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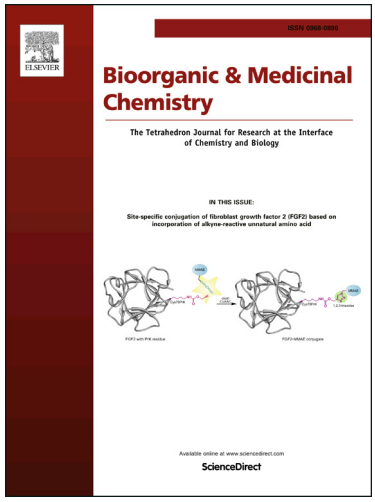

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Lipophilic methylene violet analogues as modulators of mitochondrial function and dysfunction

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A B S T R A C T

In an effort to identify methylene blue analogues having improved antioxidant activity, a series of new methylene violet analogues have been designed and synthesized. The analogues were prepared following a synthetic route that is more efficient than the previously reported methods, both in terms of yield and purity of the final products. The route involves the Smiles rearrangement as one of the crucial steps. Smiles rearrangement of suitably substituted diphenyl sulfide intermediates afforded the corresponding phenothiazine analogues in high yields, which were subsequently converted to the final products. The methylene violet analogues were evaluated for their ability to preserve mitochondrial function in Friedreich's ataxia (FRDA) lymphocytes. The analogues were shown to be efficient ROS scavengers, and able to protect cultured FRDA lymphocytes from oxidative stress resulting from inhibition of complex I. The analogues also preserved mitochondrial membrane potential and augmented ATP production. The analogues were found to be better antioxidants than the parent compounds methylene blue and methylene violet.

1. Introduction

Mitochondrial dysfunction involving disruption of the electron transport chain 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.[1-5] The broad impact of mitochondria in so many diseases makes this organelle a target for therapeutic intervention. In mammalian cells, mitochondria are the major site of ATP production via oxidative phosphorylation (OXPHOS). This process involves four respiratory chain complexes (I-IV) and ATP synthase (complex V), all of which are embedded in the inner mitochondrial membrane.[6-9] However, mitochondria are also a major source of reactive oxygen species (ROS) even in fully functional mitochondria.[10,11] Impaired oxidative phosphorylation leads to enhanced production of ROS, which can overwhelm the endogenous antioxidant systems and expose cellular macromolecules to oxidative damage.[12,13] Given the role of mitochondrial dysfunction in the development and progression of metabolic disorders, strategies for preserving mitochondrial function are of great interest for addressing mitochondrial and neurological diseases.[14-19]

Methylene blue (MB), a member of the phenothiazine family of compounds, was originally of interest as a synthetic cationic dye, but has an extensive history of medical uses for more than a century.[20] MB is approved by the US Food and Drug Administration for the treatment of methemoglobinemia and as an antidote to ifosfamide-induced encephalopathy.[21,22]

MB can readily interconvert between its oxidized and reduced forms using specific mitochondrial and cytosolic redox centers.[18,19] This property of MB enables the

redirection of electron transfer across mitochondrial electron transfer complexes, minimizing electron leakage and inhibiting superoxide production.[18,19] Thus, MB analogues may be able to act as alternative electron carriers, bypassing lesions in a damaged mitochondrial respiratory chain. In fact, methylene blue itself is being evaluated in Alzheimer's disease.[21,22]

Methylene violet (MV) is a neutral phenothiazine dye; hydrolysis of MB under strongly basic condition yields MV with increased hydrophobicity.[23] Although MV is naturally obtained in its oxidized (quinone) form, it can be reduced at the mitochondrial redox centers, generating the phenolic (quinol) form. The reduced form of MV can act as a phenolic antioxidant, similar to coenzyme Q_{10}. The goal of the study was to design novel MB/MV optimize MB analogues of potential utility as mitochondrial probes, leading to the development of novel therapeutic agents.

In earlier studies involving other heterocyclic antioxidants as potential mitochondrial therapeutic agents, we found that the presence of a lipophilic substituent was essential to activity, presumably because interaction with mitochondrial respiratory complexes embedded in the inner mitochondrial membrane represent the putative locus of action.[17,24-26] Presently we describe five lipophilic MV analogues capable of quenching ROS and diminishing the degradation of cellular macromolecules, in addition to supporting ATP synthesis in FRDA lymphocytes.

2. Results and discussion

2.1. Synthesis of the methylene violet analogues

The syntheses of MV analogues **1**-**5** having long alkyl substituents on the exocyclic nitrogen atom (Figure 1) were carried out using the Smiles rearrangement[27,28] as the key

step. Phenothiazines **1**-**5** were prepared by the intramolecular rearrangement of functionalized diphenyl sulfides under basic conditions (Schemes 1 and 2). 2-Amino-5-methoxythiophenol was prepared by the basic hydrolysis of 2-amino-6-methoxybenzothiazole at reflux in aqueous KOH. The cooled reaction mixture was used for the next step without isolation or further purification by admixture of the crude

