice of Hepatobiliary Diseases*, Vol.1, Dancygier, H. (ed.), Springer, 2010, P. 207, *The Washington Manual of*

Surgical Pathology: Department of Pathology and Immunology, by P. A. Humphrey, L.P. Dehner, J.D. Pfeifer (eds), Washington University School of Medicine,; Lippincott Williams & Wilkins, 2008, P. 209; *Robbins & Cotran Pathologic Basis of Disease*, 9th Edition, V. Kumar, A. Abbas, J. Aster, Elsevier, 2014). The mechanism of ballooning under these condition is not precisely known, although it seems that ballooning formation has similar pathways in cells of different tissues and organs. Hence, there is a need for methods and compositions for treating and preventing ballooning.

SUMMARY

The present technology provides methods and compositions for treating and preventing cell ballooning and other diseases or medical conditions. The diseases or medical conditions are characterized by cell degeneration or cell death as a part of the disease pathogenesis. Cell ballooning involves cell swelling with loss of the usual shape of the cell and inclusions in the cytoplasm filled with fat, and sometimes with dark staining cytoplasmic structures. Ballooning is a condition associated with storage of excess fat in the liver. nutritional or biochemical imbalance, damage to mitochondria, ATP depletion, or virus infection. In the present technology, a mitochondrially targeted antioxidant compound is administered to a subject in need thereof, whereby excess fat is metabolized and burned.

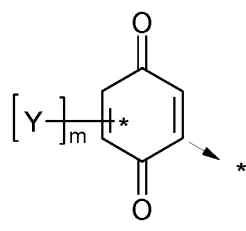
The present technology can be further summarized with the following list of features.

1. A method to aid in treating or preventing a cell ballooning-associated disease or medical condition, the method comprising the step of administering to a subject in need thereof therapeutically or prophylactically effective amount of a mitochondrially targeted antioxidant compound.
2. The method of feature 1, wherein the mitochondrially targeted antioxidant is an SkQ compound of Formula II below:



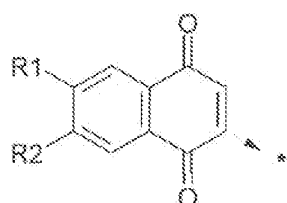
Formula II

wherein A is hydrogen or an effector moiety, which is an antioxidant optionally having a following structure:



and/or reduced forms thereof,

- wherein m is from 0 to 3; each Y is independently selected from the group consisting of: lower alkyl (1 to 6 carbons), lower alkoxy (1 to 6 carbons); or two adjacent Y groups,
 5 together with carbon atoms to which they are attached, form a following structure:



and/or reduced form thereof,

wherein R1 and R2 may be the same or different and are each independently lower alkyl (1 to 6 carbon atoms) or lower alkoxy (1 to 6 carbon atoms);

- 10 L is a linker group, comprising:

a) straight or branched hydrocarbon chain which can be optionally substituted by one or more substituents and optionally contains one or more double or triple bonds;

b) a natural isoprene chain;

n is the number of carbon atoms in the linker, which is an integer from 1 to 40; and

- 15 B is a mitochondria targeting group comprising a lipophilic cation;

and a pharmacologically acceptable anion;

and/or solvates, salts, isomers, or prodrugs thereof..

3. The method of feature 1 or feature 2, wherein the mitochondrially targeted antioxidant is selected from the group consisting of SkQ1, SkQ3, SkQ4, SkQ5, SkQR1, SkQBR1, SkQB1, and SkQBP1.
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4. The method of any of the preceding features, wherein the ballooning associated disease or medical condition comprises ballooning of hepatocytes in the subject.

5. The method of any of the preceding features, wherein the ballooning associated disease or medical condition comprises accumulation of cytosolic fat droplets in hepatocytes
 25 of the subject.

6. The method of any of the preceding features, wherein the ballooning associated disease or medical condition is selected from the group consisting of cytostatic drug and radiation injuries; kidney failure with various origin; traumatic, toxic, edematous,

neurodegenerative and ischemic brain damage; intestinal infections; toxic cardiac injuries; acute hepatitis; neonatal giant cell hepatitis; autoimmune hepatitis; chronic hepatitis B; chronic cholestasis; increased copper storage; ischemia–reperfusion injury fatty liver, alcoholic steatohepatitis, and non-alcoholic steatohepatitis (NASH).

5 7. The method of any of the preceding features, wherein the ballooning associated disease or medical condition comprises ballooning of hepatocytes, hepatic steatosis, hepatic inflammation, increase in liver weight, increase in hepatic content of fat, cholesterol, triglycerides, free fatty acids, or collagen, and/or increase in body weight.

8. The method of feature 7, wherein said administration decreases said ballooning of
10 hepatocytes, hepatic steatosis, hepatic inflammation, increase in liver weight, increase in hepatic content of fat, cholesterol, triglycerides, free fatty acids, or collagen, and/or increase in body weight.

9. The method of feature 8, wherein hepatic inflammation is decreased as measured by an inflammation or cell pathology marker, such as a marker selected from the group consisting
15 of CK-18, ALT, TNF-alpha, and IL-6.

10. The method of any of the preceding features, further comprising determining a level of a biomarker and diagnosing or prognosing the disease or medical condition based on said level.

11. The method of feature 10, wherein said determining comprises performing a liver
20 biopsy or quantifying a blood-born biomarker.

12. The method of any of the preceding features, wherein said administering reduces the likelihood of said cell ballooning-associated disease occurring or progressing in the subject.

13. The method of any of the preceding features, wherein said administering reduces ballooning of hepatocytes in the subject.

25 14. The method of any of the preceding features, wherein said administering reduces hepatic fibrosis in the subject.

15. The method of any of the preceding features, wherein the subject suffers from obesity, diabetes, or alcohol abuse and has, or is at risk of developing, a ballooning associated disease or medical condition.

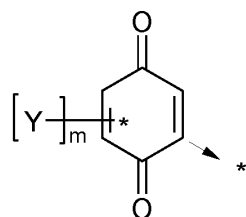
30 16. A method for altering cellular metabolism and/or storage of fat, the method comprising the step of administering to a subject in need thereof a mitochondrially targeted antioxidant compound, whereby cellular metabolism and/or storage of fat is altered in the subject.

17. The method of feature 16, wherein the mitochondrially targeted antioxidant is an SkQ compound of Formula II below:



Formula II

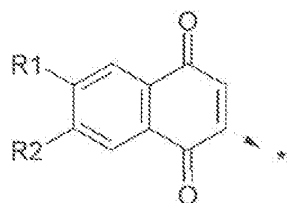
wherein A is hydrogen or an effector moiety, which is an antioxidant optionally having a following structure:



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and/or reduced forms thereof,

wherein m is from 0 to 3; each Y is independently selected from the group consisting of: lower alkyl (1 to 6 carbons), lower alkoxy (1 to 6 carbons); or two adjacent Y groups, together with carbon atoms to which they are attached, form a following structure:



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and/or reduced form thereof,

wherein R1 and R2 may be the same or different and are each independently lower alkyl (1 to 6 carbon atoms) or lower alkoxy (1 to 6 carbon atoms);

L is a linker group, comprising:

15 a) straight or branched hydrocarbon chain which can be optionally substituted by one or more substituents and optionally contains one or more double or triple bonds;

b) a natural isoprene chain;

n is the number of carbon atoms in the linker, which is an integer from 1 to 40; and

B is a mitochondria targeting group comprising a lipophilic cation;

20 and a pharmacologically acceptable anion;

and/or solvates, salts, isomers, or prodrugs thereof.

