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(54) **Title:** THERAPEUTIC COMPOUNDS

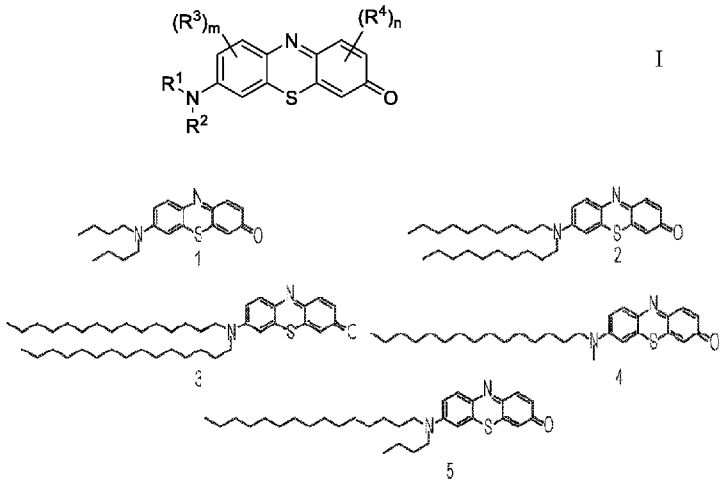


Figure 1

(57) **Abstract:** The invention provides compounds having the general formula I: and pharmaceutically acceptable salts thereof, wherein the variables R^1, R^2, R^3, R^4, subscipt m and n, have the meaning as described herein, and compositions containing such compounds and methods for using such compounds and compositions.

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THERAPEUTIC COMPOUNDS

RELATED APPLICATIONS

This application claims priority to United States Provisional Application Number 62/379,654 that was filed on 25 August 2016. The entire content of the applications referenced above are hereby incorporated by reference herein.

BACKGROUND OF THE INVENTION

Mitochondrial dysfunction and disruption of the electron transport chain (ETC) has been identified as an important factor in diseases ranging from neurodegenerative diseases including, Alzheimer's disease (AD) , Parkinson's disease (PD) and Friedreich's ataxia (FRDA), to diseases of the cardiovascular system, cancer and diabetes (Armstrong, J. S. et al. *FASEB J.* **2010**, 24, 2152; Markesbery, W. R. et al. *Brain Pathol.* **1999**, 9, 133; Barnham, K. J.; Masters, C. L. et al. *Nat. Rev Drug Discovery.* **2004**, 3, 205; Calabrese, V. et al. *J Neurol Sci.* **2005**, 233, 145; and Lin, M. T. et al. *Nature* **2006**, 443, 787). The broad impact of mitochondria in so many diseases makes them prime targets for therapeutics. As powerhouses in mammalian cells, mitochondria are responsible for the predominant mode of energy production via oxidative phosphorylation (OXPHOS) of glucose, which is performed by the four respiratory complexes (complexes I-IV) and the ATP synthase (complex V), all located in the inner mitochondrial membrane (Henze, K. et al. *Nature* **2003**, 426, 127; Saraste, M. W. *Science* **1999**, 283, 1488; Newmeyer, D. D. et al. S. *Cell* **2003**, 112, 481; and Fiore, C. et al. *Biochimie* **1998**, 80, 137). However, mitochondria are also the major sites for production of reactive oxygen species (ROS) (Turrens, J. F. *J. Physiol.* **2003**, 552, 335; and Murphy, M. P. *Biochem. J.* **2009**, 417, 1). The impaired oxidative phosphorylation function would lead to further production of ROS, which further overwhelms the endogenous antioxidant systems and exposing cellular macromolecules to oxidative damage (Mates, J. M. et al. *Clin Biochem.* **1999**, 32, 595; and Gaetani, G. F. et al. *Blood* **1989**, 73, 334). Giving the main role of mitochondrial dysfunction in the development of several metabolic disorders, extensive research has explored therapeutic strategies for preserving mitochondrial function and the treatment of mitochondrial and neurological diseases (Heller, G. et al. *Eur. J. Pharm. Biopharm.* **2012**, 82, 1; Smith, R. A. et al. *Trends in Pharmacol. Sci.* **2012**, 341; Frantz, M. C. et al. *Environ. Mol. Mutagen* **2010**, 51, 462; Arce, P. M. et al. *ACS Med. Chem. Lett.* **2011**, 2, 608; Atamna, H. et al. *FASEB. J.* **2008**, 22, 703; and Wen, Y. et al. *J. Biol. Chem.* **2011**, 286, 16504).

Methylene blue (MB), a member of the phenothiazine family, originally discovered as

a synthetic cationic dye. MB has a long-standing, extensive history of medical uses for more than a century (Wainwright, M. et al. *J Chemother.* **2002**,14,431). It is an FDA approved drug for methemoglobinemia and an antidote to ifosfamide-induced encephalopathy (Wright, R. et al. *Ann. Emerg. Med.* **1999**, 34, 646; and Zulian, G. B. et al. *N. Engl J Med.* **1995**, 332, 1239). MB was also the lead compound for successful pharmacotherapeutic derivatives such as the antimalarial agents such as quinacrine, and chloroquine (Green, R. *Lancet* **1932**, 219, 826; and Loeb, R. F. et al. *J. Am. Med. Assoc.* **1946**, 130, 1069).

MB has very unique redox property that exists in equilibrium between oxidized state in dark blue and colorless reduced state, making it both antioxidant and prooxidant under different conditions. MB with a mild redox potential, appears to readily cycle between the oxidized and reduced forms using specific mitochondrial and cytosolic redox centers. This property of MB has been reported to redirect and facilitate electron transfer across mitochondrial electron transfer complexes minimizing electron leakage, and inhibit superoxide production (mitochondrial electron-carrier bypass) (Wen, Y. et al. *J. Biol. Chem.* **2011**, 286, 16504; and Wainwright, M. et al. *J Chemother.* **2002**,14,431). Thus, MB might be able to act as an alternative electron transfer carrier that replaces the damaged mitochondrial respiratory chain.

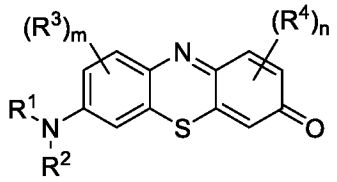
Methylene blue has limitation in term of bioavailability and tolerability (at high doses). The substantial hydrophilicity of MB may restrict its permeability across the plasma membranes of mammalian cells, which would also limit its cellular uptake. It is for this reason TauRx switch to the reduced form of methylene blue (more hydrophobic) in their phase III clinical trials (Baddeley, T.C. et al. *J Pharmacol Exp Ther.* **2015**, 352, 1; and Melis, V. et al. *Behav Pharmacol.* 2015, 26, 353). MB has a hormetic dose-response in which its beneficial effects are optimal in the lower to intermediate range (Bruchey A. K. et al. *Am J Pharm Toxicol* **2008**, 3, 72). Previous study has shown the effect and interaction of exogenous short-chain CoQ_{10} analogues on mitochondrial oxidative phosphorylation in isolated mitochondria and ROS metabolism in cultured cells. It was clearly showed that the antioxidant reactions of exogenous ubiquinones will predominantly occur within phospholipid bilayers, while the pro-oxidant reactions require an aqueous environment. It is apparent that increasing hydrophobicity limits the "bad" aqueous autoxidation of ubiquinols without diminishing its "good lipid phase antioxidant functions. This could provide explanation for the extreme hydrophobicity of endogenous CoQ_{10}. Therefore, the relative rates of these reactions can be fine-tuned by hydrophobicity, allowing a rational approach to

the design of therapeutic mitochondria-targeted redox cyclers.

Methylene violet (MV) is a neutral phenothiazine dye. Hydrolysis of MB under strongly basic condition yields MV with improved hydrophobicity (Houghtaling, M. A. et al. *Photochem. Photobiol.* **2000**, *71*, 20). MV can be looked upon as a phenolic analogue of MB where a hydroxyl group substitutes one of the two dimethylamine moieties. Although MV is naturally obtained in the oxidized (quinone) form, it can be reduced by the mitochondrial redox centers generating the phenolic (quinol) form. The reduced form of MV can act as phenolic antioxidants similar to CoQ_{10}. Currently there is a need for new antioxidant compounds with reduced cytotoxicity and side effects (e.g. by decreasing the pro-oxidant effect).

SUMMARY OF THE INVENTION

The invention provides new antioxidant compounds with reduced cytotoxicity and side effects. Accordingly the invention provides a compound of formula I:



I

or a pharmaceutically acceptable salt thereof, wherein:

R^1 is C_{1-20} alkyl, C_{2-20} alkenyl or C_{2-20} alkynyl, and wherein the C_{1-20} alkyl, C_{2-20} alkenyl and C_{2-20} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

R^2 is C_{1-20} alkyl, C_{2-20} alkenyl or C_{2-20} alkynyl, and wherein the C_{1-20} alkyl, C_{2-20} alkenyl and C_{2-20} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN; or

R^1 and R^2 taken together with the nitrogen to which they are attached form a 3-12 membered heterocycle that is optionally substituted with one or more groups independently selected from C_{1-4} alkyl, C_{1-4} haloalkyl, –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

each R^3 is independently selected from the group consisting of –F, -Cl, -Br, -I, -OH, $-OR^a$, $-SR^a$, $-NR^aR^b$, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently

selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and -CN;

each R^4 is independently selected from the group consisting of –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, -NO_2, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and -CN;

each R^a is independently hydrogen or C_{1-4} alkyl;

each R^b is independently hydrogen or C_{1-4} alkyl;

the subscript m is 0, 1, 2 or 3; and

the subscript n is 0, 1, 2, or 3.

The invention also provides a pharmaceutical composition comprising a compound of formula I or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

The invention also provides a method for treating mitochondrial disease, neurodegenerative disease, cardiovascular disease, cancer or diabetes in an animal comprising administering a compound of formula I or a pharmaceutically acceptable salt thereof to the animal.

The invention also provides a compound of formula I or a pharmaceutically acceptable salt thereof for use in medical therapy.

The invention also provides a compound of formula I or a pharmaceutically acceptable salt thereof for the prophylactic or therapeutic treatment of mitochondrial disease, neurodegenerative disease, cardiovascular disease, cancer or diabetes.

The invention also provides the use of a compound of formula I or a pharmaceutically acceptable salt thereof to prepare a medicament for treating mitochondrial disease, neurodegenerative disease, cardiovascular disease, cancer or diabetes in an animal (e.g. a mammal such as a human).

The invention also provides processes and intermediates disclosed herein that are useful for preparing a compound of formula I or a salt thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the chemical structures of the representative compounds of formula I (compounds **1-5**).

Figure 2 shows the cytotoxicity of compounds **1-5** on cultured FRDA lymphocytes by incubation for 24 hours or 48 hours in glucose free media (galactose) to force cells to rely on mitochondria to produce their ATP. Flow cytometric determination of cell viability by fluorescence labeling was used employing calcein acetoxy-methyl-ester and ethidium homodimer-1 as live and dead cell stains

Figure 3 shows the cytoprotective effect of the test compounds **1-5** in FRDA lymphocytes following preincubation with test compounds for 12 hours in glucose-free media and subsequent treatment with rotenone (50 nM) for 24 hours or 48 hours to inhibit complex I.

Figure 4 shows the percentage of cells with intact $\Delta\psi_m$ calculated using Accuri™ C6 software. In each analysis, 10000 events were recorded. Depolarization with carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), was used *t*o serve as a positive control.

Figure 5 shows flow cytometric analysis of FRDA lymphocyte cells stained with dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 minutes, following pretreatment with the test compounds **1-5** for 16 hours, and subsequent treatment with diethyl maleate (DEM) for 80 minutes to induce the production of ROS by depleting glutathione.

Figure 6 shows total ATP level in FRDA lymphocytes following incubation with the compounds **1-5** for 24 hours in glucose free media (25 mM galactose). Results are expressed as percentage of total ATP relative to untreated control.

Figure 7 shows the cytoprotective effect of compounds **1-5** in (BSO)-treated FRDA fibroblasts following preincubation with test compounds and subsequent treatment with BSO (5 mM) for 48 h. Results obtained were verified by running triplicates and repeating experiments in two independent runs.

Figure 8 shows the effect of methylene violet analogue on frataxin expression level in Friedreich ataxia cells determined by an enzyme-linked immunosorbent assay following 24 and 48 hours of treatment. In-Cell ELISA Kits were used to assess protein levels of frataxin.

Figure 9 shows the effect of methylene violet analogue on mitochondrial biogenesis in Friedreich ataxia cells determined by an enzyme-linked immunosorbent assay following 24 and 48 hours of treatment. In-Cell ELISA Kits was used to assess protein levels of succinate dehydrogenase (SDH-A), a subunit of complex II (nDNA-encoded protein) and cytochrome *c*

oxidase subunit 1 (COX-1), a subunit of complex IV (mtDNA-encoded). Interpretation: Higher the ratio of COX-I/SDH-A (%) indicated increases mitochondrial biogenesis due to mitochondrial DNA-encoded COX-I protein synthesis is higher as compared to nuclear DNA-encoded SDH-A protein.

DETAILED DESCRIPTION

The term "alkyl", by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain hydrocarbon radical, having the number of carbon atoms designated (i.e., C_{1-6} means one to six carbons). Non limiting examples of "alkyl" include methyl, ethyl, propyl, isopropyl, butyl, iso-butyl, sec-butyl, pentyl, 3-pentyl, hexyl and decyl.

The term "alkenyl" refers to an unsaturated alkyl radical having one or more double bonds. Non limiting examples of "alkenyl" include vinyl, allyl, 1-propenyl, 2-propenyl, 1-butenyl, 2-butenyl, 3-butenyl, 1,-pentenyl, 2-pentenyl, 3-pentenyl, 4-pentenyl, 1- hexenyl, 2-hexenyl, 3-hexenyl, 4-hexenyl, or 5-hexenyl; (C_2-C_6)alkynyl can be ethynyl, 1-propynyl, 2-propynyl, 1-butynyl, 2-butynyl, 3-butynyl, 1-pentynyl, 2-pentynyl, 3-pentynyl, 4-pentynyl, 1-hexynyl, 2-hexynyl, 3-hexynyl, 4-hexynyl and 5-hexynyl. Similarly, the term "alkynyl" refers to an unsaturated alkyl radical having one or more triple bonds.

The term "haloalkyl" means an alkyl that is optionally substituted with halo. Non limiting examples of "haloalkyl" include iodomethyl, bromomethyl, chloromethyl, fluoromethyl, trifluoromethyl, 2-chloroethyl, 2-fluoroethyl, 2,2,2-trifluoroethyl and pentafluoroethyl.

The term "heterocycle" refers to a saturated or partially unsaturated ring system radical having the overall having from 3-12 ring atoms that contain from one to five heteroatoms selected from N, O, and S. Unless otherwise stated, a "heterocycle" ring can be a monocyclic, a bicyclic, spirocyclic or a polycylic ring system. Non limiting examples of "heterocycle" rings include pyrrolidine, piperidine, N-methylpiperidine, imidazolidine, pyrazolidine, butyrolactam, valerolactam, imidazolidinone, hydantoin, dioxolane, phthalimide and piperidine.

The terms "treat" and "treatment" refer to both therapeutic treatment and/or prophylactic treatment or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder, such as, for example, the development or spread of cancer. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of

disease or disorder, stabilized (i.e., not worsening) state of disease or disorder, delay or slowing of disease progression, amelioration or palliation of the disease state or disorder, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the disease or disorder as well as those prone to have the disease or disorder or those in which the disease or disorder is to be prevented.

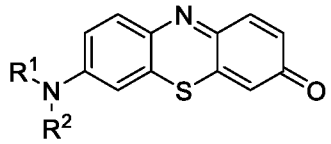
The phrase "effective amount" means an amount of a compound of the present invention that (i) treats or prevents the particular disease, condition, or disorder, (ii) attenuates, ameliorates, or eliminates one or more symptoms of the particular disease, condition, or disorder, or (iii) prevents or delays the onset of one or more symptoms of the particular disease, condition, or disorder described herein. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP) and/or determining the response rate (RR).