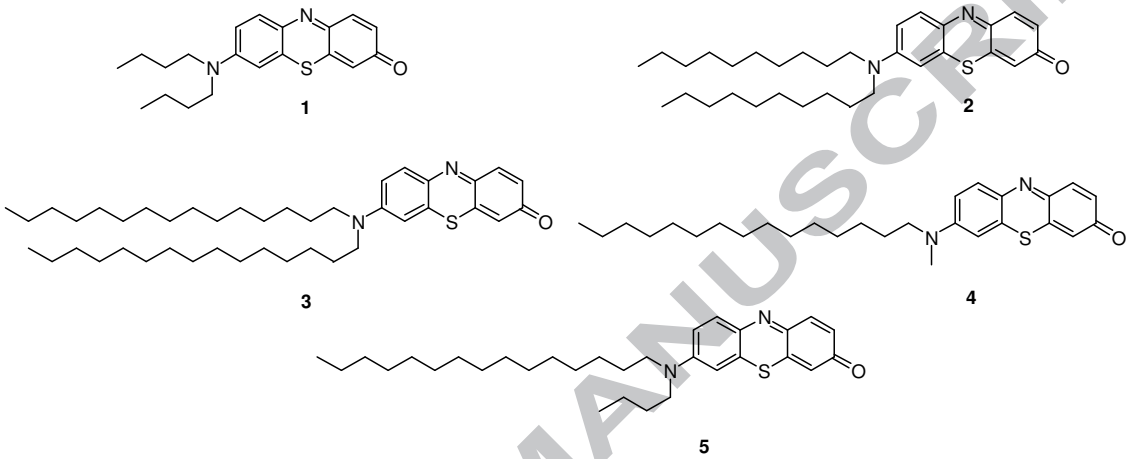
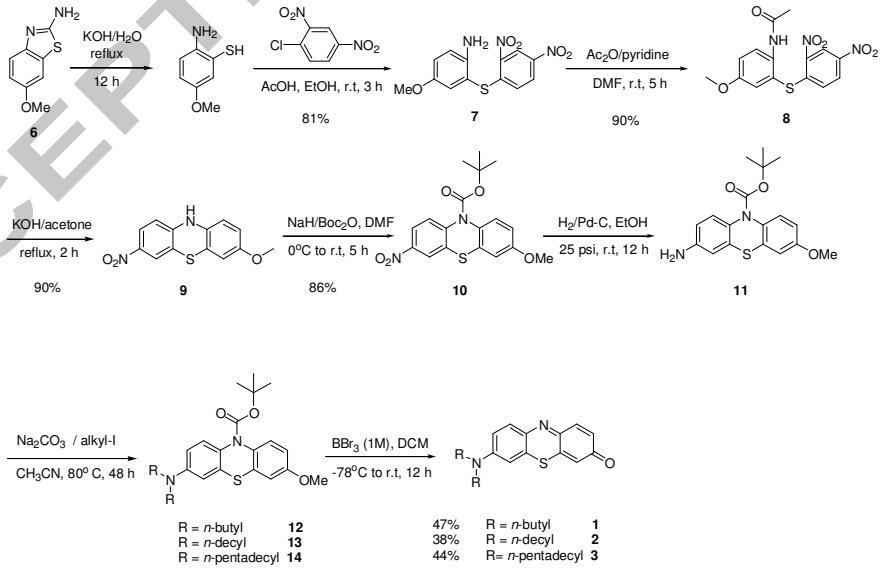


Figure 1. Chemical structures of the newly synthesized methylene violet (MV) analogues.

Scheme 1. Synthetic routes employed for compounds **1**, **2** and **3**.



solution of 2-amino-5-methoxythiophenol to a solution of 2,4-dinitrochlorobenzene in a mixture of ethanol and HOAc. Diphenyl sulfide intermediate **7** was prepared by the coupling under mildly acidic conditions (Scheme 1). The high yield diphenyl sulfide **7** (81%) indicated the efficient formation of the thiol intermediate *in situ*. Compound **7** was treated with acetic anhydride in presence of triethylamine to convert the amine to acetamide intermediate **8** in 90% yield. Smiles rearrangement of **8** under basic conditions,[27,28] followed by ring closure, yielded the functionalized phenothiazine **9** as a violet solid in 90% yield. Compound **9** was then treated with NaH (60% in mineral oil) followed by di-*tert*-butyl dicarbonate to obtain **10** in 86% yield as a bright yellow solid. Subsequently, **10** was converted to the respective to aromatic amine **11** by hydrogenation of the 7-nitro group over palladium-on-carbon. The crude amine was alkylated using different alkyl iodides/bromides under basic conditions to obtain compounds **12**-**14** (Scheme 1). Compounds **12** and **13** were purified and fully characterized prior to conversion to **1** and **2**, respectively. However, alkylation of **11** with *n*-pentadecyl bromide gave a mixture of mono- and di-*N*-alkylated products which could not be separated readily, although unreacted starting material was removed by silica gel chromatography. The crude mixture was converted to **3** directly and then purified by silica gel chromatography. Removal of the Boc and methyl groups were carried out in each case by treatment with BBr_3, affording the MV analogues **1**-**3** as violet solids in yields that ranged from 38% to 47% (Scheme 1).[29]

In order to synthesize compounds **4** and **5**, compound **11** was first alkylated using 1-bromopentadecane (Scheme 2). Mono-*N*-alkylated 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 then deprotected with 1 M BBr_3 to afford **4** and **5**, respectively.

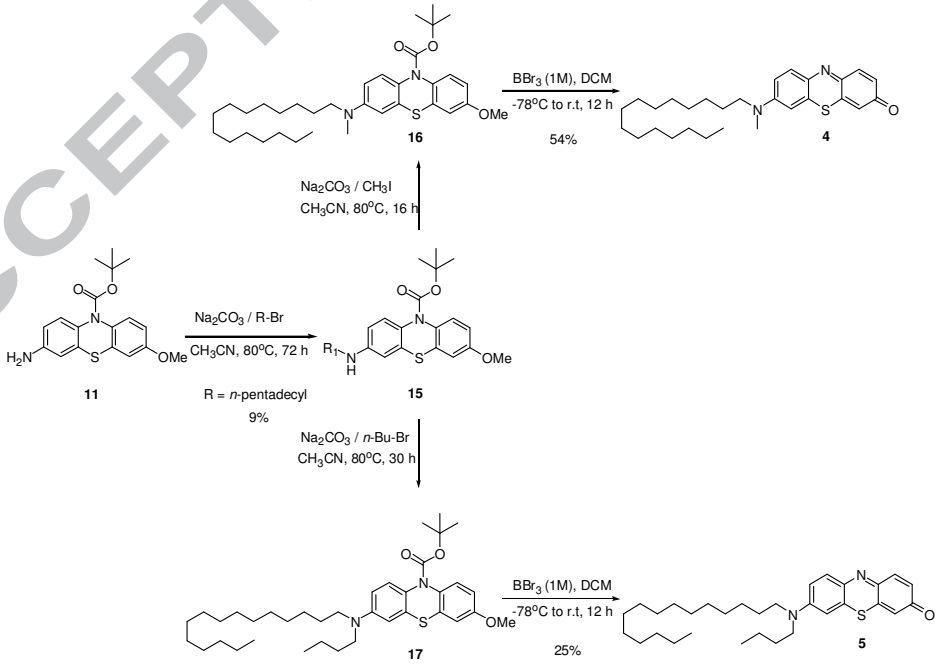
While the five new methylene violet analogues prepared were certainly insufficient in number to define any structure–activity relationships that might exist, by varying the length of alkyl substituents on the exocyclic N-atom, we hoped to establish whether these compounds interacted with the mitochondria in a fashion different from methylene violet itself, and whether there were differences between the analogues in their biological effects.