18. The method of feature 16 or 17, wherein the mitochondrially targeted antioxidant is selected from the group consisting of SkQ1, SkQ3, SkQ4, SkQ5, SkQR1, SkQBR1, SkQB1, and SkQBP1.

25 19. The method of any of features 16-18, wherein triglyceride hydrolysis and/or fatty acid

oxidation are increased and/or storage of triglycerides and/or free fatty acids is reduced in the subject.

20. The method of any of features 16-19, wherein the subject has hepatocyte ballooning and/or accumulation of cytosolic fat droplets in hepatocytes.

5 21. The method of feature 20, wherein the subject has hepatic steatosis, hepatic inflammation, increase in liver weight, increase in hepatic content of cholesterol, triglycerides, free fatty acids, or collagen, and/or increase in body weight.

22. The method of any of features 16-21, wherein the ballooning associated disease or medical condition is selected from the group consisting of cytostatic drug and radiation injuries; kidney failure with various origin; traumatic, toxic, edematous, neurodegenerative and
10 ischemic brain damage; intestinal infections; toxic cardiac injuries; acute hepatitis; neonatal giant cell hepatitis; autoimmune hepatitis; chronic hepatitis B; chronic cholestasis; increased copper storage; ischemia–reperfusion injury, fatty liver, alcoholic steatohepatitis, and non-alcoholic steatohepatitis (NASH).

15 23. The method of any of features 20-22, wherein said administration also decreases said ballooning of hepatocytes, hepatic steatosis, hepatic inflammation, increase in liver weight, increase in hepatic content of cholesterol, triglycerides, or free fatty acids, and/or increase in body weight.

24. A method for reducing body weight, or preventing an increase in body weight, of a
20 mammalian subject, the method comprising the step of administering to the subject a mitochondrially targeted antioxidant compound, whereby body weight of the subject is reduced or maintained.

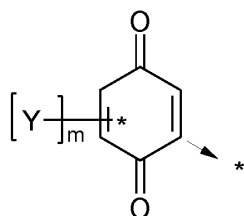
25. The method of feature 24, wherein the mitochondrially targeted antioxidant is an SkQ compound of Formula II below:



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Formula II

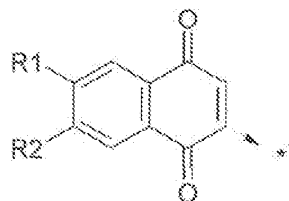
wherein A is hydrogen or an effector moiety, which is an antioxidant optionally having a following structure:



30

and/or reduced forms thereof,

wherein m is from 0 to 3; each Y is independently selected from the group consisting of: lower alkyl (1 to 6 carbons), lower alkoxy (1 to 6 carbons); or two adjacent Y groups, together with carbon atoms to which they are attached, form a following structure:



- 5 and/or reduced form thereof,
 wherein R1 and R2 may be the same or different and are each independently lower alkyl (1 to 6 carbon atoms) or lower alkoxy (1 to 6 carbon atoms);
 L is a linker group, comprising:
 a) straight or branched hydrocarbon chain which can be optionally substituted by one
 10 or more substituents and optionally contains one or more double or triple bonds;
 b) a natural isoprene chain;
 n is the number of carbon atoms in the linker, which is an integer from 1 to 40; and
 B is a mitochondria targeting group comprising a lipophilic cation;
 and a pharmacologically acceptable anion;
 15 and/or solvates, salts, isomers, or prodrugs thereof.
26. The method of feature 24 or 25, wherein the mitochondrially targeted antioxidant is selected from the group consisting of SkQ1, SkQ3, SkQ4, SkQ5, SkQR1, SkQBR1, SkQB1, and SkQBP1.
27. The method of any of features 24-26, wherein the mitochondrially targeted antioxidant
 20 is administered orally or by subcutaneous injection.
28. The method of any of features 24-27, whereby the subject's body weight is decreased by at least 5, 10, 15, or 20%.

BRIEF DESCRIPTION OF DRAWINGS

- 25 Fig. 1A is a graph of NAFLD activity score as a function of the indicated drug administration. Fig. 1B shows the amount of steatosis as a function of the indicated drug administration. Fig. 1C shows the amount of inflammation as a function of the indicated drug administration. Fig. 1D shows the amount of ballooning as a function of the indicated drug administration.
- 30 Fig. 2 shows the percentage of cells showing positive staining with Sirius Red as a function of the indicated drug administration.

Fig. 3 shows the percent positive for liver F4/80 as a function of the indicated drug administration.

Fig 4A shows the NAS score and Fig. 4B shows the ballooning score as a function of the indicated drug administration.

5 Fig. 5 shows the extent of liver fibrosis for mice treated with the indicated drug administration.

Figs. 6A-6C show the body weight (6A), fat (6B), and lean weight (6C) of mice given the indicated treatment.

Fig. 7 shows the liver weight of mice given the indicated treatment.

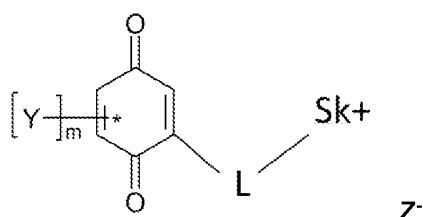
10 Fig. 8 shows the liver triglyceride level of mice given the indicated treatment.

DETAILED DESCRIPTION

The present technology provides methods and compositions to aid in treating and
15 preventing diseases or medical conditions that include cell ballooning, which may lead to cell degeneration or cell death as a part of the disease pathogenesis. As used herein, "ballooning" refers to a cellular condition, sometimes seen in hepatocytes or other cells, characterized by enlargement or swelling of the cell, such as to 1.5 to 2 times its normal diameter, with loss of the usual shape of the cell, with a pale staining cytoplasm filled with variably sized cytoplasmic
20 vacuoles filled with fat, and sometimes with dark staining cytoplasmic structures. Ballooning can be associated with storage of excess fat, such as in the liver. Ballooning also can be associated with nutritional or biochemical imbalance, damage to mitochondria, ATP depletion, or virus infection. See, e.g., Miller, M.A., and Zachary, J.F., Pathologic Basis of Veterinary Disease (2017) (doi: 10.1016/B978-0-323-35775-3.00001-1).

25 The list of diseases and medical conditions where treatment or prevention of cell ballooning would be useful includes (but is not limited to): cytostatic drug and radiation injuries, kidney failure with various origin, traumatic, toxic, edematous, neurodegenerative and ischemic brain damage, intestinal infections, toxic cardiac injuries, acute hepatitis, neonatal giant cell hepatitis, autoimmune hepatitis and chronic hepatitis B, chronic cholestasis,
30 increased copper storage, ischemia–reperfusion injury, fatty liver, alcoholic steatohepatitis, and non-alcoholic steatohepatitis. Said pathologies are termed hereafter as ballooning associated diseases.