It will be appreciated by those skilled in the art that compounds of the invention having a chiral center may exist in and be isolated in optically active and racemic forms. Some compounds may exhibit polymorphism. It is to be understood that the present invention encompasses any racemic, optically-active, polymorphic, or stereoisomeric form, or mixtures thereof, of a compound of the invention, which possess the useful properties described herein, it being well known in the art how to prepare optically active forms (for example, by resolution of the racemic form by recrystallization techniques, by synthesis from optically-active starting materials, by chiral synthesis, or by chromatographic separation using a chiral stationary phase.

When a bond in a compound formula herein is drawn in a non-stereochemical manner (e.g. flat), the atom to which the bond is attached includes all stereochemical possibilities. When a bond in a compound formula herein is drawn in a defined stereochemical manner (e.g. bold, bold-wedge, dashed or dashed-wedge), it is to be understood that the atom to which the stereochemical bond is attached is enriched in the absolute stereoisomer depicted unless otherwise noted. In one embodiment, the compound may be at least 51% the absolute stereoisomer depicted. In another embodiment, the compound may be at least 60% the absolute stereoisomer depicted. In another embodiment, the compound may be at least 80% the absolute stereoisomer depicted. In another embodiment, the compound may be at least 90% the absolute stereoisomer depicted. In another embodiment, the compound may be at

least 95 the absolute stereoisomer depicted. In another embodiment, the compound may be at least 99% the absolute stereoisomer depicted.

In one embodiment, the compound has the following formula Ia:



Ia

or a pharmaceutically acceptable salt thereof.

In one embodiment, R^1 is C_{1-20} alkyl.

In one embodiment, R^1 is *n*-butyl, *n*-decyl or *n*-pentadecyl.

In one embodiment, R^2 is C_{1-20} alkyl.

In one embodiment, R^2 is methyl, *n*-butyl, *n*-decyl or *n*-pentadecyl.

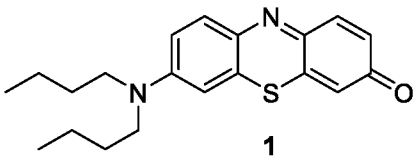
In one embodiment, m is 0 and n is 0.

In one embodiment, R^1 is C_{12-20} alkyl, C_{12-20} alkenyl or C_{12-20} alkynyl, and wherein the C_{12-20} alkyl, C_{12-20} alkenyl and C_{12-20} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

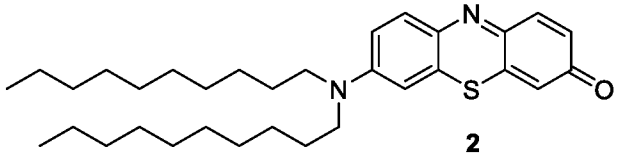
each R^a is independently hydrogen or C_{1-4} alkyl; and

each R^b is independently hydrogen or C_{1-4} alkyl.

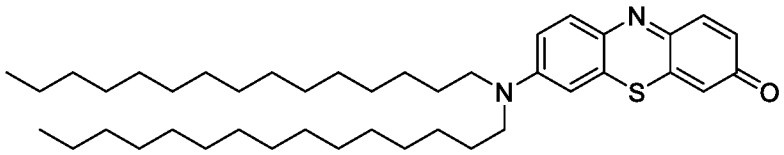
In one embodiment, the compound is selected from the group consisting of:



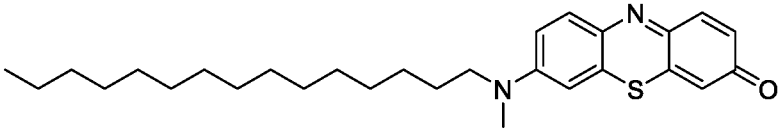
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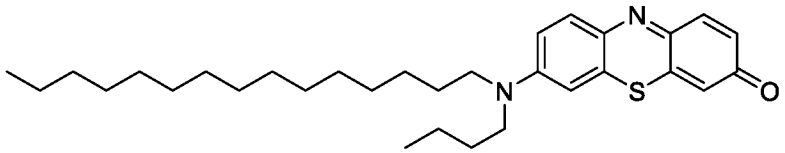


3



4

and



5 ;

and pharmaceutically acceptable salts thereof.

In one embodiment, the neurodegenerative disease is Alzheimer's disease (AD), Parkinson's disease (PD) or Friedreich's ataxia (FRDA).

In one embodiment, the invention provides a method of preserving mitochondrial function in an animal comprising administering to the animal an effective amount of compound of formula (I) or a pharmaceutically acceptable salt thereof.

In one embodiment, the invention also provides a method of protecting cells from oxidative stress in an animal comprising administering to the animal an effective amount of compound of formula (I) or a pharmaceutically acceptable.

In one embodiment, the invention also provides a method of preserving mitochondrial membrane potential and/or augmenting ATP production in an animal comprising administering to the animal an effective amount of compound of formula (I) or a pharmaceutically acceptable salt thereof.

In another embodiment, the invention provides a method of preserving mitochondrial membrane potential and/or augmenting ATP production of a cell *in vitro* comprising contacting the cell with an effective amount of compound of formula (I) or a pharmaceutically acceptable salt thereof.

In one embodiment, the invention provides a compound of formula (I) or a pharmaceutically acceptable salt thereof for use in preserving mitochondrial function.

In one embodiment the invention provides a compound of formula (I) or a pharmaceutically acceptable salt thereof for use in protecting cells from oxidative stress.

In one embodiment, the invention provides a compound of formula (I) or a pharmaceutically acceptable salt thereof for use in preserving mitochondrial membrane potential and/or augmenting ATP production.

In one embodiment, the invention provides the use of a compound of formula (I) or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for preserving mitochondrial function.

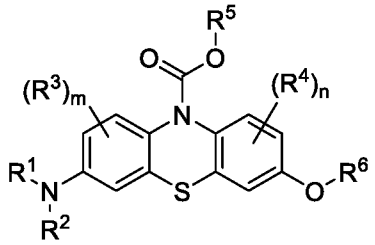
In one embodiment, the invention provides the use of a compound of formula (I) or a

pharmaceutically acceptable salt thereof for the manufacture of a medicament for protecting cells from oxidative stress.

In one embodiment, the invention provides the use of a compound of formula (I) or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for preserving mitochondrial membrane potential and/or augmenting ATP production.

Processes for preparing compounds of formula I are provided as further embodiments of the invention and are illustrated by the following procedures in which the meanings of the generic radicals are as given above unless otherwise qualified.

A compound of formula I can be prepared by converting a corresponding compound of formula II to provide the compound of formula I or a pharmaceutically acceptable salt thereof:



II

wherein:

R^1 is C_{1-20} alkyl, C_{2-20} alkenyl or C_{2-20} alkynyl, and wherein the C_{1-20} alkyl, C_{2-20} alkenyl and C_{2-20} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and -CN;

R^2 is C_{1-20} alkyl, C_{2-20} alkenyl or C_{2-20} alkynyl, and wherein the C_{1-20} alkyl, C_{2-20} alkenyl and C_{2-20} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and -CN; or

R^1 and R^2 taken together with the nitrogen to which they are attached form a 3-12 membered heterocycle that is optionally substituted with one or more groups independently selected from C_{1-4} alkyl, C_{1-4} haloalkyl, –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and -CN;

each R^3 is independently selected from the group consisting of –F, -Cl, -Br, -I, -OH, -OR^a, -SR^a, -NR^aR^b, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and -CN;

each R^4 is independently selected from the group consisting of –F, -Cl, -Br, -I, -OR^a, -

SR^a, $-NR^aR^b$, $-NO_2$, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

R^5 is C_{1-10} alkyl that is optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and –CN;

R^6 is hydrogen or C_{1-6} alkyl that is optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and –CN;

each R^a is independently hydrogen or C_{1-4} alkyl;

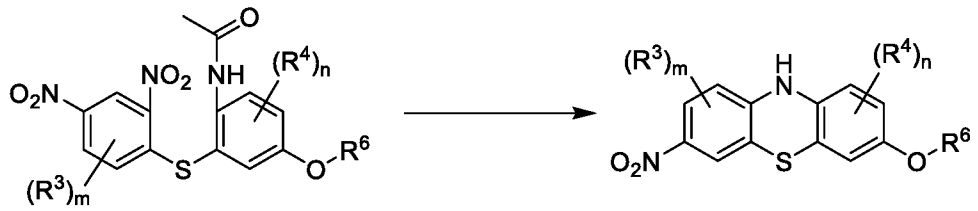
each R^b is independently hydrogen or C_{1-4} alkyl;

the subscript m is 0, 1, 2 or 3; and

the subscript n is 0, 1, 2, or 3;

or a salt thereof.

An important intermediate to provide a compound of formula II is a compound of formula III. A compound of formula III can be prepared by converting a corresponding compound of formula IV to provide the compound of formula III:



IV III

wherein:

each R^3 is independently selected from the group consisting of –F, -Cl, -Br, -I, -OH, $-OR^a$, $-SR^a$, $-NR^aR^b$, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

each R^4 is independently selected from the group consisting of –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, $-NO_2$, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

R^6 is hydrogen or C_{1-6} alkyl that is optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and –CN;

each R^a is independently hydrogen or C_{1-4} alkyl;

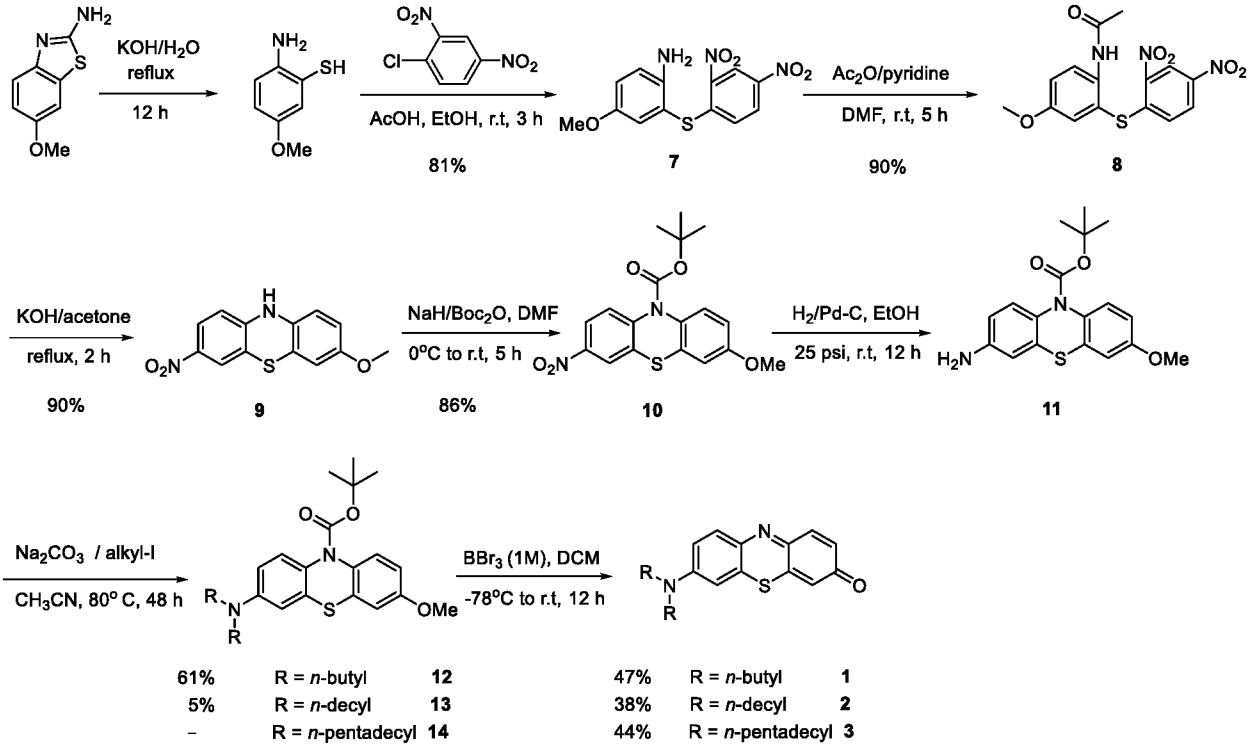
each R^b is independently hydrogen or C_{1-4} alkyl;

the subscript m is 0, 1, 2 or 3; and

the subscript n is 0, 1, 2, or 3;

or a salt thereof.

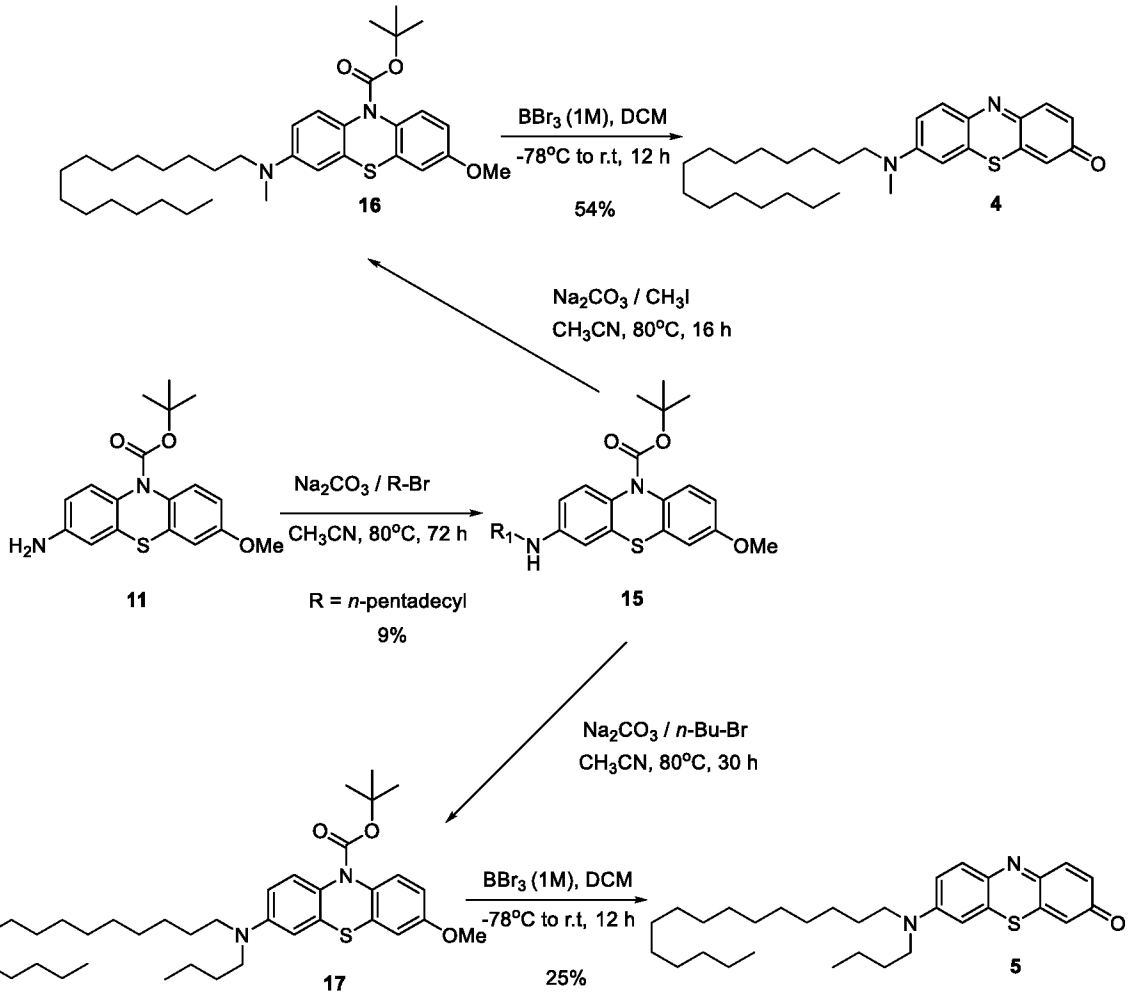
Compounds of formula (I) may be prepared by the process illustrated in Schemes 1 and 2. Representative compounds of formula (I) are compounds **1-5** (Figure 1).



Scheme 1. Synthesis of compounds **1**, **2** and **3**.