2.2. Biochemical and biological evaluation

2.2.1. Cytotoxicity

Although it is believed that methylene blue serves as an antioxidant, it has also been noted to foster toxicity by exhibiting pro-oxidant properties under certain conditions.[18] In this regard, it seemed important to ensure that the prepared analogues were not cytotoxic.

Scheme 2. Synthetic routes employed for compounds **4** and **5**.



Therefore, the MV analogues were evaluated for their cytotoxicity and respiratory chain effects by studying their effects on FRDA lymphocytes. These were grown on galactose prior to assay to increase their sensitivity to respiratory chain inhibitors.[30,31] The strong cytotoxicity of MB itself in FRDA lymphocytes at 2.5 μM concentration was surprising, given its long clinical use (Figure 2), but only one of the new analogues (**1**) exhibited any significant cytotoxicity, and only at 2.5 μM concentration after a 48-hour incubation. Measurement of cytotoxicity was carried out using the LIVE/ DEAD Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes, Eugene, OR). This assay provides information about the functional status of the cell by detecting cytoplasmic esterases activity and plasma membrane integrity.[32]

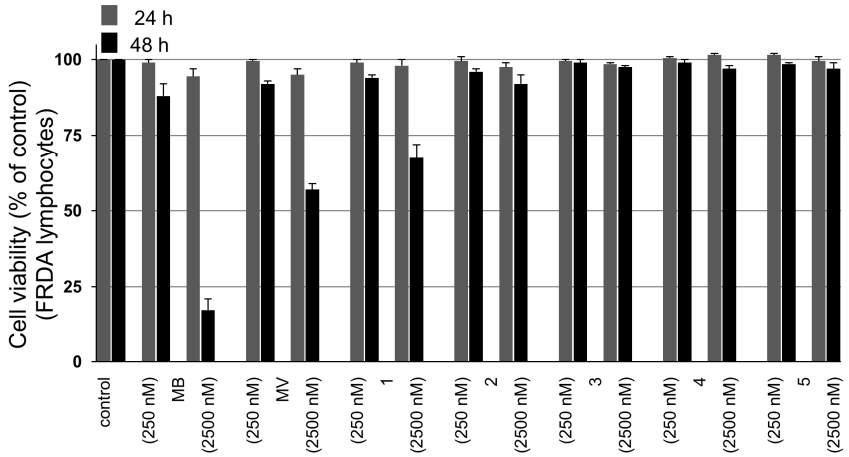


Figure 2. Methylene violet analogues (**1-5**) were evaluated for their cytotoxicity toward cultured FRDA lymphocytes by incubation for 24 h or 48 h in glucose-free media (galactose) to force the cells to rely on their mitochondria to produce ATP. Flow cytometric determination of cell viability by fluorescence labeling was used employing calcein acetoxy-methyl-ester and ethidium homodimer-1 as live and dead cell stains, respectively. The actual percentages of live cells were extracted from a two-dimensional color density dot plot. Results obtained were verified by running duplicates and repeating experiments in two independent runs. The effects of the tested compounds on the fluorescence intensities of the dyes never exceeded 0.1%.

From the results, it is evident that cytotoxicity decreased with increasing lipophilicity. While MB itself was quite cytotoxic, the modified methylene violet analogues with

longer alkyl side chains (**2**-**5**) were not cytotoxic under any tested condition. Methylene violet and short chain MV analogue **1** exhibited some relatively minor cytotoxicity.

2.2.2. Preservation of mitochondrial inner membrane potential ($\Delta\psi_m$)

Analogues **1**-**5** were next tested for their effects on inner mitochondrial membrane potential ($\Delta\psi_m$) in FRDA lymphocytes using the JC-1 probe.[33] This cationic fluorescent dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1),

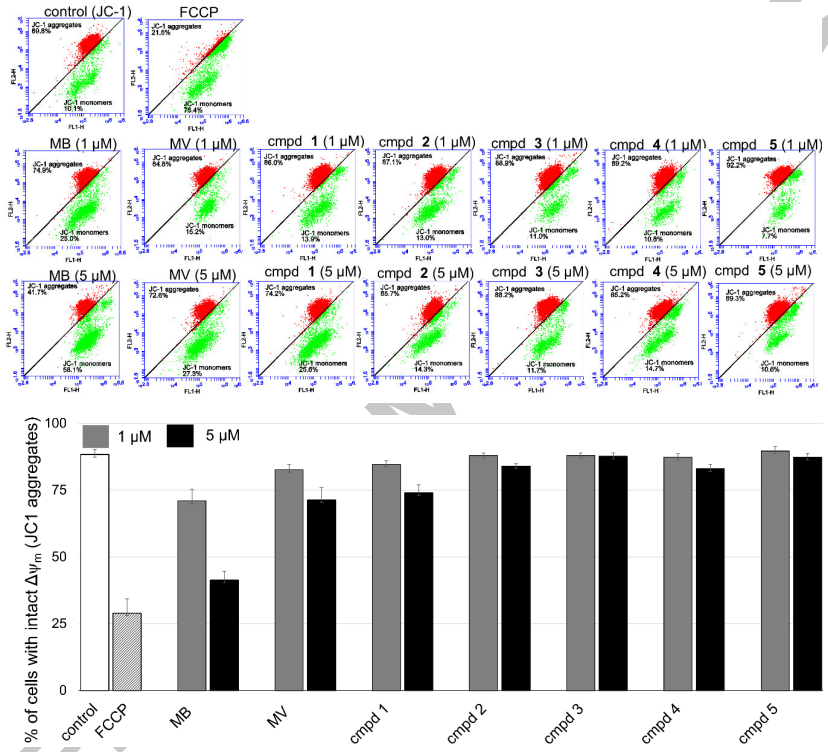


Figure 3. Flow cytometric analysis shows the effect of test compounds **1-5** on $\Delta\psi_m$ in FRDA lymphocytes using the ratiometric fluorescent probe JC-1. This probe is a cell penetrating dye that accumulates within mitochondria maintaining high $\Delta\psi_m$. The red regions represent intact mitochondrial membranes with JC-1 aggregates (red fluorescence), whereas the gated regions shown in green depict cells with loss of $\Delta\psi_m$. The bar graph shows the percentage of cells with intact $\Delta\psi_m$, calculated using Accuri™ C6 software. For each analysis, 10000 events were recorded. Depolarization with carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), served as a positive control. Results obtained were verified by running duplicates and repeating experiments in two independent runs. The effects of the tested compounds on the fluorescence intensity of JC-1 never exceeded 0.1%.

selectively accumulates in mitochondria due to the negative potential across the inner mitochondrial membrane.[34] 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.[33] Therefore, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence ratio. FACS analysis of $\Delta\psi_m$ showed that MB significantly depolarized $\Delta\psi_m$ (Figure 3) while MV had a lesser effect. Again, analogue **1** also produced significant reduction in mitochondrial membrane potential comparable to MV. In comparison, analogues **2**-**5** having 10-15 carbon side chains did not significantly affect mitochondrial membrane potential, even at 5 μM concentration. 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.