An aspect of the present technology is a method to aid in treatment and/or prevention of cell ballooning-associated diseases. The method includes administering to a patient in
35 need thereof a therapeutically or prophylactically effective amount of a mitochondrially targeted antioxidant (MTA) of the following formula:



Formula I

wherein:

Y is lower alkyl or lower alkoxy,

5 m is 0, 1, or 2

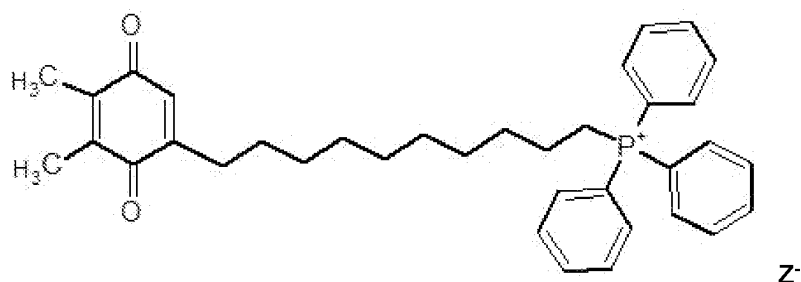
L is a linker group comprising straight or branched hydrocarbon chain of 1-40 carbon atoms, which can be optionally substituted by one or more substituents and optionally contains one or more double or triple bonds,

Sk+ is a lipophilic cation, and

10 Z- is a pharmacologically acceptable anion.

Any MTA of the above formula is included under the term "SkQ" or "SkQ compound" as used herein.

An example of SkQ is the following compound, referred to herein as "SkQ1":



15 wherein Z is pharmaceutically acceptable anion such as bromide, chloride, sulfate, mesylate etc.

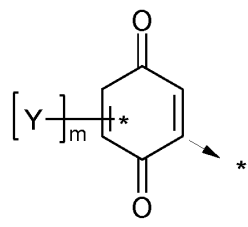
The present technology also includes methods of manufacturing or formulating a pharmaceutical product, such as a pharmaceutical dosage form, comprising a mitochondrially targeted antioxidant, for treatment or prophylaxis of ballooning associated diseases.

20 More generally, an SkQ-like compound can be a compound of the following general formula:



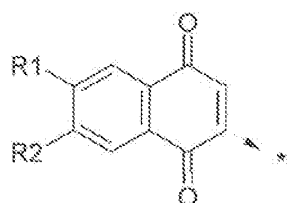
Formula II

wherein A is hydrogen or an effector moiety, which is an antioxidant optionally having a following structure:



and/or reduced forms thereof,

- 5 wherein m is from 0 to 3; each Y is independently selected from the group consisting of: lower alkyl (1 to 6 carbons), lower alkoxy (1 to 6 carbons); or two adjacent Y groups, together with carbon atoms to which they are attached, form a following structure:



and/or reduced form thereof,

- 10 wherein R1 and R2 may be the same or different and are each independently lower alkyl (1 to 6 carbon atoms) or lower alkoxy (1 to 6 carbon atoms);

L is a linker group, comprising:

a) straight or branched hydrocarbon chain which can be optionally substituted by one or more substituents and optionally contains one or more double or triple bonds;

- 15 b) a natural isoprene chain;

n is the number of carbon atoms in the linker, which is an integer from 1 to 40; and

B is a mitochondria targeting group comprising a lipophilic cation;

and a pharmacologically acceptable anion;

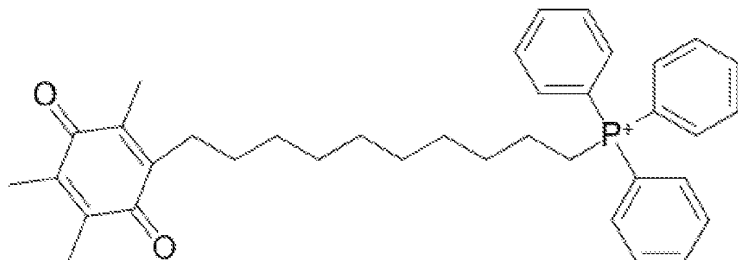
and/or solvates, salts, isomers, or prodrugs thereof.

- 20 As used herein, benzalkonium refers to a mixture of or a single-length quaternary ammonium compound represented by the general formula II wherein the alkyl group can have various even-numbered alkyl chain lengths. The structure represented in formula II is shown as a benzalkonium chloride salt, but any pharmaceutically acceptable anion can be substituted for chloride as is known in the art.

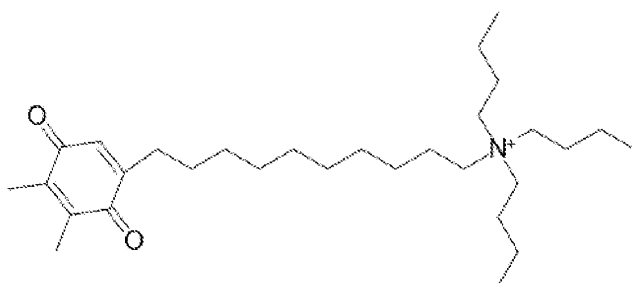
- 25 As used herein, a lower alkyl or lower alkoxy can be substituted or unsubstituted and can comprise 1 to 6 carbon atoms on the alkyl or alkoxy backbone, which can be straight-chain or branched, and all endpoints are subsumed in the range from 1 to 6.

Further non-limiting examples of SkQ compounds are the following:

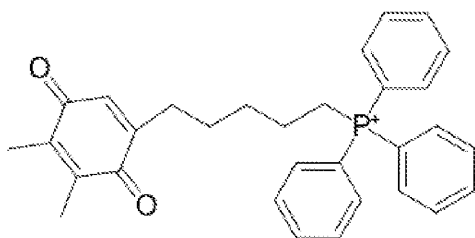
SkQ3



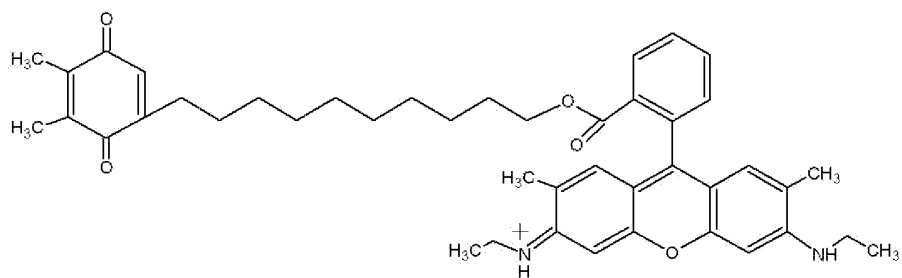
5 SkQ4



SkQ5

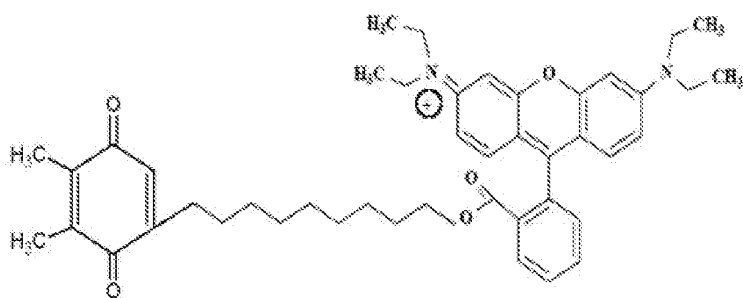


SkQR1

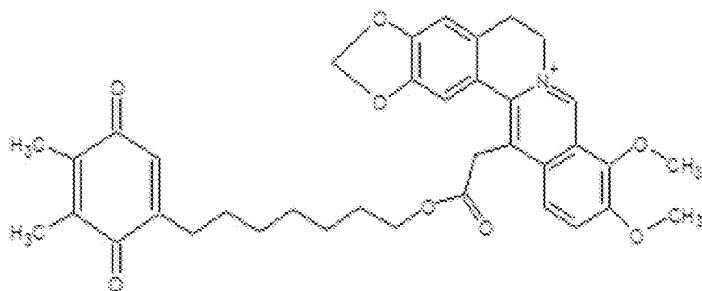


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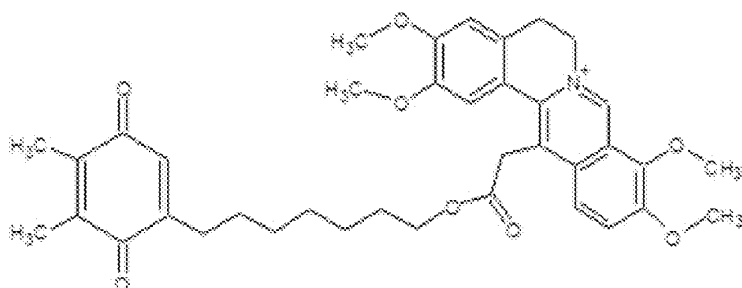
SkQRB1