Hydrolysis of 2-amino-6-methoxybenzothiazole under strongly basic conditions afforded 2-amino-5-methoxybenzothiol which was coupled with 2, 4-dinitrochlorobenzene to obtain substituted diphenylsulfide intermediate **7** in 81% yield (Yu, L. et al. *Bioorg. Med. Chem.* **2012**, 20, 4625). Compound **7** was treated with acetic anhydride in presence of triethylamine in order to activate the amine as acetamide intermediate **8** in 90% yield. Smiles rearrangement of **8** under mild basic conditions afforded the substituted phenothiazine intermediate **9** in 90% yield (Yu, L. et al. *Bioorg. Med. Chem.* **2012**, 20, 4625). Compound **9** was then treated with 60% NaH followed by di-*tert*-butyl dicarbonate to obtain **10** in 86% yield. Hydrogenolysis of **10** in presence of Pd-C afforded **11**, which was alkylated with different alkyl iodides in the presence of Na_2CO_3 as base to obtain **12-14** in varying yields. In order to remove the O-methyl group to afford the free hydroxyl group at position 7,

compounds **12-14** were treated with 1 M BBr_3 in CH_2Cl_2. The HBr, formed in situ, effected removal of the Boc group at the same time generating the desired compounds **1-3** in yields varying from 38% to 50%.



Scheme 2. Synthesis of compounds **4** and **5**.

In order to synthesize compounds **4** and **5** compound **11** was first alkylated using 1-bromopentadecane. The monoalkylated product **15** was purified on a silica gel column and was subsequently alkylated using either methyl iodide or 1-bromobutane to afford **16** and **17**, respectively. Compounds **16** and **17** were subsequently treated with 1 M BBr_3 to afford **4** and **5**, respectively.

In cases where compounds are sufficiently basic or acidic, a salt of a compound of formula I can be useful as an intermediate for isolating or purifying a compound of formula I. Additionally, administration of a compound of formula I as a pharmaceutically acceptable acid or base salt may be appropriate. Examples of pharmaceutically acceptable salts are

organic acid addition salts formed with acids which form a physiological acceptable anion, for example, tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate, α-ketoglutarate, and α-glycerophosphate. Suitable inorganic salts may also be formed, including hydrochloride, sulfate, nitrate, bicarbonate, and carbonate salts.

Pharmaceutically acceptable salts may be obtained using standard procedures well known in the art, for example by reacting a sufficiently basic compound such as an amine with a suitable acid affording a physiologically acceptable anion. Alkali metal (for example, sodium, potassium or lithium) or alkaline earth metal (for example calcium) salts of carboxylic acids can also be made.

The compounds of formula I can be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient in a variety of forms adapted to the chosen route of administration, i.e., orally or parenterally, by intravenous, intramuscular, topical or subcutaneous routes.

Thus, the present compounds may be systemically administered, e.g., orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the active compound may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of active compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be

coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum

drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

For topical administration, the present compounds may be applied in pure form, i.e., when they are liquids. However, it will generally be desirable to administer them to the skin as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid.

Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, alcohols or glycols or water-alcohol/glycol blends, in which the present compounds can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

Examples of useful dermatological compositions which can be used to deliver the compounds of formula I to the skin are known to the art; for example, see Jacquet et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

Useful dosages of the compounds of formula I can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Pat. No. 4,938,949.

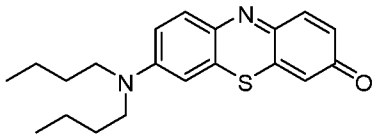
The amount of the compound, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, e.g., into a number of discrete loosely spaced

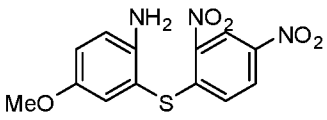
administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

The invention will now be illustrated by the following non-limiting Examples.

Example 1. Synthesis of 7-(*N*,*N*-Dibutylamino)-3*H*-phenothiazin-3-one (1)

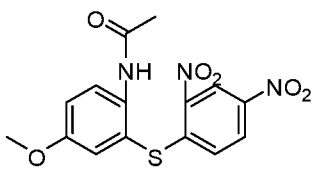


Step 1. Synthesis of 2-(2,4-Dinitrophenyl)thio)-5-methoxyaniline (7)



A sample of 2.00 g (11.1 mmol) of 2-amino-6-methoxybenzothiazole was suspended in 40 mL of water and 9.30 g (167 mmol) of solid KOH was added. The suspension was heated to reflux for 12 h. The reaction mixture was cooled to room temperature and added dropwise to a solution of 2.25 g (11.1 mmol) of 2, 4-dinitrochlorobenzene in a mixture of ethanol (30 mL)–AcOH (20 mL) in ice–water bath. The reaction mixture was stirred at room temperature for an additional 3 h. The precipitate was filtered, washed with water–ethanol (1:1, v/v) and dried to afford **7** as an orange solid: yield 2.90 g (81%); silica gel TLC R_f 0.7 (3:7 ethyl acetate–hexanes); ^{1}H NMR (CD_3COCD_3) δ 3.76 (s, 3H), 6.85 (d, 1H, J = 8.8 Hz), 6.97 (d, 1H, J = 2.8 Hz), 7.00-7.06 (m, 2H), 8.18 (dd, 1H, J = 9.2 Hz, J = 2.6 Hz) and 9.12 (d, 1H, J = 2.4 Hz); ^{13}C NMR (DMSO-d_6) δ 55.9, 111.0, 117.5, 120.4, 120.6, 121.6, 127.1, 128.6, 143.3, 144.4, 144.6, 145.8 and 153.0; mass spectrum (APCI), m/z 322.0499 $(M + H)^+$ ($C_{13}H_{12}N_3O_5S$ requires *m/z* 322.0498).

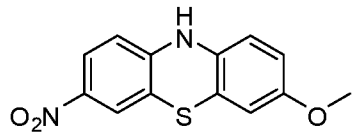
Step 2. Synthesis of *N*-(2-((2,4-Dinitrophenyl)thio)-5-methoxyphenyl)acetamide (**8**)



To a solution of 2.90 g (9.03 mmol) of **7** in 10 mL of anhydrous DMF was added 3.66 mL (2.74 g, 27.1 mmol) of anhydrous triethylamine followed by 4.30 mL (4.64 g, 45.5 mmol) of acetic anhydride. The reaction mixture was stirred for 12 h at room temperature under an argon atmosphere and was quenched by pouring into ice-cold water. The aqueous layer was

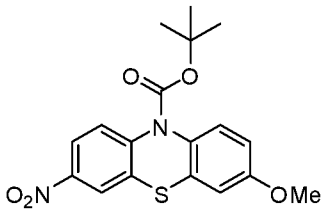
extracted with four 25-mL portions of ethyl acetate. The combined organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated under diminished pressure. The residue was purified on a silica gel column (8 × 4 cm). Elution with 3:7 ethyl acetate–hexanes afforded **8** as a bright yellow solid: yield 2.95 g (90%); silica gel TLC R_f 0.57 (3:7 ethyl acetate–hexanes); ^{1}H NMR (DMSO-d_6) δ 1.86 (s, 3H), 3.77 (s, 3H), 6.99 (d, 1H, J = 8.8 Hz), 7.18 (dd, 1H, J = 8.8 Hz, J = 2.4 Hz), 7.23 (d, 1H, J = 2.8 Hz), 7.66 (d, 1H, J = 8.8 Hz), 8.32 (dd, 1H, J = 9.0 Hz, J = 2.6 Hz), 8.88 (d, 1H, J = 2.4 Hz) and 9.43 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 22.8, 55.6, 117.7, 120.5, 121.0, 123.8, 127.5, 128.1, 129.2, 133.7, 144.05, 144.07, 145.6, 157.2 and 168.7; mass spectrum (APCI), m/z 364.0609 $(M + H)^+$ ($C_{15}H_{14}N_3O_6S$ requires *m/z* 364.0603).

Step 3. Synthesis of 3-Methoxy-7-nitro-10*H*-phenothiazine (**9**)



To a stirred solution of 2.95 g (8.13 mmol) of **8** in 20 mL of acetone, at reflux, was added in portions 0.91 g (16.2 mmol) of KOH in 10 mL of ethanol. The reaction mixture was kept at reflux for an additional 3 h and poured into ice-cold water. The aqueous layer was extracted with four 25-mL portions of ethyl acetate. The combined organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated under diminished pressure. The residue was purified on a silica gel column (8 × 2 cm). Elution with 1:1 ethyl acetate–hexanes gave **9** as a violet solid: yield 2.00 g (90%); silica gel TLC R_f 0.43 (3:7 ethyl acetate–hexanes); ^{1}H NMR (CD_3COCD_3) δ 3.70 (s, 3H), 6.57-6.70 (m, 4H), 7.73 (d, 1H, J = 2.4 Hz), 7.81-7.84 (m, 1H) and 8.47 (br s, 1H); ^{13}C NMR (CD_3COCD_3) δ 55.0, 111.7, 113.0, 113.3, 116.1, 117.0, 117.2, 121.6, 124.2, 132.4, 141.5, 148.3 and 156.6; mass spectrum (APCI), m/z 275.0488 $(M + H)^+$ ($C_{13}H_{11}N_2O_3S$ requires *m/z* 275.0490).

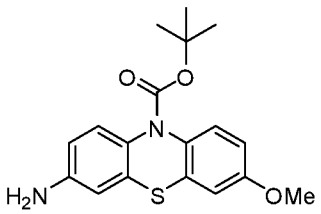
Step 4. Synthesis of *tert*-Butyl 3-Methoxy-7-nitro-10*H*-phenothiazin-10-carboxylate (**10**)



To a solution of 1.42 g (5.18 mmol) of **9** in 20 mL of anhydrous DMF at 0 °C was

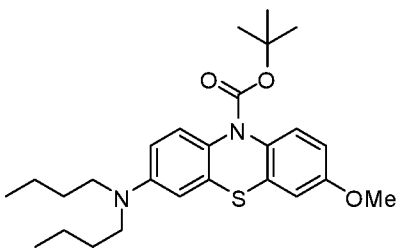
added 0.55 g (13.7 mmol) of 60% NaH. The reaction mixture was stirred at 0 °C for another 15 min and 2.40 g (11.0 mmol) of di-*tert*-butyl dicarbonate was added. The reaction mixture was stirred at room temperature for 4 h under an arogon atmosphere, and was quenched with 30 mL of water. The aqueous layer was extracted with three 20-mL portions of ethyl acetate. The combined organic layer was washed with 20 mL of brine, dried over anhydrous $MgSO_4$ and concentrated under diminished pressure. The residue was purified on a silica gel column (8 × 4 cm). Elution with 3:7 ethyl acetate–hexanes afforded **10** as a bright yellow solid: yield 1.66 g (86%); silica gel TLC R_f 0.54 (3:7 ethyl acetate–hexanes); ^{1}H NMR (CD_3COCD_3) δ 1.54 (s, 9H), 3.86 (s, 3H), 6.97 (dd, 1H, J = 9.2 Hz, J = 2.8 Hz), 7.01 (d, 1H, J = 2.8 Hz), 7.47 (d, 1H, J = 9.2 Hz), 7.84 (d, 1H, J = 8.8 Hz), 8.20 (dd, 1H, J = 9.0 Hz, J = 2.6 Hz) and 8.24 (d, 1H, J = 2.4 Hz); ^{13}C NMR (CD_3COCD_3) δ 29.2, 57.1, 84.3, 113.3, 115.6, 123.7, 124.1, 129.8, 130.0, 132.3, 133.1, 135.3, 146.9, 147.1, 153.2 and 159.9; mass spectrum (APCI), m/z 374.0932 $(M + H)^+$ ($C_{18}H_{19}N_2O_5S$ requires *m/z* 374.0936).

Step 5. Synthesis of *tert*-Butyl 3-Amino-7-methoxy-10*H*-phenothiazin-10-carboxylate (**11**)



To a suspension of 0.42 g (1.12 mmol) of **10** in 10 mL of ethanol was added 10 mg of 10% Pd on carbon. The reaction mixture was stirred at room temperature under a hydrogen atmosphere (25 psi) overnight. The reaction mixture was filtered through a Celite pad and was concentrated under diminished pressure. The crude product (**11**) was used for the next reaction without further purification.

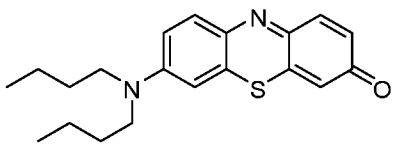
Step 6. Synthesis of *tert*-Butyl 3-(*N*,*N*-Dibutylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate (**12**)



To a solution of crude **11** in 3 mL of acetonitrile was added 1.40 g (12.8 mmol) of Na_2CO_3 followed by 0.94 g (5.12 mmol) of 1-iodobutane. The reaction mixture was sealed

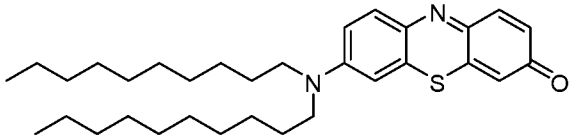
under nitrogen atmosphere and stirred at 80 °C overnight. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under diminished pressure and purified on a silica gel column (10 x 2 cm). Elution with 1:9 ethyl acetate–hexanes gave compound **12** as a pale yellow solid: yield 0.31 g (61%); silica gel TLC R_f 0.43 (1:9 ethyl acetate–hexanes); ^{1}H NMR ($CDCl_3$) δ 0.95 (t, 6H, J = 7.4 Hz), 1.32-1.37 (m, 4H), 1.49 (s, 9H), 1.51-1.57 (m, 4H), 3.23 (t, 4H, J = 7.6 Hz), 3.78 (s, 3H), 6.52-6.55 (m, 2H), 6.78 (dd, 1H, J = 8.8 Hz, J = 2.8 Hz), 6.86 (d, 1H, J = 2.8 Hz), 7.31 (d, 1H, J = 8.4 Hz) and 7.39 (d, 1H, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$) δ 13.9, 20.2, 28.1, 29.2, 50.8, 55.5, 81.2, 109.1, 110.2, 111.5, 112.6, 126.8, 127.2, 127.5, 132.3, 132.5, 133.2, 146.2, 153.2 and 157.0; mass spectrum (APCI), m/z 457.2530 $(M + H)^+$ ($C_{26}H_{37}N_2O_3S$ requires *m/z* 457.2525).

Step 7. Synthesis of 7-(*N*,*N*-Dibutylamino)-3*H*-phenothiazin-3-one (**1**)

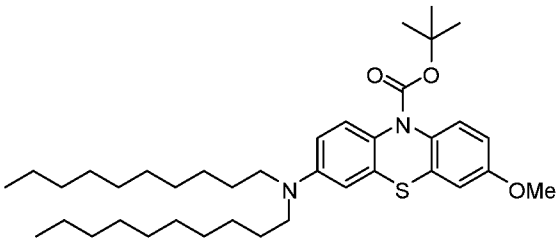


To a solution of 86.0 mg (0.19 mmol) of **12** in 2 mL of anhydrous CH_2Cl_2 was added dropwise 0.76 mL (0.76 mmol) of 1 M BBr_3 in CH_2Cl_2 at –78 °C. The reaction mixture was stirred overnight at room temperature under an argon atmosphere and was quenched with 10 mL of water. The aqueous layer was extracted with two 10-mL portions of ethyl acetate. The combined organic layer was washed with 10 mL of brine, dried over anhydrous $MgSO_4$ and concentrated under diminished pressure. The residue was purified on a silica gel column (7 x 2 cm). Elution with 1:1 ethyl acetate–hexanes afforded **1** as a violet solid: yield 30 mg (47%); silica gel TLC R_f 0.42 (1:1 ethyl acetate–hexanes); ^{1}H NMR ($CDCl_3$) δ 0.95 (t, 6H, J = 7.4 Hz), 1.26-1.37 (m, 4H), 1.51-1.57 (m, 4H), 3.23 (m, 4H), 6.52-6.55 (m, 2H), 6.76-6.87 (m, 2H), 7.31 (d, 1H, J = 8.8 Hz) and 7.38 (d, 1H, J = 8.8 Hz); mass spectrum (APCI), m/z 341.1690 $(M + H)^+$ ($C_{20}H_{25}N_2OS$ requires *m/z* 341.1688).