2.2.3. Suppression of reactive oxygen species

The ability of MV analogues **1**-**5** to suppress ROS induced by the depletion of cellular glutathione was evaluated in FRDA lymphocytes in a quantitative FACS experiment using dichlorodihydrofluorescein diacetate (DCFH-DA) as an indicator of intracellular ROS levels, as described previously.[35] The results are shown in Figure 4, and demonstrated 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. In comparison, MV analogues **1**-**3** exhibited behavior similar to MV itself.

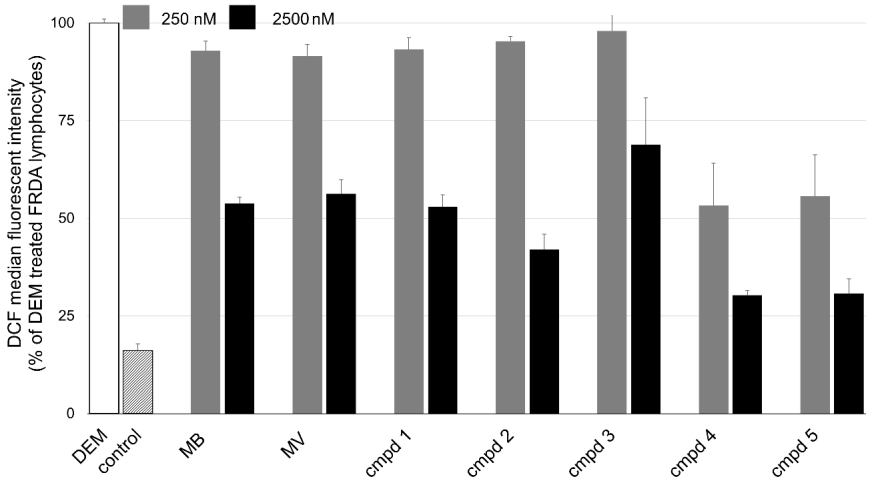


Figure 4. Flow cytometric analysis of FRDA lymphocytes stained with dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 min, following pretreatment with compounds **1-5** for 16 h, and subsequent treatment with diethyl maleate (DEM) for 80 min to induce the production of ROS by depleting glutathione. Results were expressed as a percentage of the median mean fluorescence intensity of DCF fluorescence relative to a DEM-treated control. Results obtained were verified by running duplicates and repeating experiments in two independent runs. The effects of the tested compounds on the fluorescence intensity of DCF never exceeded 0.1%. The actual fluorescence intensity data is shown in Figure S1 of the Supplementary Data.

2.2.4. Cytoprotection

The ability of the test compounds **1**-**5** to confer cytoprotection to buthionine sulfoximine (BSO)-treated FRDA fibroblasts and to rotenone-treated FRDA lymphocytes 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.[36] Oxidative damage-induced death of FRDA fibroblasts was blocked by the test compounds in a concentration dependent manner (Figure 5). 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. Again, in this assay the optimal side chain

length was 15 carbon atoms. Interestingly, compound **3**, having two 15-carbon substituents, was the least effective in this assay.

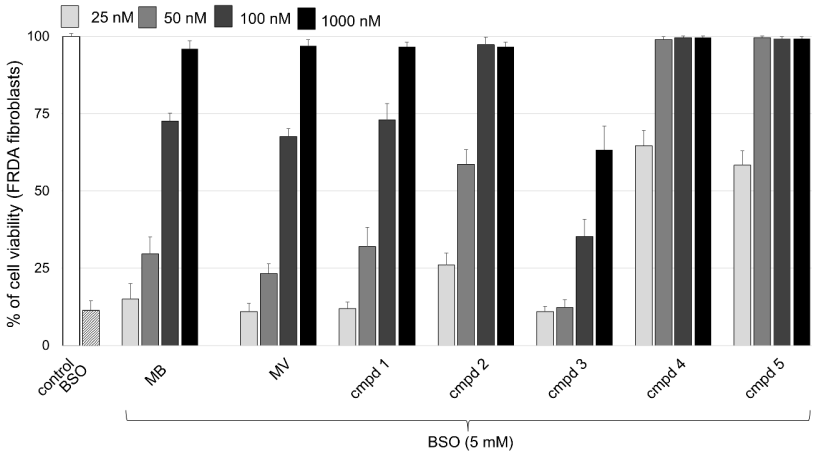


Figure 5. Cytoprotective effect of compounds **1-5** in (BSO)-treated FRDA fibroblasts following preincubation with test compounds and subsequent treatment with BSO (5 mM) for 48 h. Results obtained were verified by running triplicates and repeating experiments in two independent runs. The effects of the tested compounds on the fluorescence intensities of the dyes never exceeded 0.1%. The actual fluorescence intensity data is shown in Figure S2 of the Supplementary Data.