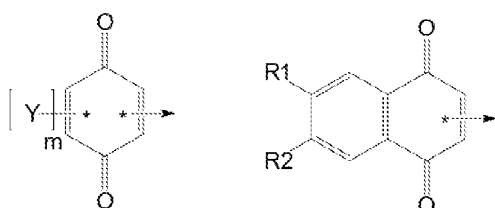
SkQB1



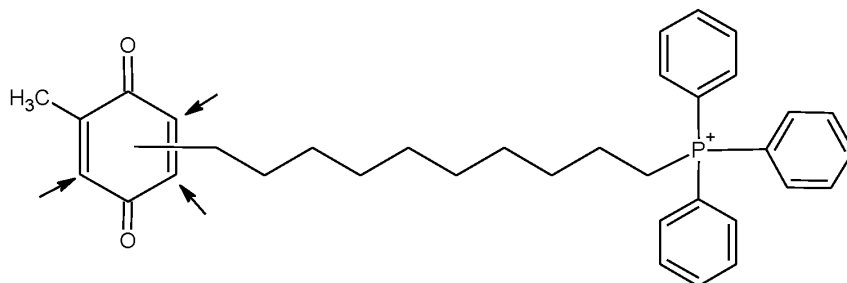
SkQBP1



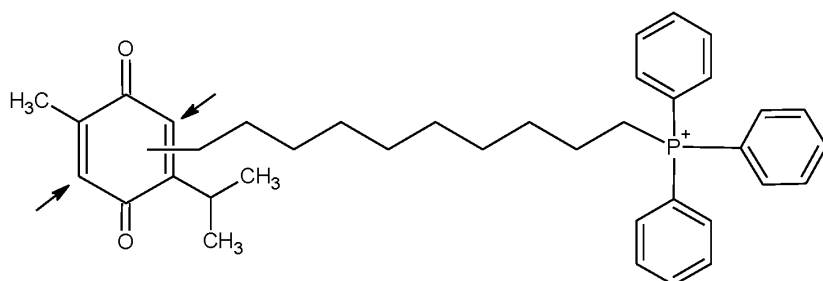
Formula I may further comprise the antioxidant below:



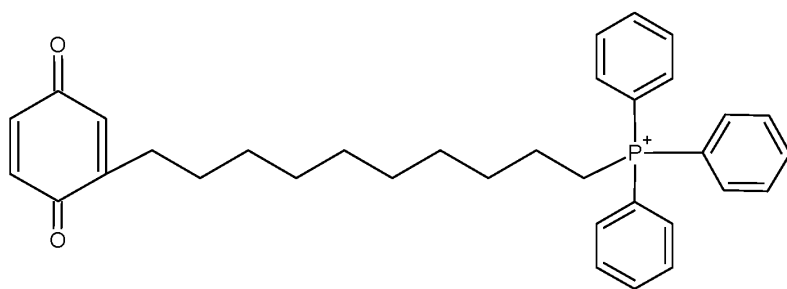
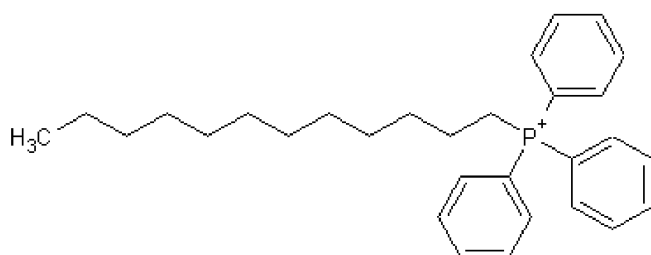
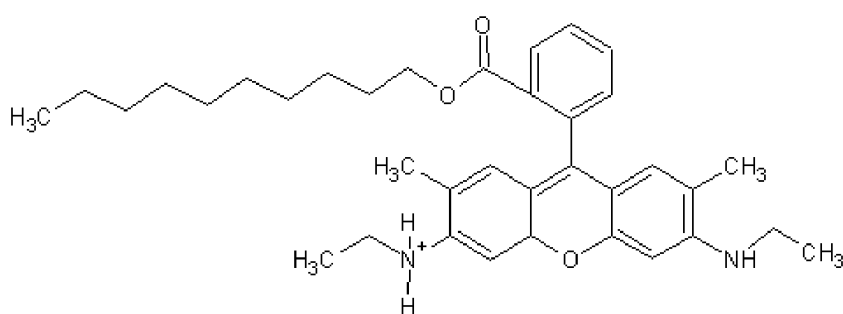
5 , or , wherein “*” indicates attachment at any ring carbon, such as to recite the example of SkQT (one isomer or a mixture of isomers that differ by the position of decyl linker, possible positions are indicated with arrows):



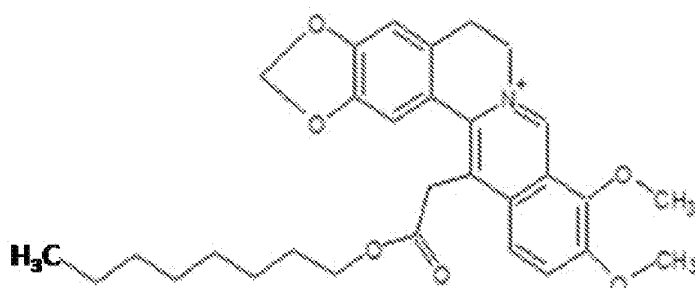
10 SkQThy (mixture of isomers that differ by the position of decyl linker, possible positions are indicated with arrows)

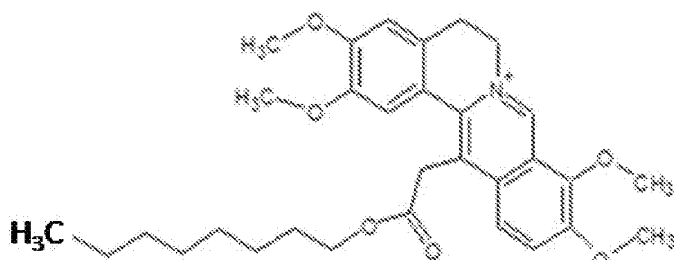


Further examples are SkQB

 C_nTPP (wherein n is from 5 to 12) C_nR1 (wherein n is from 4 to 12)

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 C_nBerb (wherein n is from 4 to 12)and C_nPalm (wherein n is from 4 to 12)



The technology described herein is not limited to the example compounds shown above, and any compound of the technology can be formulated in various pharmaceutical formulations as known in the art.

As used herein, the term “about” includes values close to the stated value as understood by one of ordinary skill. For example, the term “about” can refer to values within 10%, 5%, or 1%, of the stated value.