Example 2. Synthesis of 7-(*N*,*N*-Didecylamino)-3*H*-phenothiazin-3-one (2)

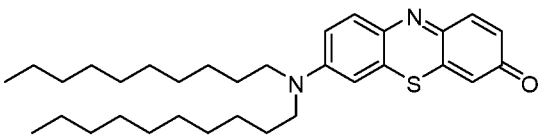


Step 1. Synthesis of *tert*-Butyl 3-(*N*,*N*-Didecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate (**13**)



0.74 g (2.90 mmol) of 1-iododecane in 2 mL of acetonitrile was added into a mixture of 0.40 g (~ 1.16 mmol) of crude **11** in 2 mL of acetonitrile and 1.23 g (11.6 mmol) of Na_2CO_3. The reaction mixture was sealed under nitrogen atmosphere and stirred at 80 °C for ~ 48 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under diminished pressure and purified on a silica gel column (8 x 2 cm). Elution with 1:19 ethyl acetate–hexanes gave compound **13** as a pale yellow solid: yield 30 mg (5%); silica gel TLC R_f 0.41 (1:19 ethyl acetate–hexanes) ^{1}H NMR ($CDCl_3$) δ 0.81 (t, 6H, J = 6.8 Hz), 1.19 (m, 28H), 1.22 (s, 9H), 1.40-1.46 (m, 4H), 3.13 (t, 4H, J = 7.6 Hz), 3.69 (s, 3H), 6.42-6.44 (m, 2H), 6.90 (dd, 1H, J = 8.6 Hz, J = 2.6 Hz), 6.70 (d, 1H, J = 2.8 Hz), 7.21 (d, 1H, J = 8.4 Hz) and 7.30 (d, 1H, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.7, 27.1, 28.2, 29.3, 29.5, 29.6, 31.9, 51.2, 55.5, 81.2, 109.1, 110.2, 111.6, 112.6, 126.8, 127.3, 127.6, 132.4, 132.6, 133.2, 146.3, 153.3 and 157.1; mass spectrum (APCI), m/z 625.4390 $(M + H)^+$ ($C_{38}H_{61}N_2O_3S$ requires *m/z* 625.4403).

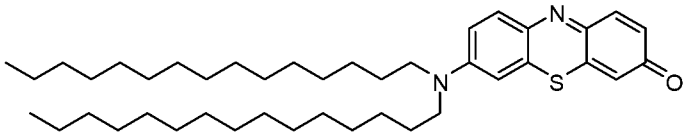
Step 2. Synthesis of 7-(*N*,*N*-Didecylamino)-3*H*-phenothiazin-3-one (**2**)



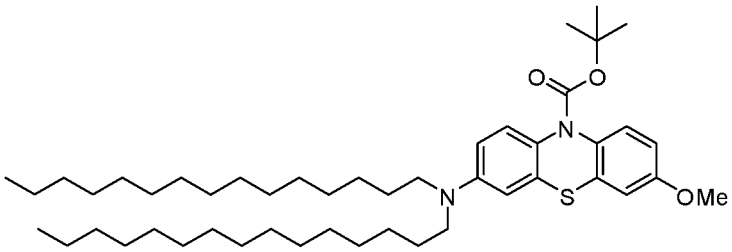
0.20 mL (0.20 mmol) of 1 M BBr_3 in CH_2Cl_2 was added dropwise into a solution of 30.0 mg (0.05 mmol) of compound **13** in 4 mL of CH_2Cl_2 at -78 °C. The reaction mixture was stirred overnight at ambient temperature and was quenched with 10 mL of water. The product was extracted with two 10-mL portions of ethyl acetate. The violet organic layer was washed

with 20 mL of brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting violet solid was purified on a silica gel column (7 x 2 cm). Elution with 1:2 ethyl acetate–hexanes afforded **2** as a violet solid: yield 9 mg (38%); silica gel TLC R_f 0.50 (1:2 ethyl acetate–hexanes); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 6H, J = 6.8 Hz), 1.24-1.34 (m, 28H), 1.63 (br s, 4H), 3.36 (t, 4H, J = 7.8 Hz), 6.51 (d, 1H, J = 2.8 Hz), 6.66 (d, 1H, J = 2.0 Hz), 6.75-6.82 (m, 2H), 7.53 (d, 1H, J = 9.6 Hz) and 7.46 (d, 1H, J = 9.2 Hz); ^{13}C NMR ($CDCl_3$) δ 14.1, 22.6, 27.0, 27.3, 29.3, 29.4, 29.50, 29.54, 29.7, 31.8, 51.4, 104.7, 113.2, 118.4, 128.6, 130.9, 132.1, 134.8, 135.9, 139.4, 139.6, 149.8 and 182.1; mass spectrum (APCI), m/z 509.3553 $(M + H)^+$ ($C_{32}H_{49}N_2OS$ requires *m/z* 509.3566).

Example 3. Synthesis of 7-(*N*,*N*-Dipentadecylamino)-3*H*-phenothiazin-3-one (3)

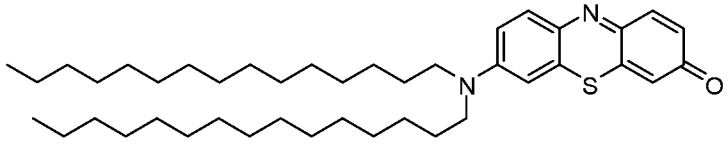


Step 1. Synthesis of *tert*-Butyl 3-(*N*,*N*-Dipentadecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate (**14**).



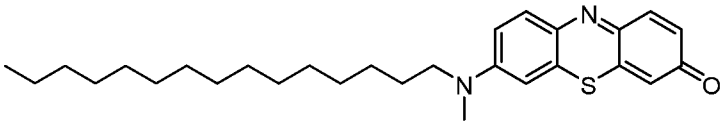
0.98 g (2.90 mmol) of 1-iodopentadecane in 2 mL of acetonitrile was added into a mixture of 0.40 g (~ 1.16 mmol) of crude **11** in 2 mL of acetonitrile and 1.23 g (11.6 mmol) of Na_2CO_3. The reaction mixture was sealed under nitrogen atmosphere and stirred at 80 °C for ~ 48 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under diminished pressure and purified on a silica gel column to remove the unreacted starting material (8 x 2 cm). Elution with 1:19 ethyl acetate–hexanes gave a mixture of mono and dialkylated product as a yellow oil.

Step 2. Synthesis of 7-(*N*,*N*-Dipentadecylamino)-3*H*-phenothiazin-3-one (**3**)

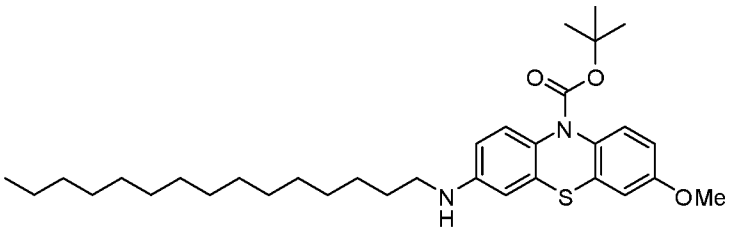


0.28 mL (0.28 mmol) of 1 M BBr_3 in CH_2Cl_2 was added dropwise into the solution of 50.0 mg of crude compound **14** in 2 mL of CH_2Cl_2 at –78 °C. The reaction mixture was stirred overnight at ambient temperature and was quenched with 10 mL of water. The product was extracted with two 10-mL portions of ethyl acetate. The combined organic layer was washed with 20 mL of brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting violet solid was purified on a silica gel column. Elution with 1:4 ethyl acetate–hexanes afforded **3** as a violet solid: yield 20 mg (44%); silica gel TLC R_f 0.50 (1:4 ethyl acetate–hexanes); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 6H, J = 6.8 Hz), 1.26-1.35 (m, 48H), 1.62-1.64 (m, 4H), 3.37 (t, 4H, J = 7.6 Hz), 6.53 (d, 1H, J = 2.8 Hz), 6.67 (d, 1H, J = 2.0 Hz), 6.76-6.84 (m, 2H), 7.55 (d, 1H, J = 9.6 Hz) and 7.66 (d, 1H, J = 9.2 Hz); ^{13}C NMR ($CDCl_3$) δ 14.3, 22.8, 27.1, 27.5, 29.50, 29.53, 29.6, 29.7, 29.77, 29.79, 29.81, 29.83, 32.1, 51.5, 104.8, 113.3, 118.7, 128.7, 131.0, 132.4, 134.9, 136.0, 139.6, 140.0, 150.0 and 182.4; mass spectrum (APCI), m/z 648.5144 $(M + H)^+$ ($C_{42}H_{69}N_2OS$ requires *m/z* 649.5131).

Example 4. Synthesis of 7-(*N*-Methyl-*N*-pentadecylamino)-3*H*-phenothiazin-3-one (4)



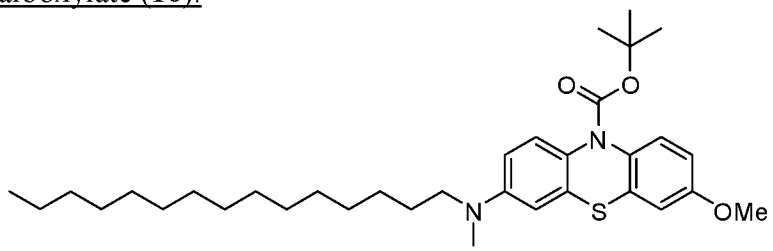
Step 1. Synthesis of *tert*-Butyl 3-(*N*-Pentadecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate (**15**).



2.11 g (7.25 mmol) of 1-bromopentadecane in 3 mL of acetonitrile was added into a mixture of 0.50 g (~ 1.45 mmol) of crude compound **11** in 2 mL of acetonitrile and 1.55 g (14.6 mmol) of Na_2CO_3. The reaction mixture was sealed under nitrogen atmosphere and stirred at 80 °C for ~ 3 days. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under diminished pressure and purified on a silica gel

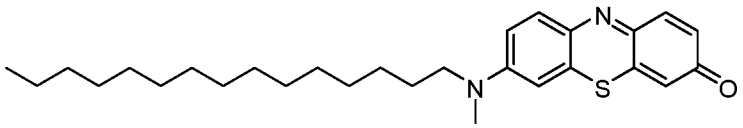
column. Elution with 1:9 ethyl acetate–hexanes gave compound **15** as a pale yellow solid: yield 0.14 g (9%); silica gel TLC R_f 0.31 (1:9 ethyl acetate–hexanes); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, J = 6.6 Hz), 1.25 (br s, 24H), 1.45 (s, 9H), 1.52-1.57 (m, 2H), 3.04 (t, 2H, J = 7.2 Hz), 3.76 (s, 3H), 6.45 (dd, 1H, J = 8.6 Hz, J = 2.6 Hz), 6.50 (d, 1H, J = 2.8 Hz), 6.76 (dd, 1H, J = 8.8 Hz, J = 2.8 Hz), 6.83 (d, 1H, J = 2.8 Hz), 7.24 (d, 1H, J = 8.4 Hz) and 7.36 (d, 1H, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 22.8, 27.2, 28.20, 28.27, 28.31, 28.4, 29.46, 29.5, 29.69, 29.70, 29.75, 29.8, 32.0, 44.2, 55.7, 81.4, 109.8, 111.65, 111.7, 112.8, 127.6, 127.8, 128.7, 132.5, 132.7, 133.3, 146.7, 153.3 and 157.3; mass spectrum (APCI), m/z 555.3629 (M + H)$^+$ ($C_{33}H_{51}N_2O_3S$ requires *m/z* 555.3620).

Step 2. Synthesis of *tert*-Butyl 3-(*N*-Methyl-*N*-pentadecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate (**16**).



7.00 μL (16.0 mg, 0.11 mmol) of iodomethane was added into a mixture of 53.0 mg (0.10 mmol) of compound **15** in 2 mL of acetonitrile and 16.0 mg (0.15 mmol) of Na_2CO_3. The reaction mixture was sealed under nitrogen atmosphere and stirred at 80 °C for ~ 16 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under diminished pressure. The crude was used to the next step without further purification.

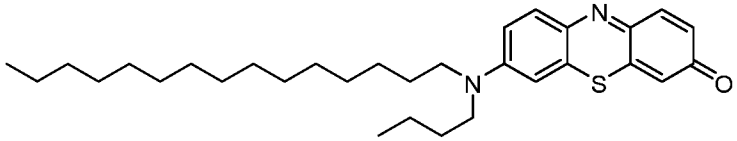
Step 3. Synthesis of 7-(*N*-Methyl-*N*-pentadecylamino)-3*H*-phenothiazin-3-one (**4**)



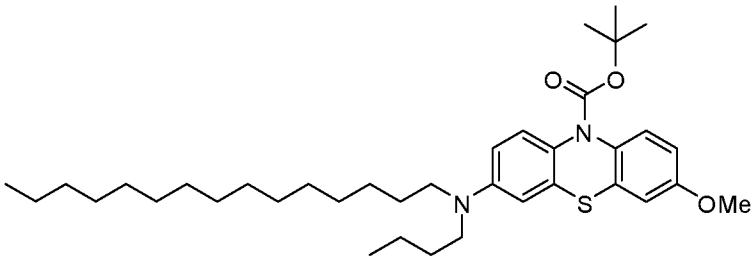
0.27 mL (0.27 mmol) of 1 M BBr_3 in CH_2Cl_2 was added dropwise into the solution of 50.0 mg of crude **16** in 2 mL of CH_2Cl_2 at -78 °C. The reaction mixture was stirred overnight at room temperature and was quenched with 10 mL of water. The product was extracted with two 10-mL portions of ethyl acetate. The combined organic layer was washed with 20 mL of brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting violet solid was purified on a silica gel column (7 x 2 cm). Elution with 3:7 ethyl acetate–hexanes afforded **5** as a violet solid: yield 22 mg (54%); silica gel TLC R_f 0.50 (1:4

ethyl acetate–hexanes); ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 3H, J = 6.4 Hz), 1.24-1.32 (m, 24H), 1.60-1.65 (m, 2H), 3.09 (s, 3H), 3.37 (t, 2H, J = 7.6 Hz), 6.55 (d, 1H, J = 2.8 Hz), 6.68 (d, 1H, J = 2.4 Hz), 6.80-6.84 (m, 2H), 7.54 (d, 1H, J = 9.6 Hz) and 7.67 (d, 1H, J = 9.2 Hz); ^{13}C NMR ($CDCl_3$) δ 14.2, 22.6, 27.1, 27.4, 29.50, 29.55, 29.6, 29.7, 29.77, 29.78, 29.81, 29.83, 51.5, 104.8, 113.3, 118.7, 128.7, 131.0, 132.4, 134.8, 136.1, 139.6, 140.2, 150.0 and 182.4; mass spectrum (APCI), m/z 453.2947 $(M + H)^+$ ($C_{28}H_{41}N_2OS$ requires *m/z* 453.2940).

Example 5. Synthesis of 7-(*N*-Butyl-*N*-pentadecylamino)-3*H*-phenothiazin-3-one (5).

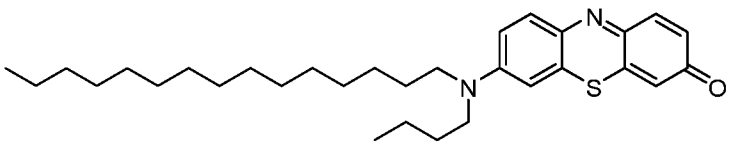


Step 1. Synthesis of *tert*-Butyl 3-(*N*-Butyl-*N*-pentadecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate (**17**)



0.11 mL (0.14 g, 1.00 mmol) of 1-bromobutane was added into a mixture of 90.0 mg (0.16 mmol) of compound **15** in 2 mL of acetonitrile and 0.17 g (1.60 mmol) of Na_2CO_3. The reaction mixture was sealed under nitrogen atmosphere and stirred at 80 °C for ~ 30 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under diminished pressure. The crude product was used to the next step without further purification.