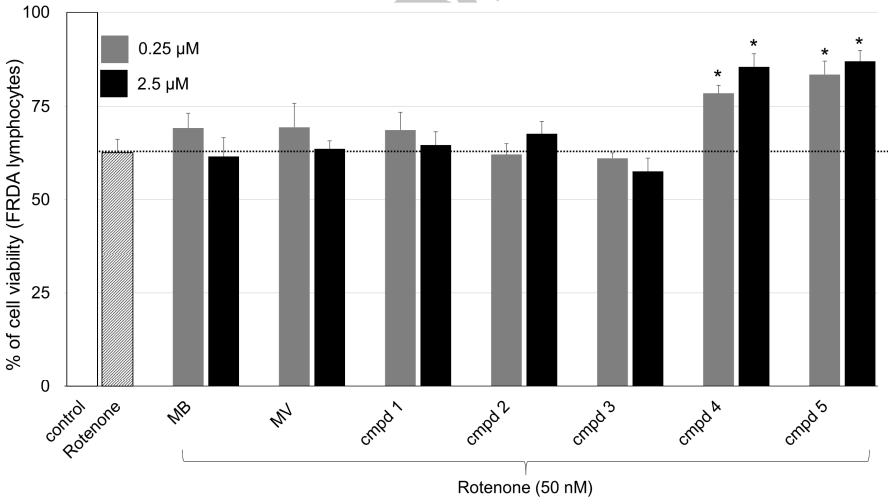


Figure 6. Cytoprotective effect of the compounds **1-5** in FRDA lymphocytes following preincubation with the test compounds for 12 h in glucose-free media and subsequent treatment with rotenone (50 nM) for 24 h to inhibit complex I. Cell viability was expressed as the percentage of cells relative to control. The actual percentages of live cells were extracted from a two-dimensional color density dot plot. Results are an average of two independent trials run in duplicate. Statistically significant differences, where observed, are also indicated. Statistical analyses were performed with a two-tailed Student's *t*-test, and data are mean values ± SD (n = 2). * $P > 0.05$, as compared to rotenone treated control.

The compounds were also tested for their ability to confer cytoprotection to rotenone-treated FRDA lymphocytes. The cells were treated with rotenone to induce cytotoxicity by inhibiting complex I. As shown in Figure 6, the results closely paralleled those found for BSO-treated FRDA fibroblasts. Compounds **4** and **5** were able to protect the cells against rotenone-induced cytotoxicity more efficiently than MB, MV or analogues **1**-**3**.

2.2.5. NADH: ubiquinone oxidoreductase activity (complex I activity)

An important characteristic of any CoQ_{10} surrogate is its ability to act as an electron carrier between the individual mitochondrial complexes in the respiratory chain. Accordingly, we evaluated MB, MV, and compound **5** for their effects on complex I function in presence and absence of rotenone (Figure 7). Both MB and MV were able to

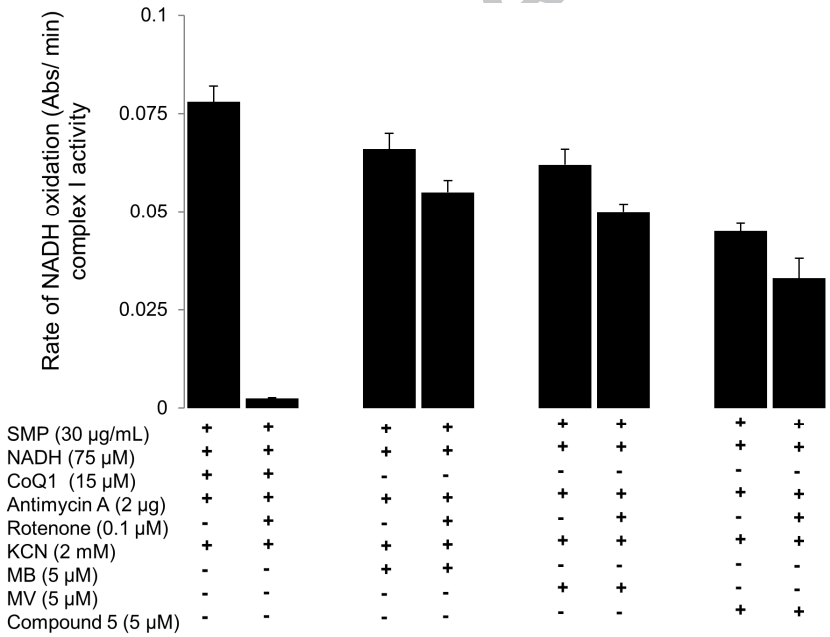


Figure 7. Effect of compound **5** on bovine heart mitochondrial complex I (SMP) activity in the presence of rotenone. In common with methylene blue and methylene violet, analogue **5** was able redirect electron flow from complex I resulting from NADH oxidation, even when the normal electron flow from complex I was completely blocked by (50 nM) rotenone. Results obtained were verified by running duplicates and repeating experiments in two independent runs.

maintain electron flow in the respiratory chain resulting from NADH oxidation even if complex I was blocked by rotenone. While compound **5** was not as efficient as MV or MB in the presence or absence of rotenone, it did support the flow of electrons. These results are in agreement with earlier studies which demonstrated that MB-mediated electron transfer is insensitive to either rotenone or antimycin A inhibition, consistent with the suggestion that MB provides an alternative route for electron transfer,[18,19,37] especially involving cytochrome *c*. MB may accept electrons from NADH at mitochondrial complex I and transfer them to cytochrome *c*, thus maintaining aerobic respiration. The alternative electron transfer through MB prevents electron leakage induced by complex I/III inhibition, thus consistent with a potential role in neuroprotection. The apparent ability of MV analogue **5** to participate in the alternative electron transfer mechanism, coupled with its lesser intrinsic cytotoxicity than MB and MV (Figure 2), and its superior properties in maintaining mitochondrial membrane potential (Figure 3), suppressing ROS (Figure 4) and conferring cytoprotection (Figures 5 and 6) seems fully consistent with its possible utility in maintaining the function of partially dysfunctional mitochondria. Indeed, a recent study demonstrated that MB itself rescues heart defect in a frataxin-depleted drosophila model of FRDA.[38]

2.2.6. Determination of ATP levels

Since the transfer of electrons directly from complex I to cytochrome *c* would logically involve the concomitant pumping of fewer protons into the intermembrane space, it might be anticipated that this pathway would result in the production of less ATP. Accordingly, MV analogues **1**–**5** were evaluated for their ability to support ATP production in FRDA lymphocytes. As shown in Figure 8, MB strongly diminished ATP

levels in a concentration dependent fashion (0.5 and 5 μM) in Friedreich's ataxia lymphocytes and had no effect at 100 nM concentration. Similar results were observed