As used herein, “to aid in treating” or “treating” refers to producing any outcome that in any part or in whole reduces or eliminates the symptoms or underlying causation of a disease or medical condition already present at the time of the stated intervention. As used herein, “to aid in preventing” or “preventing” refers to producing any outcome that in any part or in whole reduces or eliminates the likelihood of development or worsening of the symptoms or underlying causation of a disease or medical condition at some future time after the stated intervention.

The animal studies summarized in the examples below indicate that administering a mitochondrially targeted antioxidant can both treat and prevent hepatocellular ballooning and related conditions, including hepatic inflammation, hepatic fibrosis, hepatic collagen deposition, NASH, weight gain, fatty liver, and obesity. Monitoring of the treatment or prevention of these conditions can be performed by liver biopsy, determination of body weight, hepatocyte ballooning, hepatic fibrosis or collagen content, hepatic fat content, and/or levels of systemic markers of inflammation or cell pathology, such as CK-18, ALT, TNF-alpha, or IL-6, which can be measured in blood plasma.

Example 1. Effects of SkQ1 in Mouse Model of Non-Alcoholic Steatohepatitis (NASH).

1. STUDY OBJECTIVE

To examine the effects of SkQ1 in a model of non-alcoholic steatohepatitis.

2. MATERIALS AND METHODS

2.1. Test substance

SkQ1 stock solution was diluted 10x in saline according to the study protocol.

5 2.2. Induction of NASH

NASH was induced in 24 male mice by a single subcutaneous injection of low-dose streptozotocin solution 2 days after birth and feeding with high fat diet after 4 weeks of age.

2.3. Route of drug administration

10 SkQ1 was administered subcutaneously in a volume of 5 mL/kg.

2.4. Treatment doses

SkQ1 was administered at 2 dose levels of 0.3 or 1.7 mg/kg, twice daily.

15 2.5. Animals

C57BL/6 mice (14-day-pregnant females) were used in the study.

2.5.1. Environment

The animals were maintained in a SPF facility under controlled conditions of temperature ($23 \pm 2^{\circ}\text{C}$), humidity ($45 \pm 10\%$), lighting (12-hour artificial light and dark cycles; light from 8:00 to 20:00) and air exchange. A high pressure was maintained in the experimental room to prevent contamination of the facility.

2.5.2. Animal husbandry

The animals were housed in TPX cages (CLEA Japan) with a maximum of 4 mice per cage. Sterilized Paper-Clean (Japan SLC) was used for bedding and replaced once a week.

2.5.3. Food and drink

Sterilized solid HFD was provided *ad libitum*, being placed in a metal lid on the top of the cage. Pure water was provided *ad libitum* from a water bottle equipped with a rubber stopper and a sipper tube. Water bottles were replaced once a week, cleaned, and sterilized in an autoclave and reused.

2.5.4. Animal and cage identification

Mice were identified by ear punch. Each cage was labeled with a specific identification code.

35 2.6. Histological analyses

For HE staining, sections were cut from paraffin blocks of liver tissue prefixed in Bouin's solution and stained with Lillie-Mayer's Hematoxylin (Muto Pure Chemicals Co., Ltd., Japan) and eosin solution (Wako Pure Chemical Industries). NAFLD Activity score (NAS) was calculated according to the criteria of Kleiner (Kleiner DE. *Et al.*, *Hepatology*, 2005;41:1313).

To visualize collagen deposition, Bouin's fixed liver sections were stained using picro-Sirius red solution (Waldeck, Germany). For quantitative analysis of fibrosis area, bright field images of Sirius red-stained sections were captured around the central vein using a digital camera (DFC295; Leica, Germany) at 200-fold magnification, and the positive areas in 5 fields/section were measured using ImageJ software (National Institute of Health, USA).

For F4/80-immunohistochemistry, sections were cut from frozen liver tissues embedded in Tissue-Tek O.C.T. compound and fixed in acetone. Endogenous peroxidase activity was blocked using 0.03% H₂O₂ for 5 minutes, followed by incubation with Block Ace (Dainippon Sumitomo Pharma Co. Ltd., Japan) for 10 minutes. The sections were incubated with a 100-fold dilution of anti-F4/80 antibody (BMA Biomedicals, Switzerland) at room temperature for 1 hour. The sections were then incubated with biotin-conjugated secondary antibody (VECTASTAIN Elite ABC kit, Vector laboratories, Inc., USA) followed by ABC reagent each for 30 minutes at room temperature. Enzyme-substrate reactions were performed using 3, 3'-diaminobenzidine/H₂O₂ solution (Nichirei Bioscience Inc.).

2.7. Statistical tests

Statistical analyses were performed using Bonferroni Multiple Comparison Test on GraphPad Prism 6 (GraphPad Software Inc., USA). *P* values < 0.05 were considered statistically significant. A trend or tendency was assumed when a one-tailed t-test returned *P* values < 0.1. Results were expressed as mean ± SD.

3. EXPERIMENTAL DESIGN AND TREATMENT

3.1. Study groups

Group 1: Vehicle

Eight NASH mice were subcutaneously administered vehicle [5% propylene glycol in saline] in a volume of 5 mL/kg twice daily from 6 to 9 weeks of age.

Group 2: SkQ1 Low Dose

Eight NASH mice were subcutaneously administered vehicle supplemented with SkQ1 at a dose of 0.3 mg/kg twice daily from 6 to 9 weeks of age.

Group 3: SkQ1 High Dose

Eight NASH mice were subcutaneously administered vehicle supplemented with SkQ1 at a dose of 1.7 mg/kg twice daily from 6 to 9 weeks of age.

The table below summarizes the treatment schedule:

Group	No. mice	Test substance	Dose (mg/kg)	Volume (mL/kg)	Regimen	Sacrifice (wks)
1	8	Vehicle	-	5	SC, BID, 6 – 9 wks	9
2	8	SkQ1	0.3	5	SC, BID, 6 – 9 wks	9
3	8	SkQ1	1.7	5	SC, BID, 6 – 9 wks	9

5

4. RESULTS

4.1. Histological analyses

4.1.1. HE staining and NAFLD Activity Score (NAS) (Figures 1A-D).

10 Liver sections from the Vehicle group exhibited micro- and macrovesicular fat deposition, hepatocellular ballooning and inflammatory cell infiltration. The NAFLD activity score (NAS), which is a composite score of steatosis, lobular inflammation, cytological ballooning, and fibrosis, was determined for Groups 1-3. The SkQ1 High Dose group showed a significant decrease in NAS compared with the Vehicle group. The SkQ1 Low Dose group tended to decrease NAS compared with the Vehicle group. Both SkQ1 High 15 Dose group and SkQ1 Low Dose group showed a significant decrease in ballooning score compared with the Vehicle group.

Definition of NAS Components

Steatosis	0	<5%
	1	5-33%
	2	>33-66%
	3	>66%
Lobular Inflammation	0	No foci
	1	<2 foci/200x
	2	2-4 foci/200x
	3	>4 foci/200x
Hepatocyte Ballooning	0	None
	1	Few balloon cells
	2	Many cells/prominent ballooning

20 4.1.2. Sirius red staining (Figure 2)

Liver sections from the Vehicle group showed increased collagen deposition in the pericentral region of liver lobule. The SkQ1 High Dose group had significantly decreased fibrosis area (Sirius red-positive area) compared with the Vehicle group. There was no

significant difference in the fibrosis area between the Vehicle group and the SkQ1 Low Dose group.