Step 2. Synthesis of 7-(*N*-Butyl-*N*-pentadecylamino)-3*H*-phenothiazin-3-one (**5**)



0.48 mL (0.48 mmol) of 1 M BBr_3 in CH_2Cl_2 was added dropwise into the solution of 100 mg (~ 0.16 mmol) of crude **17** in 2 mL of CH_2Cl_2 at -78 °C. The reaction mixture was stirred overnight at room temperature and was quenched with 10 mL of water. The crude

product was extracted with two 10-mL portions of ethyl acetate. The combined organic layer was washed with 20 mL of brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting violet solid was purified on a silica gel column (7 x 2 cm). Elution with 3:7 ethyl acetate–hexanes afforded **6** as a violet solid: yield 20 mg (25%); silica gel TLC R_f 0.50 (1:4 ethyl acetate–hexanes); ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, J = 6.6 Hz), 1.00 (t, 3H, J = 7.2 Hz), 1.26-1.43 (m, 26H), 1.62-1.64 (m, 4H), 3.36-3.41 (m, 4H), 6.55 (d, 1H, J = 2.4 Hz), 6.71 (s, 1H), 6.79-6.85 (m, 2H), 7.57 (d, 1H, J = 9.6 Hz) and 7.68 (d, 1H, J = 9.2 Hz); ^{13}C NMR ($CDCl_3$) δ 14.3, 22.8, 27.1, 27.5, 29.50, 29.53, 29.6, 29.7, 29.77, 29.79, 29.81, 29.83, 32.1, 51.5, 104.8, 113.3, 118.7, 128.7, 131.0, 132.4, 134.9, 136.0, 139.6, 140.0, 150.0 and 182.4; mass spectrum (APCI), m/z 495.3330 $(M + H)^+$ ($C_{31}H_{47}N_2OS$ requires *m/z* 495.3331).

Example 6. Cytotoxicity and cytoprotection evaluation

Cytotoxicity was assessed by the dye exclusion method. Live cells have intact membranes, which exclude a variety of dyes that easily penetrate the damaged, permeable membranes of non-viable cells. Propidium iodide, a membrane impermeant dye, is generally excluded from viable cells, although it can enter non-viable cells and bind to double-stranded DNA by intercalating between base pairs. Compounds **1-5** were tested for their cytotoxicity in FRDA lymphocytes using FACS analysis with propidium iodide. The results are depicted in Figure 2. From the results it is evident that cytotoxicity decreases with increasing lipophilicity in lower as well as higher concentrations for compounds **1-5**.

The ability of compounds **1-5** to demonstrate cyotoprotection was measured in FRDA lymphocytes. The cells were treated with rotenone to induce cytotoxicity by inhibiting complex I. The results are shown in Figure 3. Compounds **4** and **5** were able to protect the cells against rotenone cytotoxicity more efficiently than the parent compound MV.

Cytotoxicity Assay

Evaluation of compounds **1-5** for their cytotoxicity and their ability to function within the mitochondrial respiratory chain was carried out by incubation of the prepared compounds for 24 or 48 h with FRDA lymphocytes. A nutrient-sensitized screening strategy has been used by culturing FRDA cells in galactose as the sole sugar source which forces mammalian cells to rely on mitochondrial oxidative phosphorylation (OXPHOS) to produce their ATP; they also become more sensitive to mitochondrial respiratory chain inhibitors than cells

grown in glucose medium. Compounds **1-5** were tested for their cytotoxicity in FRDA lymphocytes using a simultaneous staining with a two-color fluorescence assay, the Live/ Dead® Viability/Cytotoxicity Kit (Molecular Probes). This assay is used to measure two recognized parameters of cell viability, intracellular esterase activity and plasma integrity. The membrane-impermeant DNA dye ethidium homodimer-1 (EthD-1) was used to identify dead cells whose plasma membrane integrity was disrupted. The membrane-permeant dye calcein-AM was used to label live cells. It penetrates into the cells where it is metabolized by cytoplasmic esterases and becomes a fluorescent but membrane-impermeant probe which is retained in viable cells. One mL of FRDA lymphocyte cells (5×10^5 cells) was plated in a 24-well plate in glucose free media (galactose 25 mM), treated with the test compounds and incubated at 37 °C for 24 h or 48 h in a humidified atmosphere containing 5% CO_2 in air. Cells were collected by centrifugation at 300 × g for 3 min and washed with phosphate buffered saline. Cells were resuspended in phosphate buffered saline containing 25 mM galactose. Cell suspension was stained with 0.1 μM calcein AM and 0.2 μM EthD-1 and incubated in the dark at 37 °C for 15 minutes. Cells were collected by centrifugation at 300 × g for 3 min and then washed with PBS. The samples were analyzed immediately by flow cytometry (C6 Accuri, BD Biosciences, San Jose, CA), using a 488 nm excitation laser and the and the FL1-H channel 530 ±15 nm emission filter and the FL2-H channel 585 ±15 nm. For each analysis 10,000 events were recorded and analyzed using C6 Accuri software (BD Biosciences).

Example 7. Preserving mitochondrial inner membrane potential ($\Delta\Psi_m$)

The ability of **1-5** to maintain mitochondrial membrane potential ($\Delta\Psi_m$) compared to the parent compound MV was studied. $\Delta\Psi_m$ was determined using the cationic fluorescent dye 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1), which is a lipophilic cation that selectively accumulates in mitochondria due to the negative potential across the inner mitochondrial membrane. The dye exists as a monomer at low concentrations giving a green fluorescence. At higher concentrations the dye forms J-aggregates, which exhibit red fluorescence. Therefore, mitochondrial depolarization is indicated by a decrease in red/green fluorescence ratio. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a commonly used uncoupler of oxidative phosphorylation in mitochondria, was used as a negative control to dissipate the chemiosmotic proton gradient, which results in depolarization of mitochondrial membrane

potential. Figure 4 summarizes the results of this study. Clearly compounds **2-5** were more effective in preserving mitochondrial membrane potential than the parent compound MV.

JC-1 Mitochondrial Membrane Potential ($\Delta\psi_m$) Assay

The ability of the test compounds to depolarize or maintains mitochondrial inner membrane potential ($\Delta\psi_m$) was assessed using the JC-1 probe. JC-1 is a cationic dye which exhibits potential-dependent accumulation in mitochondria. JC-1 is a dual stain, which can identify high membrane potential through J-aggregates (red fluorescence) and low membrane potential through J-monomers (green fluorescence). When the $\Delta\psi_m$ collapses, the reagent (JC-1) no longer accumulates inside the mitochondria; instead, it is diffuses throughout the cell cytosol in the monomeric form which fluoresces green. The detection of mitochondrial depolarization using JC-1was accomplished by flow cytometry as described before (Arce *et al.* (2012) *Bioorg. Med. Chem.*20, 5188). Briefly, FRDA lymphocytes cells (5×10^5 cells) were pre-treated with or without the test compounds for 16 h. The cells were incubated at 37 °C in the dark for 20 min with 1 μM JC-1. Cells were collected by centrifugation at 300 × g for 3 min and washed with phosphate buffered saline. Cells were resuspended in phosphate buffered saline supplemented with 20 mM glucose and were analyzed immediately by FACS (C6 Accuri, BD Biosciences, San Jose, CA), using a 488 nm excitation laser and the FL1-H channel 530 ±15 nm emission filter and the FL2-H channel 585 ±15 nm. For each analysis 10,000 events were recorded and analyzed using C6 Accuri software (BD Biosciences). FCCP (carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone), a mitochondrial uncouple, was used to produce a negative control.

Example 8. Suppression of reactive oxygen species

The ability of compounds **1-5** to suppress ROS induced by the depletion of cellular glutathione was evaluated in FRDA lymphocytes in a quantitative FACS experiment using dichlorofluorescein diacetate (DCFH-DA) as an indicator of intracellular ROS levels. In cells DCFH-DA is hydrolyzed by esterases to afford 2, 7-dichlorodihydrofluorescein (DCFH). The non-fluorescent DCFH can be oxidized by cellular oxidants generating fluorescent dichlorofluorescein (DCF). The results are shown in Figure 5, and demonstrate that compounds **4** and **5** were more potent and effective in protecting FRDA lymphocytes than the parent compound MV and did so in a concentration dependent manner.

Reactive Oxygen Species (ROS) Assay

Intracellular ROS production was measured in FRDA lymphocyte cells (GM15850, Coriell Cell Repositories, Camden, NJ) using the oxidant sensitive fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes) as described previously. One mL of FRDA lymphocyte cells (5×10^5 cells) was plated in a 24-well plate, treated with the test compounds and incubated at 37 °C for 16 h in a humidified atmosphere containing 5% CO_2 in air. Cells were treated with or without 5 mM diethyl maleate (DEM) for 80 min, collected by centrifugation at 300 × g for 3 min and then washed with phosphate buffered saline (PBS) (Life Technologies). Cells were resuspended in PBS containing 20 mM glucose and incubated at 37 °C in the dark for 25 min with 10 μM DCFH-DA. Cells were collected by centrifugation at 300 × g for 3 min and then washed with PBS. The samples were analyzed immediately by flow cytometry (C6 Accuri, BD Biosciences, San Jose, CA), using a 488 nm excitation laser and the FL1-H channel 530 ±15 nm emission filter for DCF and FL2-H channel 585 ±15 nm emission filter for propidium iodide. The generation of ROS, mainly peroxides, was detected as a result of the oxidation of DCFH. In each analysis, 10,000 events were recorded after dead cell and debris were gated out. Apoptotic and dead cells were excluded by using propidium iodide (1 μg/mL) as counter stain dye for nucleic acid. ROS production was evaluated only in living cells which are propidium iodide negative. Propidium iodide is excluded by viable cells but can penetrate cell membranes of dying or dead cells. Results obtained were verified by running duplicates and repeating experiments in three independent runs. Results were expressed as percentage of ROS scavenging activity or as percentage of the median fluorescence intensity of DCF.

Example 9. Determination of ATP levels

ATP levels in FRDA lymphocytes were measured using firefly luciferase. The cells were grown on glucose-free media supplemented with galactose for 24 h prior to the addition of the test compounds. Since cells grown in galactose rely mostly on oxidative phosphorylation (OX-PHOS) to produce their ATP, they become more sensitive to mitochondrial respiratory chain inhibitors than cells grown in glucose medium. The results are shown in Figure 6. MV strongly reduces cellular ATP levels in a concentration dependent manner. Compounds **1** and **2** reduces cellular ATP levels in all concentrations. Compounds **3-5** maintain ATP levels up to 2.5 μM concentration, beyond that they reduce ATP levels significantly.

Measurement of Cellular ATP Concentration

Total ATP levels in FRDA lymphocytes with or without partially blocking complex I activity with rotenone (5 nM) were measured using firefly luciferase as a means to measure ATP level. The cells were grown on glucose-free media supplemented with galactose. Since cells grown in galactose rely mostly on oxidative phosphorylation (OXPHOS) to produce their ATP, they become more sensitive to mitochondria respiratory chain inhibitors than cells grown in glucose medium. Briefly, lymphocytes (2×10^5cell/mL) were plated (1 mL) in 24-well plates, treated with the test compounds, and then incubated at 37 °C for 48 h in a humidified atmosphere containing 5% CO_2 in air. FRDA lymphocytes with partial inhibition of complex I, cells were preincubated with test compounds for overnight before rotenone (5 nM) treatment for 24 h. Cells in each well were mixed and transferred (100 µL) to 96-well microtiter black-walled cell culture plates (Costar, Corning, NY). The total intracellular ATP level was measured in a luminator (Clarity™ luminescence microplate reader) using an ATP Bioluminescence Assay Kit (ViaLight®-Plus ATP monitoring reagent kit, Lonza) following the manufacturer's protocol. The total ATP level was expressed as a percentage of untreated control. Data are reported as the mean of at least three independent experiments.

Example 10. Methylene Violet Analogues as Mitochondrial Therapeutic Agents

(a) Cytoprotective effect of compounds 1-5 in (BSO)-treated FRDA fibroblasts (Figure 7).

The ability of test compounds **1-5** to confer cytoprotection to buthionine sulfoximine (BSO)-treated FRDA fibroblasts from oxidative damage-induced death was determined by using simultaneous staining with a two-color fluorescence assay, the Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). The cells were subjected to L-buthionine (*S,R*)-sulfoximine, an inhibitor of *de novo* glutathione (GSH) biosynthesis. Oxidative damage-induced death of FRDA fibroblasts was blocked by the test compounds in a concentration dependent manner (Figure 7). Compounds **4** and **5**, each having a single longer alkyl substituent, exhibited better potency than **1** or **2** and were by far the most efficient when tested at lower concentrations. The optimal side chain length was 15 carbon atoms. Interestingly, compound **3**, having two 15-carbon substituents, was the least effective in this assay.

Detailed Method

The ability of the test compounds to confer cytoprotection to BSO-treated FRDA

fibroblasts was determined by assessing plasma membrane integrity and intracellular esterase activity using the LIVE/ DEAD Viability/Cytotoxicity Kit for mammalian cells according to the manufacturer's protocol. Briefly, FRDA fibroblasts (GM04078) were plated in 96-well microtiter plates at a density of 3000 cell/well (50 µL) (Costar, Corning, NY). The plates were incubated at 37 °C for 24 h in an atmosphere having 95% humidity and 5% CO_2 to allow attachment of the cells to the culture plates and to allow the cell density reach 50-60% confluency. The next day the test compounds were dissolved in DMSO, diluted to the appropriate concentrations in fresh cell culture media and the cells were then treated with the test compounds (final DMSO concentration was <0.5%). Plates were incubated at 37 °C overnight in a humidified atmosphere containing 5% CO_2 in air. The following day BSO solution (in culture media) was added to each well to achieve a final BSO concentration of 5 mM. After 48 hours of BSO treatment, the media was removed, and cells were stained with the Live/Dead® reagent (4 µM ethidium bromide homodimer, 1.2 µM calcein-AM) and then incubated at 37 °C for 60 min in the dark to allow the dye to enter the cell and be hydrolyzed by esterases. Fluorescence intensities were measured with a Spectramax M5 spectrofluorometer (Molecular Devices, Sunnyvale, CA) using excitation and emission wavelengths of 485 nm and 525 nm, respectively. The test compounds were assayed in triplicate. The viability of non-BSO treated fibroblasts was set as 100%, and the viability of the BSO treated and sample-treated cells was calculated relative to this value. Cell viability was expressed as the percentage of control.

(b) Effect of methylene violet analogue on frataxin expression level and on mitochondrial biogenesis in Friedreich ataxia cells (Figures 8 and 9).

Method:

Cell lines and culture conditions:

Human Friedreich's ataxia affected B-lymphocytes cell line (cat#: GM16216) and clinically unaffected Friedreich's ataxia B-lymphocytes cell line (cat#: GM16213) were obtained from Coriell Cell Repositories (Camden, NJ). Lymphocytes were routinely cultured in RPMI-1640 medium (Gibco, Life Technologies, Grand Island, NY) with added 15% fetal calf serum (FBS) and 1% penicillin–streptomycin antibiotic supplement (Cellgro, Manassas, VA). Cells were passaged every other day to maintain them in log phase growth and kept at a nominal concentration range of 5–10 x 10^5.

Treatment and measurements of Frataxin level and mitochondrial biogenesis:

Lymphocytes were seeded at density of 5 x 10^5 cell/mL in 24-well plate and treated with the test compounds and incubated at 37 °C for 24 and 48 hr in a humidified atmosphere containing 5% CO2 in air. After treatment period, frataxin levels were measured using the Frataxin Human Simple Step enzyme-linked immunosorbent assay (ELISA) kit (cat#: ab176112, Abcam, UK) according to manufacturer's protocol. For mitochondrial biogenesis, the treated suspension lymphocytes cells were then transferred to the amine assay plate (cat# 354717, Corning, USA) and follow immediately the fixation step and quantitative immunocytochemistry assay to measure protein levels of succinate dehydrogenase (SDH-A), a subunit of complex II (nuclear DNA-encoded protein) and cytochrome *c* oxidase subunit 1 (COX-1), a subunit of complex IV (mitochondrial DNA-encoded) according to protocol describe in In-Cell mitochondrial biogenesis ELISA Kit (cat#: ab110217, Abcam, UK).

Example 11. The following illustrate representative pharmaceutical dosage forms, containing a compound of formula I (compound X), for therapeutic or prophylactic use in humans.