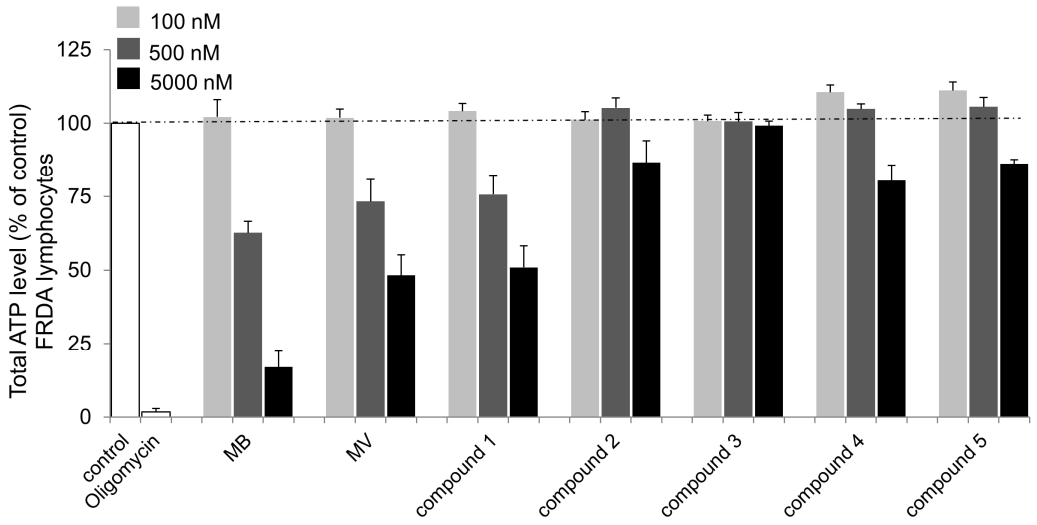


Figure 8. Total ATP level in FRDA lymphocytes following incubation with compounds **1-5** for 24 h in glucose free media (25 mM galactose). Results are expressed as the percentage of total ATP relative to an untreated control. Results obtained were verified by running triplicates and repeating experiments in three independent runs. The actual chemiluminescence intensity data is shown in Figure S3 of the Supplementary Data.

for MV and analogue **1**, the latter having a short alkyl substituent chain. Compounds **2** and **3** had little effect on the ATP level. In comparisons, compound **4** and **5** clearly increased the ATP level when employed at 0.1 and 0.5 μM concentrations, although they suppressed ATP levels when used at 5 μM concentration. The mechanism by which ATP levels were increased is presently unclear, but could plausibly reflect more efficient redox cycling by the analogues between complex I and cytochrome *c*, at a level sufficient to compensate for reduced proton pumping in a single cycle.

3. Conclusions

In conclusion, five lipophilic MV analogues were designed and synthesized. The synthetic route involved Smiles rearrangement as one of the key steps to obtain a substituted phenothiazine analogue. The final compounds were obtained in a good state

of purity. While the number of analogues studied was too small to allow detailed conclusions to be reached regarding structure–activity relationships, the specific side chain modifications did affected the biological activity of individual MV derivatives. Compounds **4** and **5** were more effective than MB and MV in almost all of the assays, and exhibited better antioxidant activity than the parent compounds. Surprisingly, compounds **4** and **5** were able to increase ATP levels in cultured FRDA lymphocytes, consistent with their potential utility for therapeutic intervention in mitochondrial disorders. Compounds **4** and **5** were also superior to **1** – **3** in most of the bioassays, suggesting that more detailed investigation may reveal more systematic structure–activity relationships.

4. Experimental section

4.1. General experimental procedures

Reagent grade chemicals and solvents were purchased from Sigma-Aldrich Chemicals and were used without further purification. All the reactions were performed under an argon atmosphere, unless otherwise specified. Thin layer chromatography (TLC) plates (precoated glass plates with silica gel 60 F254, 0.25 mm thickness) were used for analytical TLC and were visualized by UV irradiation (254 nm). Flash chromatography was carried out using Silicycle 200–400 mesh silica gel. ^{1}H and ^{13}C NMR spectra were obtained using a Varian 400 MHz NMR spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) and are referenced to residual $CHCl_3$ (δ 7.26 ppm for ^{1}H NMR and δ 77.16 for ^{13}C NMR) as the internal standard. Splitting patterns are designated as s, singlet; d, doublet; m, multiplet. High resolution mass spectra were obtained at the

Facility or the Arizona State University CLAS High Resolution Mass Spectrometry Facility.

Human mitochondrial disease cell lines, Friedreich's ataxia lymphocytes (GM15850), and Friedreich's ataxia skin fibroblast (GM04078), were obtained from Coriell Cell Repositories (Camden, NJ).

4.1.1. 2-(2,4-Dinitrophenyl)thio)-4-methoxyaniline (7). A sample containing 2.00 g (11.1 mmol) of 2-amino-6-methoxybenzothiazole (**6**) was suspended in 40 mL of water and treated with 9.30 g (167 mmol) of solid KOH. The resulting suspension was heated at reflux for 12 h. The cooled reaction mixture was added dropwise to a solution of 2.25 g (11.1 mmol) of 2, 4-dinitrochlorobenzene in a mixture of 30 mL of ethanol and 20 mL of HOAc in an ice–water bath. The reaction mixture was then stirred at room temperature for an additional 3 h. The precipitate was filtered, washed with 1:1 water–ethanol 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 (acetone-d_6) 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 and 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).

4.1.2. *N*-(2-(2,4-Dinitrophenyl)thio-4-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 then 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 $MgSO_4$, then 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 and 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 and 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).

4.1.3. 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 portionwise 0.91 g (16.2 mmol) of KOH in 10 mL of ethanol. The reaction mixture was maintained 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 $MgSO_4$, then 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 (acetone-d_6) 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 (acetone-d_6) 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).

4.1.4. *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 NaH (60% in mineral oil). 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 argon atmosphere, and was then 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 (acetone-d_6) δ 1.54 (s, 9H), 3.86 (s, 3H), 6.97 (dd, 1H, J = 9.2 and 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 and 2.6 Hz) and 8.24 (d, 1H, J = 2.4 Hz); ^{13}C NMR (acetone-d_6) 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).

4.1.5. *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% palladium-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 the filtrate was concentrated under diminished pressure. The crude product (**11**) was used for the next reaction without further purification.

4.1.6. *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 a nitrogen atmosphere and stirred at 80 °C overnight. The cooled reaction mixture was filtered and the filtrate was concentrated under diminished pressure and purified on a silica gel column (10 × 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 and 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).