4.1.4. F4/80 immunostaining (Figure 3)

- 5 F4/80 immunostaining of liver sections from the Vehicle group demonstrated accumulation of F4/80⁺ cells in the liver lobule. The SkQ1 High Dose group had significantly decreased number and size of F4/80⁺ cells (F4/80-positive area) compared with the Vehicle group. There was no significant difference in the number and size of F4/80⁺ cells between the Vehicle group and the SkQ1 Low Dose group.

10 5. SUMMARY

SkQ1 Low Dose

Treatment with SkQ1 Low Dose showed statistically significant decreases in NAS in ballooning score compared with the Vehicle group.

SkQ1 High Dose

- 15 Treatment with SkQ1 High Dose showed a significant decrease in NAS, steatosis score, ballooning score, fibrosis score and inflammation area compared with the Vehicle group.
- Treatment with the SkQ1 significantly decreased NAS in a dose dependent manner. Importantly, because NAS is one of the clinical endpoints for assessing the activity of NASH (Sanyal AJ. *Et al.*, *Hepatology*, 2011;**54**:344), the observed changes in the treatment group suggest clinical efficacy of SkQ1 as an anti-NASH therapeutics. The improvement in NAS was attributable to the reduction in hepatocyte ballooning, which was significantly decreased compared with the Vehicle group. Rangwala reported the close association of hepatocyte ballooning and NASH-related fibrosis (Rangwala F. *et al.*, *J. Pathol.*, 2011;**224**:401). The
- 20 SkQ1 High Dose group also significantly decreased the pathological deposition of collagen in the liver as demonstrated by Sirius red staining. Thus, reduction of hepatocyte ballooning in the SkQ1 group may underlie the anti-fibrotic effect observed in this study.

- 30 Example 2. Effects of an increased dose of SkQ1 in Mouse Model of Non-Alcoholic Steatohepatitis (NASH).

1. STUDY OBJECTIVE

To examine the effects of increased dose of SkQ1 in a model of non-alcoholic steatohepatitis.

35

2. MATERIALS AND METHODS

2.1. Test substance

SkQ1 stock solution was diluted 10x in saline according to the study protocol.

2.2. Induction of NASH

NASH was induced in 24 male mice by a single subcutaneous injection of low-dose streptozotocin solution 2 days after birth and feeding with high fat diet after 4 weeks of age.

5 2.3. Route of drug administration

SkQ1 was administered subcutaneously in a volume of 5 mL/kg.

2.4. Treatment doses

SkQ1 was administered at a dose level of 3 mg/kg twice daily. Positive control Telmisartan was administered orally at a dose level of 10 mg/kg once daily.

10 2.5. Animals

C57BL/6 mice (14-day-pregnant female) were used in the study.

2.5.1. Environment

The animals were maintained in a SPF facility under controlled conditions of temperature ($23 \pm 2^{\circ}\text{C}$), humidity ($45 \pm 10\%$), lighting (12-hour artificial light and dark cycles; light from 8:00 to 20:00) and air exchange. A high pressure was maintained in the experimental room to prevent contamination of the facility.

2.5.2. Animal husbandry

The animals were housed in TPX cages (CLEA Japan) with a maximum of 4 mice per cage. Sterilized Paper-Clean (Japan SLC) was used for bedding and replaced once a week.

2.5.3. Food and drink

Sterilized solid HFD was provided *ad libitum*, being placed in a metal lid on the top of the cage. Pure water was provided *ad libitum* from a water bottle equipped with a rubber stopper and a sipper tube. Water bottles were replaced once a week, cleaned, and sterilized in an autoclave and reused.

2.5.4. Animal and cage identification

Mice were identified by ear punch. Each cage was labeled with a specific identification code.

30 2.6. Histological analyses

For HE staining, sections were cut from paraffin blocks of liver tissue prefixed in Bouin's solution and stained with Lillie-Mayer's Hematoxylin (Muto Pure Chemicals Co., Ltd., Japan) and eosin solution (Wako Pure Chemical Industries). NAFLD Activity score (NAS) was calculated according to the criteria of Kleiner (Kleiner DE. *Et al.*, *Hepatology*, 2005;41:1313).

To visualize collagen deposition, Bouin's fixed liver sections were stained using picro-Sirius red solution (Waldeck, Germany). For quantitative analysis of fibrosis area, bright field

images of Sirius red-stained sections were captured around the central vein using a digital camera (DFC295; Leica, Germany) at 200-fold magnification, and the positive areas in 5 fields/section were measured using ImageJ software (National Institute of Health, USA).

For F4/80-immunohistochemistry, sections were cut from frozen liver tissues
5 embedded in Tissue-Tek O.C.T. compound and fixed in acetone. Endogenous peroxidase activity was blocked using 0.03% H₂O₂ for 5 minutes, followed by incubation with Block Ace (Dainippon Sumitomo Pharma Co. Ltd., Japan) for 10 minutes. The sections were incubated with a 100-fold dilution of anti-F4/80 antibody (BMA Biomedicals, Switzerland) at room
10 temperature for 1 hour. The sections were then incubated with biotin-conjugated secondary antibody (VECTASTAIN Elite ABC kit, Vector laboratories, Inc., USA) followed by ABC reagent each for 30 minutes at room temperature. Enzyme-substrate reactions were performed using 3, 3'-diaminobenzidine/H₂O₂ solution (Nichirei Bioscience Inc.).

2.7. Statistical tests

15 Statistical analyses were performed using Bonferroni Multiple Comparison Test on GraphPad Prism 6 (GraphPad Software Inc., USA). *P* values < 0.05 were considered statistically significant. A trend or tendency was assumed when a one-tailed t-test returned *P* values < 0.1. Results were expressed as mean ± SD.

20 3. EXPERIMENTAL DESIGN AND TREATMENT

3.1. Study groups

Group 1: Vehicle

Eight NASH mice were subcutaneously administered vehicle [5% propylene glycol in saline] in a volume of 5 mL/kg twice daily from 6 to 10 weeks of age.

25 Group 2: SkQ1

Eight NASH mice were subcutaneously administered vehicle supplemented with SkQ1 at a dose of 3 mg/kg twice daily from 6 to 10 weeks of age.

Group 3: Telmisartan (positive control)

30 Eight NASH mice were orally administered Telmisartan at a dose of 10 mg/kg once daily from 6 to 10 weeks of age.

The table below summarizes the treatment schedule:

Group	No. mice	Test substance	Dose (mg/kg)	Volume (mL/kg)	Regimen	Sacrifice (wks)
1	8	Vehicle	-	5	SC, BID, 6 – 10 wks	10
2	8	SkQ1	3	5	SC, BID, 6 – 10 wks	10

3	8	Telmisartan	10	5	PO, QD, 6 – 10 wks	10
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4. RESULTS

4.1. Histological analyses

5 4.1.1. HE staining and NAFLD Activity score (Figures 4A-B).

Liver sections from the Vehicle group exhibited micro- and macrovesicular fat deposition, hepatocellular ballooning and inflammatory cell infiltration. The SkQ1 group and Telmisartan group showed significant decreases in NAS compared with the Vehicle group. Only SkQ1 group group showed a significant decrease in ballooning score compared with the Vehicle group.

Definition of NAS Components

Steatosis	0	<5%
	1	5-33%
	2	>33-66%
	3	>66%
Lobular Inflammation	0	No foci
	1	<2 foci/200x
	2	2-4 foci/200x
	3	>4 foci/200x
	0	None
	1	Few balloon cells
	2	Many cells/prominent ballooning

4.1.2. Sirius red staining (Figure 5)

Liver sections from the Vehicle group showed increased collagen deposition in the pericentral region of liver lobule. The SkQ1 group had significantly decreased fibrosis area (Sirius red-positive area) compared with the Vehicle group.