(i) Tablet 1	mg/tablet
Compound X=	100.0
Lactose	77.5
Povidone	15.0
Croscarmellose sodium	12.0
Microcrystalline cellulose	92.5
Magnesium stearate	3.0
	300.0

(ii) Tablet 2	mg/tablet
Compound X=	20.0
Microcrystalline cellulose	410.0
Starch	50.0
Sodium starch glycolate	15.0
Magnesium stearate	5.0
	500.0

(iii) Capsule	mg/capsule
Compound X=	10.0
Colloidal silicon dioxide	1.5
Lactose	465.5
Pregelatinized starch	120.0
Magnesium stearate	3.0
	600.0

(iv) Injection 1 (1 mg/mL)	mg/mL
Compound X= (free acid form)	1.0
Dibasic sodium phosphate	12.0
Monobasic sodium phosphate	0.7
Sodium chloride	4.5
1.0 N Sodium hydroxide solution (pH adjustment to 7.0-7.5)	q.s.
Water for injection	q.s. ad 1 mL

(v) Injection 2 (10 mg/mL)	mg/mL
Compound X= (free acid form)	10.0
Monobasic sodium phosphate	0.3
Dibasic sodium phosphate	1.1
Polyethylene glycol 400	200.0
1.0 N Sodium hydroxide solution (pH adjustment to 7.0-7.5)	q.s.
Water for injection	q.s. ad 1 mL

(vi) Aerosol	mg/can
Compound X=	20.0
Oleic acid	10.0
Trichloromonofluoromethane	5,000.0
Dichlorodifluoromethane	10,000.0
Dichlorotetrafluoroethane	5,000.0

The above formulations may be obtained by conventional procedures well known in the pharmaceutical art.

Discussion

Compounds **1-5** were synthesized using the Smiles rearrangement where functionalized phenothiazines are prepared by the intramolecular rearrangement of functionalized diphenyl sulfides under basic conditions (Schemes 1 and 2). The diphenyl sulfide intermediate **7** was prepared by the coupling of 2-amino-5-methoxybenzothiol and 2, 4-dinitrochlorobenzene under mildly acidic condition. The highly unstable 2-amino-5-methoxybenzothiol was prepared by the basic hydrolysis of 2-amino-5-methoxybenzothiazole at reflux (Arce, P. M. et al. *ACS Med. Chem. Lett.* **2011**, 2, 608). The thiol was used for the coupling step without isolation or further purification from the reaction mixture. The high yield of **7** (81%) indicates the efficient formation of the thiol intermediate. Compound **7** was then activated as its acetamide derivative (**8**) by treatment with acetic anhydride. Smiles rearrangement of **8** under mildly basic condition yielded the functionalized phenothiazine **9** in

90% yield. Subsequently the nitro group at position 7 was reduced to afford an aromatic amine, which was alkylated using different alkyl iodides/bromides under basic conditions to obtain compounds **12-17**. Removal of the Boc and methyl groups by BBr_3 afforded the final compounds **1-5** in varying yields.

A number of representative compounds of formula I have been designed and synthesized with variations in their redox core and their side chain that could enable the compounds to protect against oxidative stress, augment ATP levels and enhance mitochondrial function. The strong cytotoxicity of MB itself in FRDA lymphocytes at 0.5-2.5 μM concentrations was surprising, given its long clinical use. A nutrient-sensitized screening strategy was used by culturing the FRDA cells on galactose as the sole sugar source, forcing them to rely on mitochondrial OX-PHOS to produce their ATP; consequently they became more sensitive to respiratory chain inhibitors than cells grown on glucose. Figure 2 shows that MB was cytotoxic when used at 2.5 μM concentration for 48 h. The compounds of formula I with long alkyl side chains (**3-5**) were not significantly cytotoxic under any tested condition, although compounds of formula I with shorter side chains (**1** and **2**) were somewhat cytotoxic. Shown in Figure 5 are the abilities of the compounds of formula I to suppress ROS production in FRDA lymphocytes depleted of glutathione by using 5 mM diethyl maleate. The compounds of formula I preserved the mitochondrial membrane potential of FRDA lymphocytes. FACS analysis of $\Delta\psi_m$ showed that MB and MV significantly depolarized $\Delta\psi_m$, while compounds of formula I did not (Figure 4). The three long alkyl chain compounds of formula I also restored ATP levels in FRDA lymphocytes (Figure 6). In contrast, MB itself inhibited ATP production, especially at higher concentrations. The side chain modifications also had a dramatic effect on their ability to afford cytoprotection to rotenone-treated FRDA lymphocytes (Figure 3).

In conclusion, five representative compounds of formula I were designed and synthesized. The synthetic route involved Smiles rearrangement as one of the key steps to obtain a substituted phenothiazine compounds. The yields for the final compounds ranged from 25-50% with high purity. When the compounds were tested for their ability to act as multifunctional radical quenchers in FRDA lymphocytes they exhibit better antioxidant activity than MB and MV compounds.

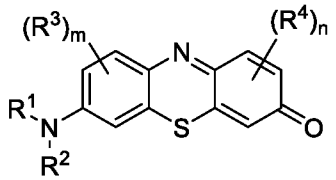
All publications, patents, and patent documents are incorporated by reference herein, as though individually incorporated by reference. The invention has been described with

reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

CLAIMS

What is claimed is:

1. A compound of formula I:



I

or a pharmaceutically acceptable salt thereof, wherein:

R^1 is C_{1-20} alkyl, C_{2-20} alkenyl or C_{2-20} alkynyl, and wherein the C_{1-20} alkyl, C_{2-20} alkenyl and C_{2-20} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

R^2 is C_{1-20} alkyl, C_{2-20} alkenyl or C_{2-20} alkynyl, and wherein the C_{1-20} alkyl, C_{2-20} alkenyl and C_{2-20} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN; or

R^1 and R^2 taken together with the nitrogen to which they are attached form a 3-12 membered heterocycle that is optionally substituted with one or more groups independently selected from C_{1-4} alkyl, C_{1-4} haloalkyl, –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

each R^3 is independently selected from the group consisting of –F, -Cl, -Br, -I, -OH, $-OR^a$, $-SR^a$, $-NR^aR^b$, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

each R^4 is independently selected from the group consisting of –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, $-NO_2$, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

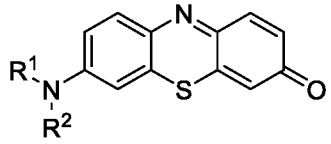
each R^a is independently hydrogen or C_{1-4} alkyl;

each R^b is independently hydrogen or C_{1-4} alkyl;

the subscript m is 0, 1, 2 or 3; and

the subscript n is 0, 1, 2, or 3.

2. The compound of claim 1 that is a compound of formula Ia:



Ia

or a pharmaceutically acceptable salt thereof.

3. The compound of claim 1 or claim 2, wherein R^1 is C_{1-20} alkyl.

4. The compound of any one of claims 1-3, wherein R^1 is *n*-butyl, *n*-decyl or *n*-pentadecyl.

5. The compound of any one of claims 1-4, wherein R^2 is C_{1-20} alkyl.

6. The compound of any one of claims 1-5, wherein R^2 is methyl, *n*-butyl, *n*-decyl or *n*-pentadecyl.

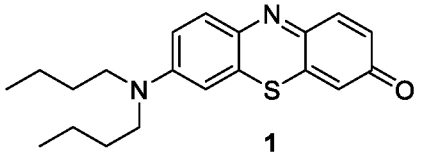
7. The compound of any one of claims 1 and 3-6, wherein m is 0 and n is 0.

8. The compound of claim 1, wherein R^1 is C_{12-20} alkyl, C_{12-20} alkenyl or C_{12-20} alkynyl, and wherein the C_{12-20} alkyl, C_{12-20} alkenyl and C_{12-20} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and
-CN;

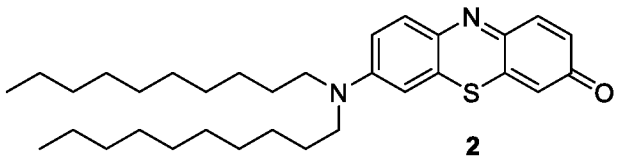
each R^a is independently hydrogen or C_{1-4} alkyl; and

each R^b is independently hydrogen or C_{1-4} alkyl.

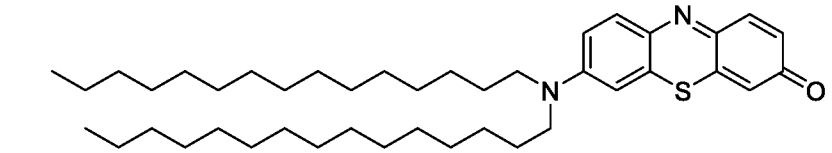
9. The compound of claim 1 that is selected from the group consisting of:



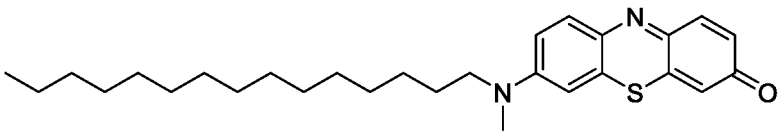
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2

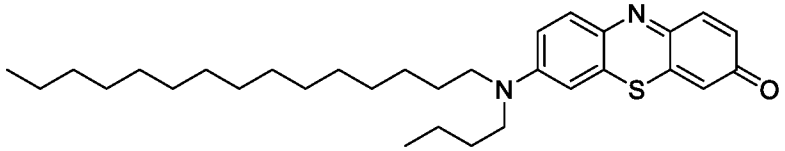


3



and

4



5

;

and pharmaceutically acceptable salts thereof.

10. A pharmaceutical composition comprising a compound of formula (I) as described in any one of claims 1-9, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

11. A method of treating mitochondrial disease, neurodegenerative disease, cardiovascular disease, cancer or diabetes in an animal comprising administering to the animal an effective amount of compound of formula (I) or a pharmaceutically acceptable salt thereof as described in any one of claims 1-9.

12. The method of claim 11, wherein the neurodegenerative disease is Alzheimer's disease (AD) , Parkinson's disease (PD) or Friedreich's ataxia (FRDA).

13. A method of preserving mitochondrial function in an animal comprising administering to the animal an effective amount of compound of formula (I) or a pharmaceutically acceptable salt thereof as described in any one of claims 1-9.

14. A method of protecting cells from oxidative stress in an animal comprising administering to the animal an effective amount of compound of formula (I) or a pharmaceutically acceptable salt thereof as described in any one of claims 1-9.

15. A method of preserving mitochondrial membrane potential and/or augmenting ATP production in an animal comprising administering to the animal an effective amount of compound of formula (I) or a pharmaceutically acceptable salt thereof as described in any one of claims 1-9.

16. A method of preserving mitochondrial membrane potential and/or augmenting ATP production of a cell *in vitro* comprising contacting the cell with an effective amount of compound of formula (I) or a pharmaceutically acceptable salt thereof as described in any one of claims 1-9.

17. A compound as described in any one of claims 1-9 or a pharmaceutically acceptable salt thereof for use in medical therapy.

18. A compound as described in any one of claims 1-9 or a pharmaceutically acceptable salt thereof for use in treating mitochondrial disease, neurodegenerative disease, cardiovascular disease, cancer or diabetes.

19. A compound as described in any one of claims 1-9 or a pharmaceutically acceptable salt thereof for use in preserving mitochondrial function.

20. A compound as described in any one of claims 1-9 or a pharmaceutically acceptable salt thereof for use in protecting cells from oxidative stress.

21. A compound as described in any one of claims 1-9 or a pharmaceutically acceptable salt thereof for use in preserving mitochondrial membrane potential and/or augmenting ATP production.

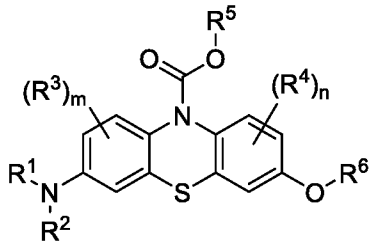
22. The use of a compound as described in any one of claims 1-9 or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for treating mitochondrial disease, neurodegenerative disease, cardiovascular disease, cancer or diabetes.

23. The use of a compound as described in any one of claims 1-9 or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for preserving mitochondrial function.

24. The use of a compound as described in any one of claims 1-9 or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for protecting cells from oxidative stress.

25. The use of a compound as described in any one of claims 1-9 or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for preserving mitochondrial membrane potential and/or augmenting ATP production.

26. A method to prepare a compound of formula I as described in any one of claims 1-9 comprising converting a corresponding compound of formula II to provide the compound of formula I or a pharmaceutically acceptable salt thereof:



II

wherein:

R^1 is C_{1-20} alkyl, C_{2-20} alkenyl or C_{2-20} alkynyl, and wherein the C_{1-20} alkyl, C_{2-20} alkenyl and C_{2-20} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and -CN;

R^2 is C_{1-20} alkyl, C_{2-20} alkenyl or C_{2-20} alkynyl, and wherein the C_{1-20} alkyl, C_{2-20} alkenyl and C_{2-20} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and -CN; or

R^1 and R^2 taken together with the nitrogen to which they are attached form a 3-12 membered heterocycle that is optionally substituted with one or more groups independently selected from C_{1-4} alkyl, C_{1-4} haloalkyl, –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and -CN;

each R^3 is independently selected from the group consisting of –F, -Cl, -Br, -I, -OH, -

OR^a, $-SR^a$, $-NR^aR^b$, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

each R^4 is independently selected from the group consisting of –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, $-NO_2$, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

R^5 is C_{1-10} alkyl that is optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and –CN;

R^6 is hydrogen or C_{1-6} alkyl that is optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and –CN;

each R^a is independently hydrogen or C_{1-4} alkyl;

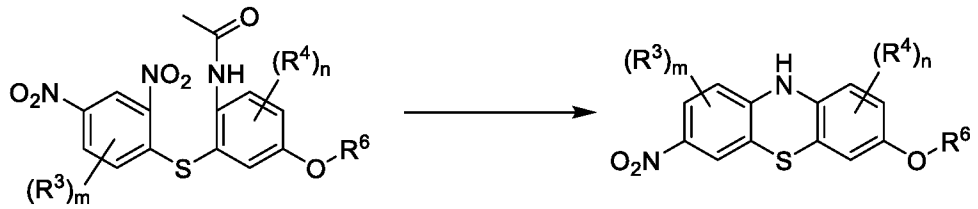
each R^b is independently hydrogen or C_{1-4} alkyl;

the subscript m is 0, 1, 2 or 3; and

the subscript n is 0, 1, 2, or 3;

or a salt thereof.

27. A method to prepare a compound of formula III comprising converting a corresponding compound of formula IV to provide the compound of formula III:



IV III

wherein:

each R^3 is independently selected from the group consisting of –F, -Cl, -Br, -I, -OH, $-OR^a$, $-SR^a$, $-NR^aR^b$, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

each R^4 is independently selected from the group consisting of –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, $-NO_2$, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently

selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and -CN;

R^6 is hydrogen or C_{1-6} alkyl that is optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and –CN;

each R^a is independently hydrogen or C_{1-4} alkyl;

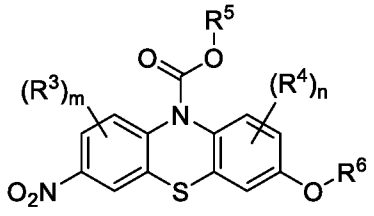
each R^b is independently hydrogen or C_{1-4} alkyl;

the subscript m is 0, 1, 2 or 3; and

the subscript n is 0, 1, 2, or 3;

or a salt thereof.

28. The method of claim 27 further comprising converting the compound of formula III or a salt thereof to provide a corresponding compound of formula IIa:



IIa

wherein:

each R^3 is independently selected from the group consisting of –F, -Cl, -Br, -I, -OH, -OR^a, -SR^a, -NR^aR^b, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and -CN;

each R^4 is independently selected from the group consisting of –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, -NO_2, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and -CN;

R^5 is C_{1-10} alkyl that is optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and –CN;

R^6 is hydrogen or C_{1-6} alkyl that is optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, -OR^a, -SR^a, -NR^aR^b, oxo, -NO_2 and –CN;

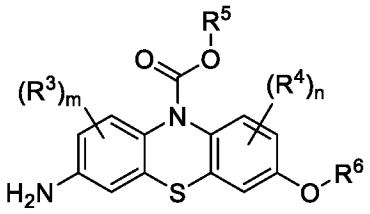
each R^a is independently hydrogen or C_{1-4} alkyl;

each R^b is independently hydrogen or C_{1-4} alkyl;

the subscript m is 0, 1, 2 or 3; and
the subscript n is 0, 1, 2, or 3;

or a salt thereof.