4.1.7. 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 then 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 × 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).

4.1.8. *tert*-Butyl 3-(*N*,*N*-Didecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate (13). A sample containing 0.74 g (2.90 mmol) of 1-iododecane in 2 mL of acetonitrile was added to 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 a nitrogen atmosphere and stirred at 80 °C for 48 h. The cooled reaction mixture was filtered and the filtrate was concentrated under diminished pressure and purified on a silica gel column (8 × 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 and 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).

4.1.9. 7-(*N*,*N*-Didecylamino)-3*H*-phenothiazin-3-one (2). A sample containing 0.20 mL (0.20 mmol) of 1 M BBr_3 in CH_2Cl_2 was added dropwise to a solution containing 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 then quenched with 10 mL of water. The product was extracted with two 10-mL portions of ethyl acetate. The violet combined organic phase was washed with 20 mL of brine, dried over anhydrous Na_2SO_4 and concentrated under diminished pressure. The resulting violet solid was purified on a silica

gel column (7 × 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).

4.1.10. *tert*-Butyl 3-(*N*,*N*-Dipentadecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate (14). A sample containing 0.98 g (2.90 mmol) of 1-iodopentadecane in 2 mL of acetonitrile was added to 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 a nitrogen atmosphere and stirred at 80 °C for ~ 48 h. The cooled reaction mixture was filtered and the filtrate was concentrated under diminished pressure. The crude product was purified on a silica gel column (8 × 2 cm) to separate unreacted starting material. Elution with 1:19 ethyl acetate–hexanes gave a mixture of mono and dialkylated products as a yellow oil. The crude product (**14**) was used for the next reaction without further purification.

4.1.11. 7-(*N*,*N*-Dipentadecylamino)-3*H*-phenothiazin-3-one (3). A sample containing 0.28 mL (0.28 mmol) of 1 M BBr_3 in CH_2Cl_2 was added dropwise to a 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 then 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 diminished 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).

4.1.14. *tert*-Butyl 3-(*N*-Pentadecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate (15). A sample containing 2.11 g (7.25 mmol) of 1-bromopentadecane in 3 mL of acetonitrile was added to 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 a nitrogen atmosphere and stirred at 80 ^{o}C for 3 days. The cooled reaction mixture was filtered and 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 and 2.6 Hz), 6.50 (d, 1H, J = 2.8 Hz), 6.76 (dd, 1H, J = 8.8 and 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).

4.1.15. *tert*-Butyl 3-(*N*-Methyl-*N*-pentadecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate (16). A sample containing 7.00 μL (16.0 mg, 0.11 mmol) of iodomethane was added to 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 a nitrogen atmosphere and stirred at 80 °C for 16 h. The cooled reaction mixture was filtered, and the filtrate was concentrated under diminished pressure. The crude product was used in the next step without further purification.

4.1.16. 7-(*N*-Methyl-*N*-pentadecylamino)-3*H*-phenothiazin-3-one (4). A sample containing 0.27 mL (0.27 mmol) of 1 M BBr_3 in CH_2Cl_2 was added dropwise to a 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 then quenched with 10 mL of water. The product was extracted with two 10-mL portions of ethyl acetate. The combined organic extract was washed with 20 mL of brine, dried over anhydrous Na_2SO_4 and concentrated under diminished pressure. The resulting violet solid was purified on a silica gel column (7 × 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).

4.1.17. *tert*-Butyl 3-(*N*-Butyl-*N*-pentadecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate (17). A sample containing 0.11 mL (0.14 g, 1.00 mmol) of 1-bromobutane was added to 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 a nitrogen atmosphere and stirred at 80 °C for 30 h. The cooled reaction mixture was filtered and the filtrate was concentrated under diminished pressure. The crude product was used in the next step without further purification.

4.1.18. 7-(*N*-Butyl-*N*-pentadecylamino)-3*H*-phenothiazin-3-one (5). A sample containing 0.48 mL (0.48 mmol) of 1 M BBr_3 in CH_2Cl_2 was added dropwise to a 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 then quenched with 10 mL of water. The crude product was extracted with two 10-mL portions of ethyl acetate. The combined organic extract was washed with 20 mL of brine, dried over anhydrous Na_2SO_4 and concentrated under diminished pressure. The resulting violet solid was purified on a silica gel column (7 × 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).

4.2. Biochemical and biological evaluation of the MV analogues

4.2.1. Cytotoxicity Assay. Evaluation of methylene blue analogues 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. We have used a nutrient-sensitized screening strategy 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.[30,31] Methylene blue analogues 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). Results obtained were verified by running duplicates and repeating experiments in two independent runs.

4.2.2. 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.[24] 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. Results expressed as the percentage of cells with intact $\Delta\psi_m$ were verified by running duplicates and repeating the experiments in two independent runs.

4.2.3. Reactive Oxygen Species (ROS). 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.[17,35] 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. 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 cell debris was electronically gated out. Results obtained were verified by running duplicates and

repeating experiments in two independent runs. Results were expressed as a percentage of the median mean fluorescence intensity of DCF fluorescence relative to a DEM-treated control.

4.2.4. NADH: ubiquinone oxidoreductase activity (complex I activity)

The inhibition of NADH-Q oxidoreductase activity was determined using the same experimental conditions described previously.[39] SMPs (30 µg/mL) were incubated at 30 °C for 5 min with the test compound in 1 mL of 50 mM phosphate buffer, pH 7.4, containing 0.25 M sucrose, 1 mM $MgCl_2$, 2 µM antimycin A and 2 mM KCN. The reaction was initiated by the addition of 75 µM NADH and 15 µM coenzyme Q_1. Enzymatic activity, measured by the decrease in NADH absorbance, was monitored at 340 nm. Data are reported as the mean of two independent experiments run in duplicate.

4.2.5. Effects of methylene violet analogues on cell viability in BSO-treated FRDA fibroblasts

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 as previously described.[17] 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.

4.2.6. Effects of methylene violet analogues on cell viability in rotenone-treated FRDA lymphocytes

The cytoprotection conferred by the prepared compounds was determined in rotenone treated FRDA lymphocyte using a simultaneous staining with a two-color fluorescence assay, the Live/ Dead® Viability/Cytotoxicity Kit (Molecular Probes). 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 4 h. Cells were treated with 50 nM rotenone and incubated at 37 °C for 24 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). Results obtained were verified by running duplicates and repeating experiments in two independent runs.