5. SUMMARY

Treatment with SkQ1 showed a significant decrease in NAS, steatosis score, ballooning score and fibrosis score compared with the Vehicle group.

Importantly, because NAS is one of the clinical endpoints for assessing the activity of NASH (Sanyal AJ. *Et al.*, *Hepatology*, 2011;**54**:344), the observed changes in the treatment group suggest potential clinical efficacy of SkQ1 as an anti-NASH therapeutics. The improvement in NAS was attributable to the changes in hepatocyte ballooning, which was completely suppressed and in a statistically significant manner compared with the Vehicle group. Rangwala reported the close association of hepatocyte ballooning and NASH-related

fibrosis (Rangwala F. *et al.*, *J. Pathol.*, 2011;**224**:401). The SkQ1 group also significantly decreased the pathological deposition of collagen in the liver as demonstrated by Sirius red staining. Thus, reduction of hepatocyte ballooning in the SkQ1 group may underlie the anti-fibrotic effect observed in this study.

5

Example 3. Effects of SkQ1 Dosing in Diet Induced Obese (DIO) Mice.

1. STUDY OBJECTIVE

10 To examine the effects of increased dose of SkQ1 in a model of obesity and fatty liver.

2. MATERIALS AND METHODS

2.1. Test substance

15 SkQ1 stock solution was diluted 10x in saline according to the study protocol.

2.2. Induction of obesity and fatty liver

After exposure to the high fat diet for circa 32 weeks, eight male mice were singly housed and placed on a reverse phase light/ dark cycle. Mice continued to be provided with
20 high fat diet and filtered water ad libitum during the study.

2.3. Route of drug administration

SkQ1 was administered subcutaneously in a volume of 5 mL/kg.

2.4. Treatment doses

25 SkQ1 was administered subcutaneously at a dose level of 1 mg/kg on days 1-10 and at a dose level of 0.5 mg/kg on days 16-21 once daily.

2.5. Animals

30 Male C57BL/6J mice were used in the study. Animals were group housed with ad libitum access to a high fat diet and filtered tap water. The mice were housed in polypropylene cages with sawdust-coated floors, red house, red tunnel, sizzle nest and nestlet at a temperature of 22±2°C. Relative humidity was typically 55±15% with prolonged periods below 40% RH or above 70% RH avoided.

35

2.6. Body composition analyses

Body composition analysis was performed by DEXA on mice anaesthetised with isoflurane using the Lunar PIXImus Densitometer.

2.7. Statistical tests

5 Daily body weight and weekly and overall body weight gain were analyzed by analysis of covariance (ANCOVA) with Day 1 body weight as a covariate.

 DEXA data was analyzed by analysis of covariance with Day 1 body weight as a covariate. A log transformation and/or robust regression were used, if appropriate.

10 3. EXPERIMENTAL DESIGN AND TREATMENT

3.1. Study groups

Group 1: Vehicle

Four DIO mice were subcutaneously administered vehicle [5% propylene glycol in saline] in a volume of 5 mL/kg once daily with no treatment on days 11-15 of the study.

15 Group 2: SkQ1

Four DIO mice were subcutaneously administered vehicle supplemented with SkQ1 at a dose of 1 mg/kg once daily on days 1-10 of the study, then no dosing was performed on days 11-15, and SkQ1 was dosed at a level of 0.5 mg/kg once daily on days 16-21.

20 The table below summarizes the treatment schedule:

Group	No. mice	Test substance	Dose (mg/kg)	Volume (mL/kg)	Regimen
1	4	Vehicle	-	5	SC, for 21 days with no treatment days 11-15
2	4	SkQ1	1 (days 1-10), 0.5 (days 16-21)	5	SC, for 21 days with no treatment days 11-15

4. RESULTS

4.1. Weights (Figures 6A-C)

25 Animals in SkQ1 group exhibited steady weight loss over the first 10 days of drug administration. Animals in Vehicle group expectedly gained weight throughout the study. Statistically significant difference between weights in the two groups was achieved at day 3 of the study and stayed significant at all of the following days. Treatment withdrawal between days 11-15 kept weights unchanged in SkQ1 group with no weight increase during

30 withdrawal, when animals in Vehicle group kept gaining weight as expected. After the treatment was reintroduced at a lower dose on day 16, animals in SkQ1 group lost weight on average by the end of the study compared to day 16, when animals in Vehicle group gained

weight compared to day 16. The weight difference between the two groups achieved 15% by day 21.

DEXA measurements indicated that weight difference between the groups was achieved by SkQ1 significantly reducing fat mass compared to Vehicle while preserving lean
5 mass.

4.2 Liver measurements (Figures 7-8)

Measurements at study termination showed statistically significant reduction of liver weights in SkQ1 group compared to Vehicle group. As liver lipid measurements
10 demonstrated, weight difference was driven by statistically significant reduction of liver lipids in SkQ1 group compared to Vehicle group.

5. SUMMARY

Treatment with SkQ1 showed a significant decrease in body weight, fat mass, liver
15 weight and liver lipids compared with the Vehicle group.

Importantly, body weights did not increase in SkQ1 group when treatment was paused for 5 days of the study. Body weight loss was driven by significant loss of fat mass, while lean (muscle and bone) mass was protected.

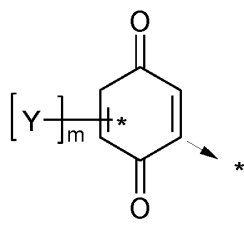
CLAIMS

1. A method to aid in treating or preventing a cell ballooning-associated disease or medical condition, the method comprising the step of administering to a subject in need thereof therapeutically or prophylactically effective amount of a mitochondrially targeted antioxidant compound.
2. The method of claim 1, wherein the mitochondrially targeted antioxidant is an SkQ compound of Formula II below:



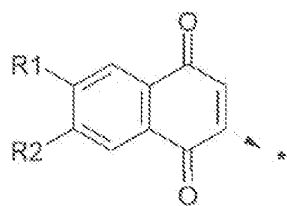
Formula II

wherein A is hydrogen or an effector moiety, which is an antioxidant optionally having a following structure:



and/or reduced forms thereof,

wherein m is from 0 to 3; each Y is independently selected from the group consisting of: lower alkyl (1 to 6 carbons), lower alkoxy (1 to 6 carbons); or two adjacent Y groups, together with carbon atoms to which they are attached, form a following structure:



and/or reduced form thereof,

wherein R1 and R2 may be the same or different and are each independently lower alkyl (1 to 6 carbon atoms) or lower alkoxy (1 to 6 carbon atoms);

L is a linker group, comprising:

- a) straight or branched hydrocarbon chain which can be optionally substituted by one or more substituents and optionally contains one or more double or triple bonds;

b) a natural isoprene chain;
n is the number of carbon atoms in the linker, which is an integer from 1 to 40; and
B is a mitochondria targeting group comprising a lipophilic cation;
and a pharmacologically acceptable anion;
and/or solvates, salts, isomers, or prodrugs thereof..

3. The method of claim 1, wherein the mitochondrially targeted antioxidant is selected from the group consisting of SkQ1, SkQ3, SkQ4, SkQ5, SkQR1, SkQBR1, SkQB1, and SkQBP1.

4. The method of claim 1, wherein the ballooning associated disease or medical condition comprises ballooning of hepatocytes in the subject.

5. The method of claim 1, wherein the ballooning associated disease or medical condition comprises accumulation of cytosolic fat droplets in hepatocytes of the subject.