29. The method of claim 28 further comprising converting the compound of formula IIa or a salt thereof to provide a corresponding compound of formula IIb:



IIb

wherein:

each R^3 is independently selected from the group consisting of –F, -Cl, -Br, -I, -OH, $-OR^a$, $-SR^a$, $-NR^aR^b$, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

each R^4 is independently selected from the group consisting of –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, $-NO_2$, -CN, C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl, and wherein the C_{1-4} alkyl, C_{2-4} alkenyl and C_{2-4} alkynyl are optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and -CN;

R^5 is C_{1-10} alkyl that is optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and –CN;

R^6 is hydrogen or C_{1-6} alkyl that is optionally substituted with one or more groups independently selected from –F, -Cl, -Br, -I, $-OR^a$, $-SR^a$, $-NR^aR^b$, oxo, $-NO_2$ and –CN;

each R^a is independently hydrogen or C_{1-4} alkyl;

each R^b is independently hydrogen or C_{1-4} alkyl;

the subscript m is 0, 1, 2 or 3; and

the subscript n is 0, 1, 2, or 3;

or a salt thereof.

30. The method of claim 29 further comprising converting the corresponding compound of formula IIb or a salt thereof to provide the compound of formula II or a salt thereof described in claim 26.

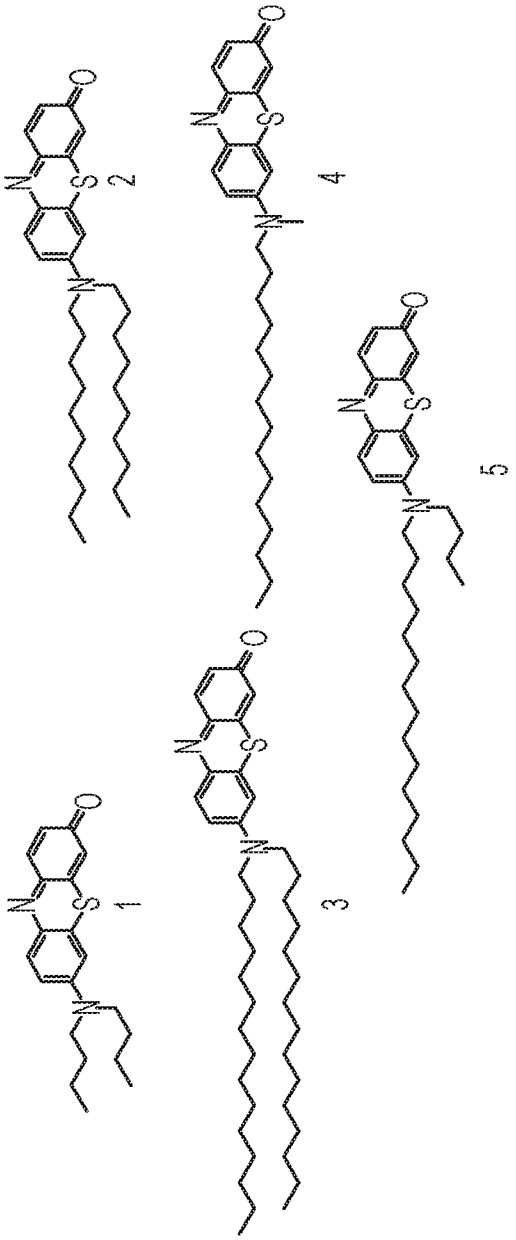


Figure 1

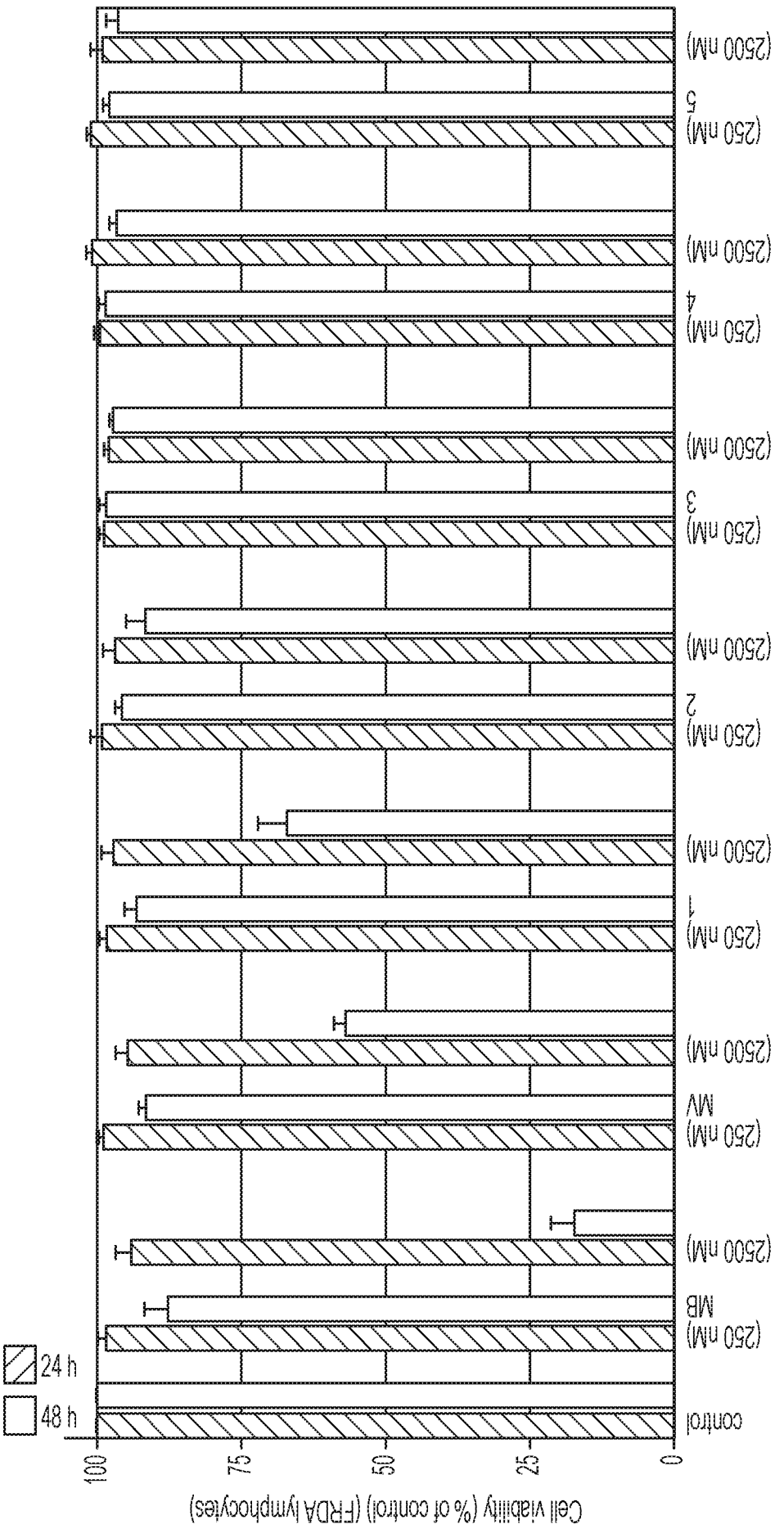

Cell viability (% of control) (FRDA lymphocytes)
100
75
50
25
0
24 h
48 h
control
(250 nM) MB
(2500 nM)
(250 nM) MV
(2500 nM)
(250 nM) 1
(2500 nM)
(250 nM) 2
(2500 nM)
(250 nM) 3
(2500 nM)
(250 nM) 4
(2500 nM)
(250 nM) 5
(2500 nM)

Figure 2

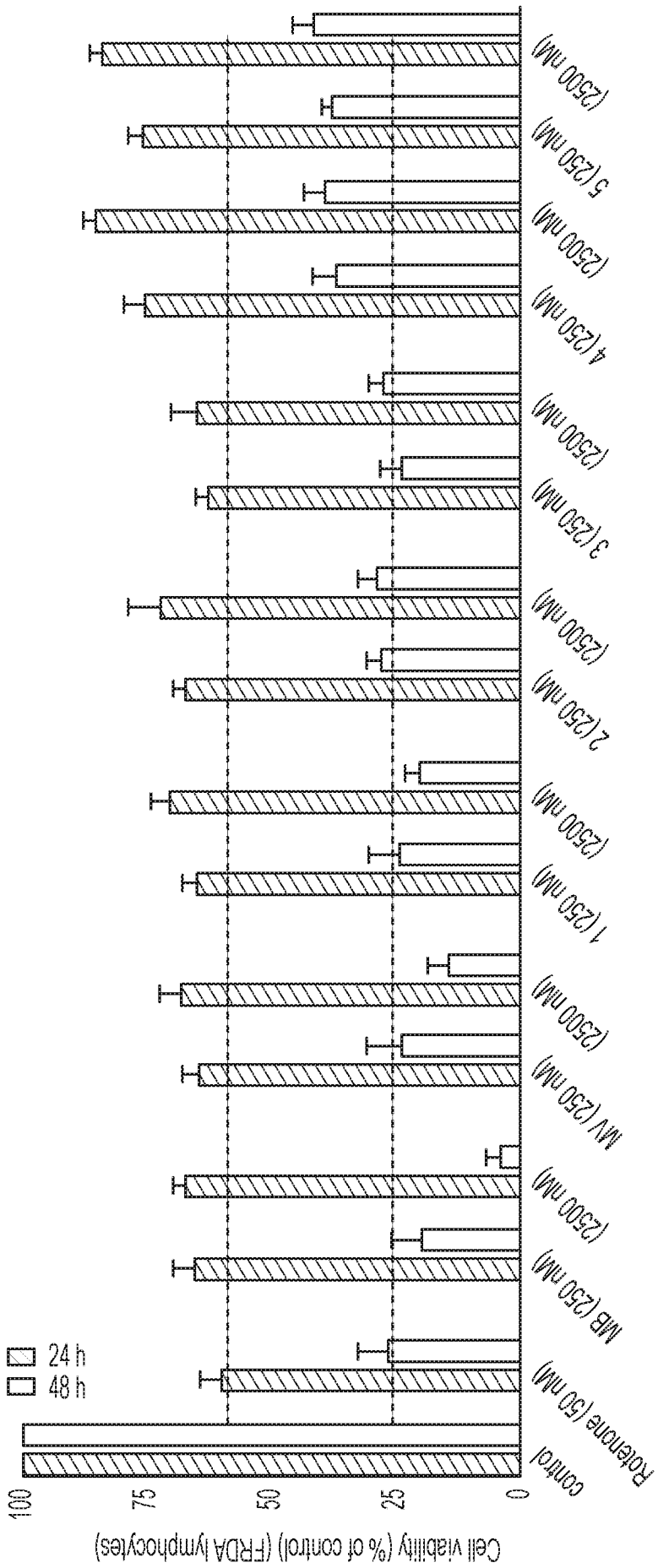

Cell viability (% of control) (FRDA lymphocytes)
100
75
50
25
0
24 h
48 h
control
Rotenone (50 nM)
MB (250 nM)
(2500 nM)
MV (250 nM)
(2500 nM)
1 (250 nM)
(2500 nM)
2 (250 nM)
(2500 nM)
3 (250 nM)
(2500 nM)
4 (250 nM)
(2500 nM)
5 (250 nM)
(2500 nM)

Figure 3

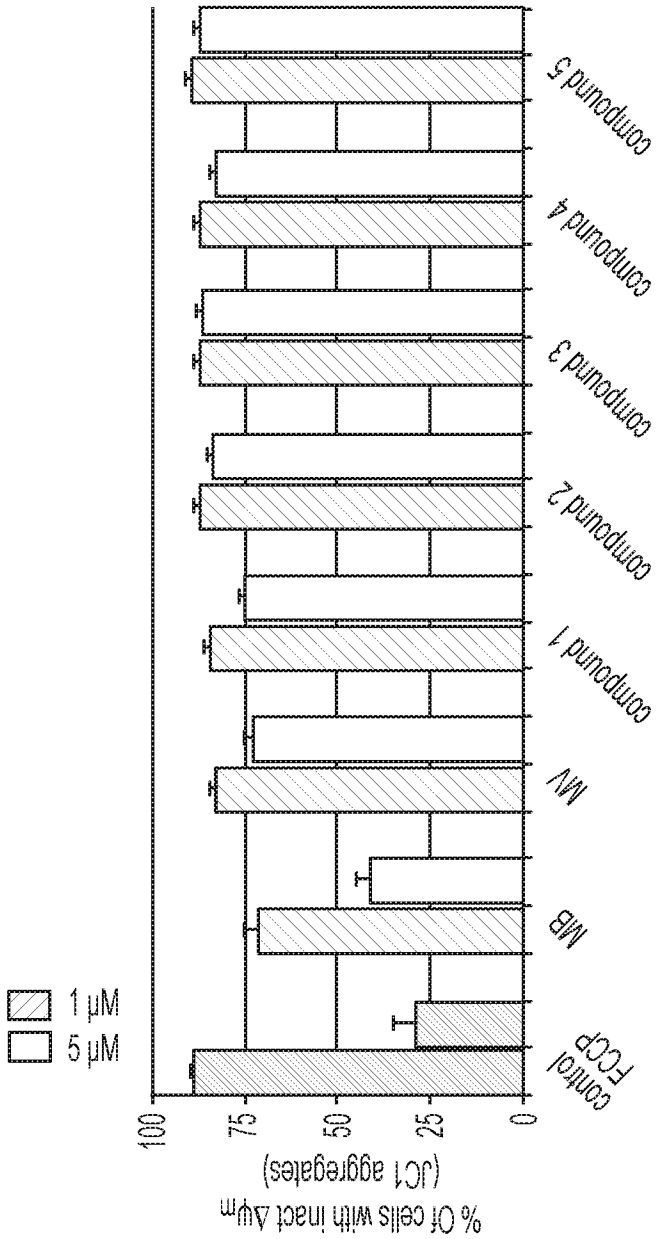

% Of cells with inact $\Delta\psi_m$
(JC1 aggregates)
100
75
50
25
0
control FCCP
MB
MV
compound 1
compound 2
compound 3
compound 4
compound 5
1 µM
5 µM

Figure 4

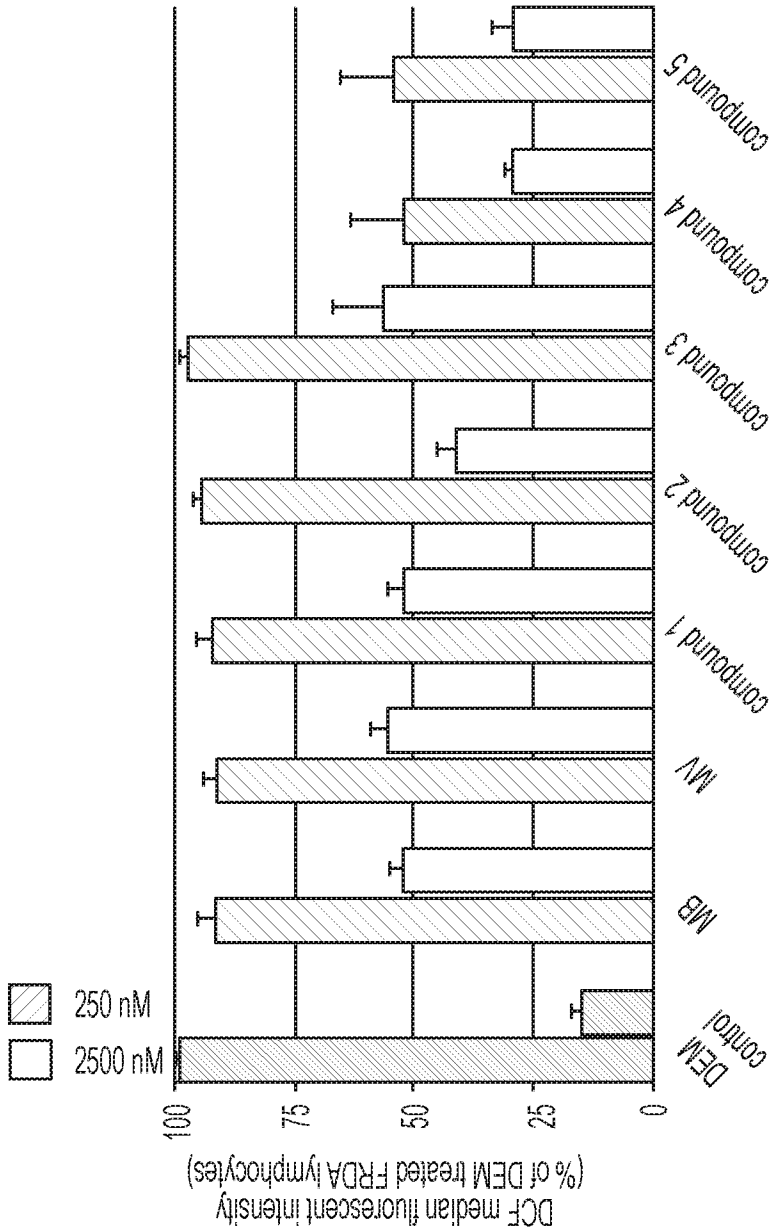

DCF median fluorescent intensity
(% of DEM treated FRDA lymphocytes)
100
75
50
25
0
250 nM
2500 nM
DEM control
MB
MV
compound 1
compound 2
compound 3
compound 4
compound 5