4.2.7. Measurement of cellular ATP concentration. Total ATP levels in FRDA lymphocytes were measured as described before.[31] Briefly, lymphocytes (2×10^5 cell/mL) were plated (1 mL) in 24-well plates, treated with the test compounds (dissolved in DMSO and added to the assay medium at final DMSO concentrations <0.5%) at final concentrations of 0.1, 0.5 and 5 μM, and then incubated at 37 °C for 24 h in a humidified atmosphere containing 5% CO_2 in air. 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.

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References

1. Armstrong JS, Khdour OM, Hecht SM. *FASEB J*. 2010;24:2152.
2. Markesbery WR, Carney JM. *Brain Pathol*. 1999;9:133.
3. Barnham KJ, Masters CL, Bush AI. *Nat Rev Drug Discovery*. 2004;3:205.
4. Calabrese V, Lodi R, Tonon C, D'Agata V, Sapienza M, Scapagnini G, Mangiameli A, Pennisi G, Stella AM, Butterfield DA. *J Neurol Sci.* 2005;233:145.
5. Lin MT, Beal MF. *Nature.* 2006;443:787.
6. Henze K, Martin W. *Nature.* 2003;426:127.
7. Saraste MW. *Science.* 1999;283:1488.
8. Newmeyer DD, Ferguson-Miller S. *Cell.* 2003;112:481.
9. Fiore C, Trezeguet V, Le Saux A, Roux P, Schwimmer C, Dianoux AC, Noel F, Lauquin GJM, Brandolin G, Vignais PV. *Biochimie.* 1998;80:137.
10. Turrens JF. *J Physiol*. 2003;552:335.
11. Murphy MP. *Biochem J*. 2009;417:1.
12. Mates JM, Perez-Gomez C, Nunez de Castro I. *Clin Biochem*. 1999;32:595.
13. Gaetani GF, Galiano S, Canepa L, Ferraris AM, Kirkman H. *Blood.* 1989;73:334.
14. Heller G, Brockhoff A, Goepferich A. *Eur J Pharm Biopharm*. 2012;82:1.
15. Smith RA, Hartley RC, Cocheme HM, Murphy MP. *Trends Pharmacol Sci.* 2012;33:341.
16. Frantz M-C, Wipf P. *Environ Mol Mutagen.* 2010;51:462.
17. Arce PM, Khdour OM, Goldschmidt R, Armstrong JS, Hecht SM. *ACS Med Chem Lett*. 2011;2:608.
18. Atamna H, Nguyen A, Schultz C, Boyle K, Newberry J, Kato H, Ames BN. *FASEB*

J. 2008;22:703.

19. Wen Y, Li W, Poteet EC, Xie L, Tan C, Yan LJ, Ju X, Liu R, Qian H, Marvin MA, Goldberg MS, She H, Mao Z, Simpkins JW, Yang SH. *J Biol Chem.* 2011;286:16504.
20. Wainwright M, Crossley KB. *J Chemother*. 2002;14:431.
21. Baddeley TC, McCaffrey J, Storey JMD, Cheung JKS, Melis V, Horsley D, Harrington CR, Wischik CM. *J Pharmacol Exp Ther*. 2015;352:1.
22. Melis V, Magbagbeolu M, Rickard JE, Horsley D, Davidson K, Harrington KA, Goatman K, Goatman EA, Deiana S, Close SP, Zabke C, Stamer K, Dietze S, Schwab K, Storey JM, Harrington CR, Wischik CM, Theuring F, Riedel G. *Behav Pharmacol.* 2015;26:353.
23. Houghtaling MA, Perera R, Owen KE, Wagner S, Kuhn RJ, Morrison H. *Photochem Photobiol.* 2000;71:20.
24. Arce PM, Goldschmidt R, Khdour OM, Madathil, MM, Jaruvangsanti J, Dey S, Fash DM, Armstrong JS, Hecht SM. *Bioorg Med Chem.* 2012;20:5188.
25. Khdour OM, Arce, PM, Roy B, Hecht SM. ACS Med Chem Lett. 2013;4:724.
26. Chevalier A, Khdour OM, Schmierer M, Bandyopadhyay I, Hecht SM. *Bioorg Med Chem.* 2017;25:1703.
27. Li JJ, Corey EJ. Editors. *Name Reactions in Heterocyclic Chemistry II*. John Wiley & Sons, Inc.; 2011.
28. Smith MB. *March's Advanced Organic Chemistry.* 7th Edition. John Wiley & Sons, Inc.; 2013; pp 800-802.
29. Yu L, Cui J, Padakanti PK, Engel L, Bagchi DP, Kotzbauer PT, Tu Z. *Bioorg Med*

Chem. 2012;20:4625.

30. Aguer C, Gambarotta D, Mailloux RJ, Moffat C, Dent R, McPherson R, Harper ME. *PLoS One.* 2011;6:e28536.
31. Robinson BH, Petrova-Benedict R, Buncic JR, Wallace DC. *Biochem Med Metab Biol*. 1992;48:122.
32. Papadopoulos NG, Dedoussis GV, Spanakos G, Gritzapis AD, Baxevanis CN, Papamichail M. *J Immunol Methods*. 1994;177:101.
33. Smiley ST, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith TW, Steele GD Jr, Chen LB. *Proc Natl Acad Sci U.S.A.* 1991;88:3671.
34. Ehrenberg B, Montana V, Wei MD, Wuskell JP, Loew LM. *Biophys J.* 1988;53:785.
35. Chevalier A, Alam MP, Khdour OM, Schmierer M, Arce PM, Cripe CD, Hecht SM. *Bioorg Med Chem*. 2016;24:5206.
36. Griffith OW, Meister A. *J Biol Chem*. 1979;254:7558.
37. Poteet E, Winters A, Yan LJ, Shufelt K, Green KN, Simpkins JW, Wen Y, Yang SH. *PLoS One*. 2012;7:e48279.
38. Tricoire H, Palandri A, Bourdais A, Camadro JM, Monnier V. *Hum Mol Genet.* 2014;23:968.
39. Goldschmidt R, Arce PM, Khdour OM, Collin VC, Dey S, Jaruvangsanti J, Fash DM, Hecht SM. *Bioorg Med Chem.* 2013;21:969.

Graphical abstract