6. The method of claim 1, wherein the ballooning associated disease or medical condition is selected from the group consisting of cytostatic drug and radiation injuries; kidney failure with various origin; traumatic, toxic, edematous, neurodegenerative and ischemic brain damage; intestinal infections; toxic cardiac injuries; acute hepatitis; neonatal giant cell hepatitis; autoimmune hepatitis; chronic hepatitis B; chronic cholestasis; increased copper storage; ischemia–reperfusion injury fatty liver, alcoholic steatohepatitis, and non-alcoholic steatohepatitis (NASH).

7. The method of claim 1, wherein the ballooning associated disease or medical condition comprises ballooning of hepatocytes, hepatic steatosis, hepatic inflammation, increase in liver weight, increase in hepatic content of fat, cholesterol, triglycerides, free fatty acids, or collagen, and/or increase in body weight.

8. The method of claim 7, wherein said administration decreases said ballooning of hepatocytes, hepatic steatosis, hepatic inflammation, increase in liver weight, increase in hepatic content of fat, cholesterol, triglycerides, free fatty acids, or collagen, and/or increase in body weight.

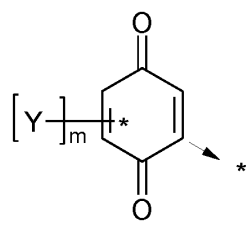
9. The method of claim 8, wherein hepatic inflammation is decreased as measured by an inflammation or cell pathology marker, such as a marker selected from the group consisting of CK-18, ALT, TNF-alpha, and IL-6.

10. The method of claim 1, further comprising determining a level of a biomarker and diagnosing or prognosing the disease or medical condition based on said level.
11. The method of claim 10, wherein said determining comprises performing a liver biopsy or quantifying a blood-born biomarker.
12. The method of claim 1, wherein said administering reduces the likelihood of said cell ballooning-associated disease occurring or progressing in the subject.
13. The method of claim 1, wherein said administering reduces ballooning of hepatocytes in the subject.
14. The method of claim 1, wherein said administering reduces hepatic fibrosis in the subject.
15. The method of claim 1, wherein the subject suffers from obesity, diabetes, or alcohol abuse and has, or is at risk of developing, a ballooning associated disease or medical condition.
16. A method for altering cellular metabolism and/or storage of fat, the method comprising the step of administering to a subject in need thereof a mitochondrially targeted antioxidant compound, whereby cellular metabolism and/or storage of fat is altered in the subject.
17. The method of claim 16, wherein the mitochondrially targeted antioxidant is an SkQ compound of Formula II below:



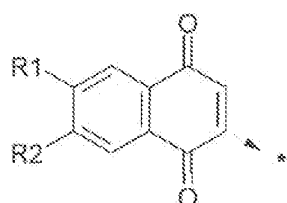
Formula II

wherein A is hydrogen or an effector moiety, which is an antioxidant optionally having a following structure:



and/or reduced forms thereof,

wherein m is from 0 to 3; each Y is independently selected from the group consisting of: lower alkyl (1 to 6 carbons), lower alkoxy (1 to 6 carbons); or two adjacent Y groups, together with carbon atoms to which they are attached, form a following structure:



and/or reduced form thereof,

wherein R1 and R2 may be the same or different and are each independently lower alkyl (1 to 6 carbon atoms) or lower alkoxy (1 to 6 carbon atoms);

L is a linker group, comprising:

a) straight or branched hydrocarbon chain which can be optionally substituted by one or more substituents and optionally contains one or more double or triple bonds;

b) a natural isoprene chain;

n is the number of carbon atoms in the linker, which is an integer from 1 to 40; and

B is a mitochondria targeting group comprising a lipophilic cation;

and a pharmacologically acceptable anion;

and/or solvates, salts, isomers, or prodrugs thereof.

18. The method of claim 16, wherein the mitochondrially targeted antioxidant is selected from the group consisting of SkQ1, SkQ3, SkQ4, SkQ5, SkQR1, SkQBR1, SkQB1, and SkQBP1.

19. The method of claim 16, wherein triglyceride hydrolysis and/or fatty acid oxidation are increased and/or storage of triglycerides and/or free fatty acids is reduced in the subject.

20. The method of claim 16, wherein the subject has hepatocyte ballooning and/or accumulation of cytosolic fat droplets in hepatocytes.

21. The method of claim 20, wherein the subject has hepatic steatosis, hepatic inflammation, increase in liver weight, increase in hepatic content of cholesterol, triglycerides, free fatty acids, or collagen, and/or increase in body weight.

22. The method of claim 16, wherein the ballooning associated disease or medical condition is selected from the group consisting of cytostatic drug and radiation injuries; kidney failure with various origin; traumatic, toxic, edematous, neurodegenerative and ischemic brain damage; intestinal infections; toxic cardiac injuries; acute hepatitis; neonatal giant cell hepatitis; autoimmune hepatitis; chronic hepatitis B; chronic cholestasis; increased copper storage; ischemia–reperfusion injury, fatty liver, alcoholic steatohepatitis, and non-alcoholic steatohepatitis (NASH).

23. The method of claim 20, wherein said administration also decreases said ballooning of hepatocytes, hepatic steatosis, hepatic inflammation, increase in liver weight, increase in hepatic content of cholesterol, triglycerides, or free fatty acids, and/or increase in body weight.

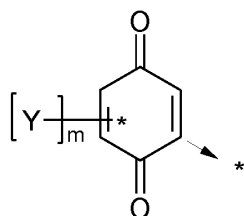
24. A method for reducing body weight, or preventing an increase in body weight, of a mammalian subject, the method comprising the step of administering to the subject a mitochondrially targeted antioxidant compound, whereby body weight of the subject is reduced or maintained.

25. The method of claim 24, wherein the mitochondrially targeted antioxidant is an SkQ compound of Formula II below:



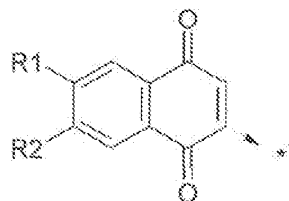
Formula II

wherein A is hydrogen or an effector moiety, which is an antioxidant optionally having a following structure:



and/or reduced forms thereof,

wherein m is from 0 to 3; each Y is independently selected from the group consisting of: lower alkyl (1 to 6 carbons), lower alkoxy (1 to 6 carbons); or two adjacent Y groups, together with carbon atoms to which they are attached, form a following structure:



and/or reduced form thereof,

wherein R1 and R2 may be the same or different and are each independently lower alkyl (1 to 6 carbon atoms) or lower alkoxy (1 to 6 carbon atoms);

L is a linker group, comprising:

a) straight or branched hydrocarbon chain which can be optionally substituted by one or more substituents and optionally contains one or more double or triple bonds;

b) a natural isoprene chain;

n is the number of carbon atoms in the linker, which is an integer from 1 to 40; and

B is a mitochondria targeting group comprising a lipophilic cation;

and a pharmacologically acceptable anion;

and/or solvates, salts, isomers, or prodrugs thereof.

26. The method of claim 24, wherein the mitochondrially targeted antioxidant is selected from the group consisting of SkQ1, SkQ3, SkQ4, SkQ5, SkQR1, SkQBR1, SkQB1, and SkQBP1.

27. The method of claim 24, wherein the mitochondrially targeted antioxidant is administered orally or by subcutaneous injection.

28. The method of claim 24, whereby the subject's body weight is decreased by at least 5, 10, 15, or 20%.