Figure 5

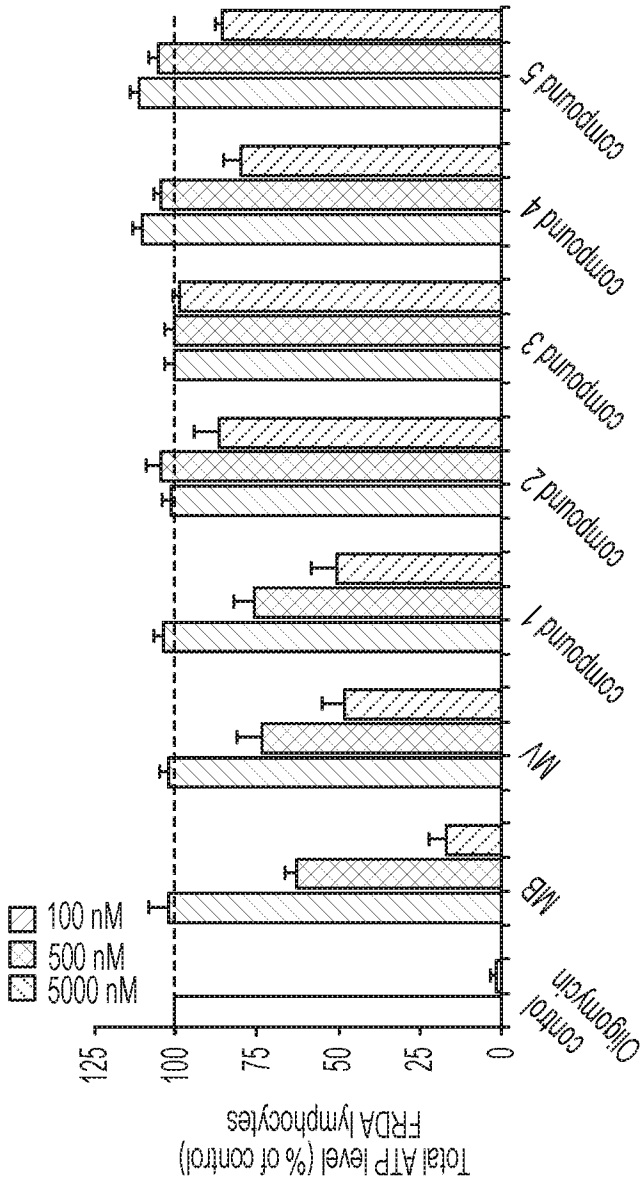

Total ATP level (% of control)
FRDA lymphocytes
125
100
75
50
25
0
100 nM
500 nM
5000 nM
control
Oligomycin
MB
MV
compound 1
compound 2
compound 3
compound 4
compound 5

Figure 6

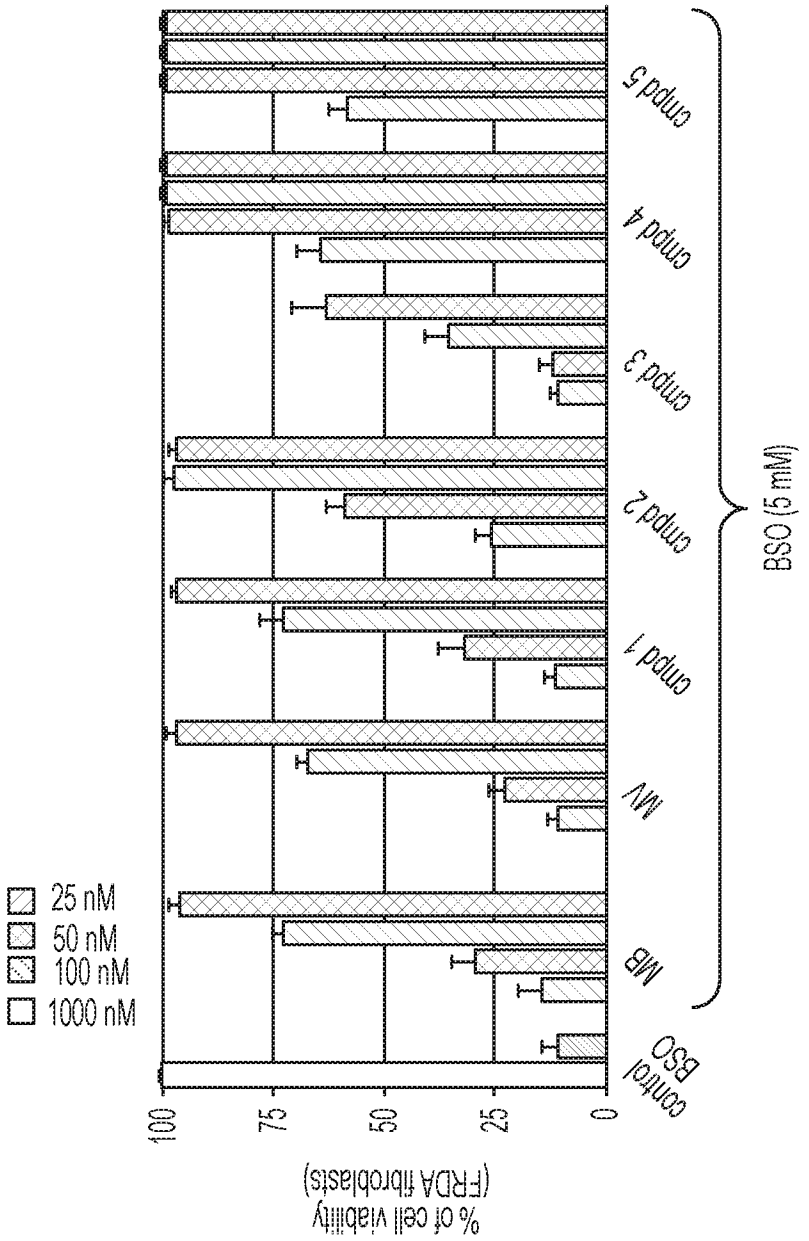

25 nM
50 nM
100 nM
1000 nM
% of cell viability
(FRDA fibroblasts)
100
75
50
25
0
control
BSO
MB
MV
cmpd 1
cmpd 2
cmpd 3
cmpd 4
cmpd 5
BSO (5 mM)

Figure 7

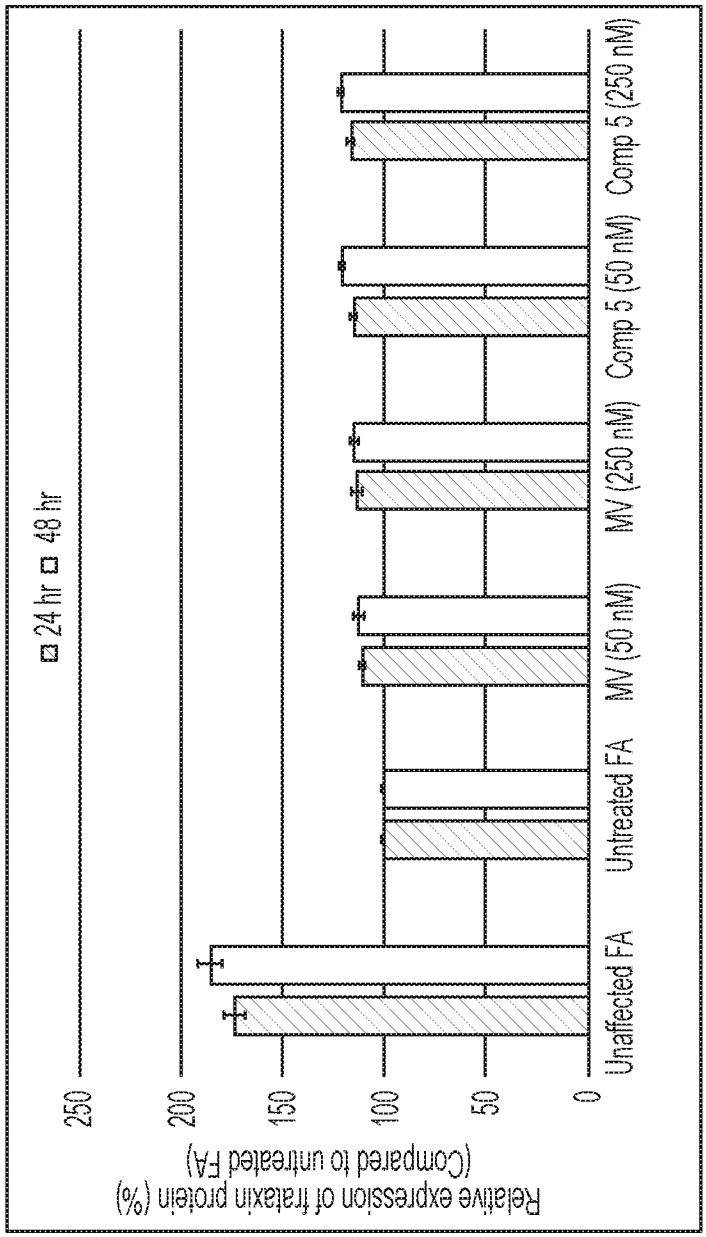
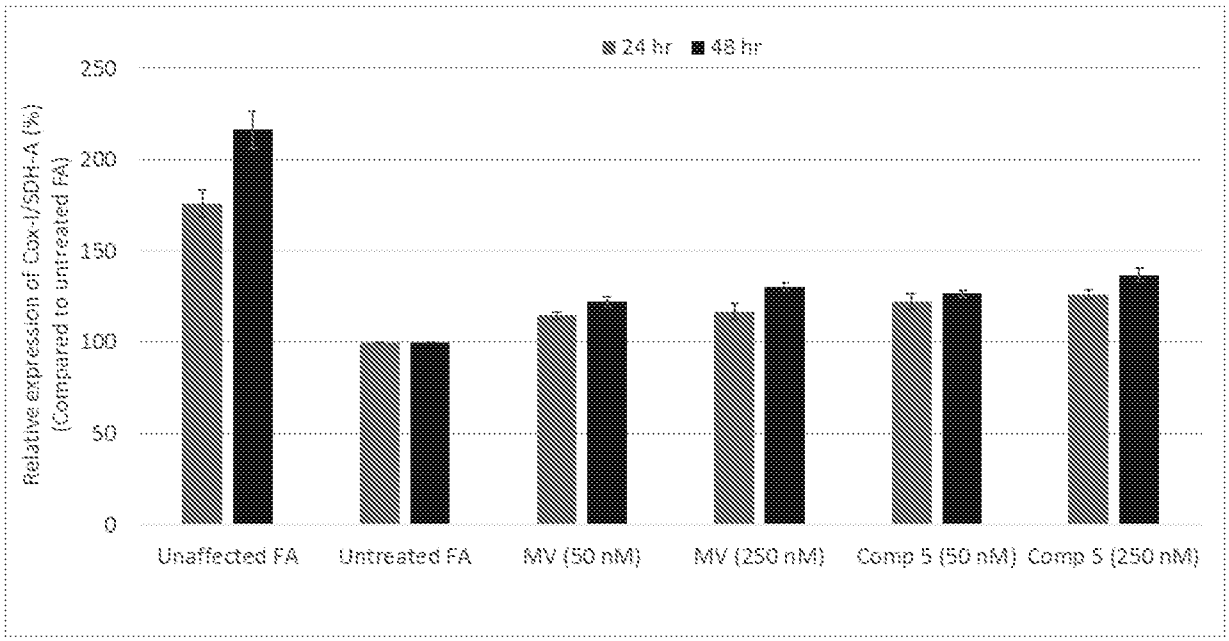

24 hr
48 hr
Relative expression of frataxin protein (%)
(Compared to untreated FA)
250
200
150
100
50
0
Unaffected FA
Untreated FA
MV (50 nM)
MV (250 nM)
Comp 5 (50 nM)
Comp 5 (250 nM)

Figure 8

Figure 9


24 hr
48 hr
Relative expression of Cox-1/SDH-A (%)
(Compared to untreated FA)
250
200
150
100
50
0
Unaffected FA
Untreated FA
MV (50 nM)
MV (250 nM)
Comp 5 (50 nM)
Comp 5 (250 nM)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/048482

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/54; A61K 31/5415; A61P 39/06; C07D 279/14 (2017.01)
CPC - A61K 31/5415; A61K 31/54; C07D 279/14 (2017.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PUBCHEM, Compound Summary for SID 224730291, Available Date: 02 February 2015 [retrieved on 26 September 2017]. Retrieved from the Internet: <URL: https://pubchem.ncbi.nlm.nih.gov/substance/224730291> entire document	1-3
A	US 9,102,626 B2 (ARIZONA BOARD OF REGENTS A BODY CORPORATE OF THE STATE OF ARIZONA) 11 August 2015 (11.08.2015) entire document	1-3
A	WO 2014/145119 A1 (EDISON PHARMACEUTICALS INC) 18 September 2014 (18.09.2014) entire document	1-3
A	US 2013/0317012 A1 (WISTA LABORATORIES LTD) 28 November 2013 (28.11.2013) entire document	1-3

☐ 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
21 November 2017	07 DEC 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/048482

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

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

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

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

3. ☒ Claims Nos.: 4-7, 10-26, 30
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 Extra Sheet

Claims 1-3 have been analyzed subject to the restriction that the claims read on a compound of formula I or a pharmaceutically acceptable salt thereof, wherein: R1 is unsubstituted C1 alkyl; R2 is unsubstituted C1 alkyl; R3 is absent; R4 is absent; m is 0; and n is 0.

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3

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.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/048482

Continued from Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-3, 8, and 9 are drawn to compounds of formula (I) or a pharmaceutically acceptable salt thereof.

Group II: claims 27-29 are drawn to methods to prepare a compound of formula III.

The first invention of Group I+ is restricted to a compound of formula I or a pharmaceutically acceptable salt thereof, wherein: R1 is unsubstituted C1 alkyl; R2 is unsubstituted C1 alkyl; R3 is absent; R4 is absent; m is 0; and n is 0. It is believed that claims 1-3 read on this first named invention and thus these claims will be searched without fee to the extent that they read on the above embodiment.

Applicant is invited to elect additional formula(e) for each additional compound to be searched in a specific combination by paying an additional fee for each set of election. Each additional elected formula(e) requires the selection of a single definition for each compound variable. An exemplary election would a compound of formula I or a pharmaceutically acceptable salt thereof, wherein: R1 is unsubstituted C2 alkyl; R2 is unsubstituted C1 alkyl; R3 is absent; R4 is absent; m is 0; and n is 0. Additional formula(e) will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The special technical features of Group I+, compounds of formula (I) or a pharmaceutically acceptable salt thereof, are not present in Group II; and the special technical features of Group II, methods to prepare a compound of formula III, are not present in Group I+.

The Groups I+ and II formulae do not share a significant structural element requiring the selection of alternatives for the compound variables R1, R2, R3, R4, m, and n.

The Groups I+ and II share the technical features of a compound having the core structure of formula I or a pharmaceutically acceptable salt thereof. However, these shared technical features do not represent a contribution over the prior art.

Specifically, Compound Summary for SID 224730291 to PubChem teaches a compound of formula I (Pg. 3;...see shown structure...).

The inventions listed in Groups I+ and II therefore lack unity under Rule 13 because they do not share a same or corresponding special technical feature.