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Phenothiazine antioxidants increase mitochondrial biogenesis and frataxin levels in Friedreich's ataxia cells†

Omar M. Khmour, ^{*a} Indrajit Bandyopadhyay, ^{ab} Nishant P. Visavadiya,^a
Sandipan Roy Chowdhury^{ab} and Sidney M. Hecht ^{ab}

Friedreich's ataxia (FRDA) is a progressive neurodegenerative disease that is linked to transcriptional repression of the nuclear FXN gene encoding the essential mitochondrial protein frataxin (FXN). Compounds that increase frataxin levels may enable effective therapeutic intervention for blunting disease progression. Recently, we showed that lipophilic methylene violet (MV) and methylene blue (MB) analogues both conferred benefit to cultured FRDA cells in several regards, including ROS suppression, maintenance of mitochondrial membrane potential and increased ATP production. Some of the MB analogues were also shown to promote increased frataxin levels and mitochondrial biogenesis. Presently, we report that two of the MV analogues studied previously (**1** and **2**) also increased frataxin levels and mitochondrial biogenesis significantly. Because the substitution pattern in the two series of compounds was not the same, we also prepared new MV derivatives having the same substitution pattern as the original MB derivatives studied to enable a more direct comparison. Two of the new MV compounds, **4b** and **6b**, exhibited enhanced antioxidant capability, increased frataxin levels and mitochondrial biogenesis, and improved aconitase activity. These encouraging findings demonstrated that the MV analogues had better overall activity with less cytotoxicity.

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1. Introduction

Friedreich's ataxia (FRDA) is an autosomal recessive neurodegenerative disease that is caused predominantly by a homozygous GAA repeat expansion in the first intron of the FXN gene, impairing expression levels of fully functional frataxin protein (FXN).^{1–5} Frataxin is a nuclear-encoded mitochondrial protein widely conserved among eukaryotes; it is known to play a significant role in both cellular and mitochondrial iron metabolism, iron-sulfur (Fe-S) cluster, and heme synthesis.^{6–12} The deficiency in frataxin is associated with iron accumulation in the mitochondria, impairment of Fe-S cluster containing enzymes, increased sensitivity to oxidative stress, and deficits of respiratory chain complex activities.^{13–18} Given the putative role of frataxin, the potential of small molecules to treat FRDA might well reflect their ability to increase frataxin expression, and relieve mitochondrial oxidative stress.

Phenothiazines are a class of heterocyclic compounds characterized by a tricyclic aromatic ring with sulfur and ni-

trogen atoms. This motif has been employed successfully in the design of a variety of pharmaceuticals with a broad spectrum of biological activities.^{19–21} As the phenothiazine ring substituents substantially influence their properties, methods for the preparation of a diverse range of substituted phenothiazines are highly desirable. Various phenothiazine derivatives such as methylene blue (MB) and methylene violet (MV) have been prepared and these have become important due to their varied biological as well as chemical properties.^{19–25} We and others have shown that MB and their analogues increase frataxin levels in FRDA lymphoblasts.^{26,27} Methylene blue has been shown to rescue heart defects in a drosophila model of FRDA ataxia.²⁸ MV is a neutral phenothiazine accessible by hydrolysis of MB under strongly basic conditions. MV can be regarded as a phenolic analogue of MB in which an OH group replaces 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 a phenolic antioxidant analogous to CoQ₁₀.

MB and MV can act as alternative electron carriers, rerouting mitochondrial electron flow from NADH to cytochrome *c*.^{22–26} This process can bypass the complex I/III blockage under pathological conditions and thus can potentially suppress ROS production from the mitochondrial electron transport chain. In earlier studies involving MV,

^a Biodesign Center for BioEnergetics, Arizona State University, Tempe, AZ 85287, USA. E-mail: Khdour@asu.edu

^b School of Molecular Sciences, Arizona State University, Tempe, AZ 85287, USA

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derivatives with varied alkyl side chains were attached to the exocyclic amine (position 3). The increased lipophilicity was associated with enhanced cytoprotective activity, but only to a certain extent. This was presumably because the interaction with mitochondrial respiratory complexes embedded in the inner mitochondrial membrane represented the locus of action. We postulated that MV modification might lead to analogues with optimal properties. Presently, we describe a comparative analysis focused on the previously reported MB analogues (3a–6a) and their newly synthesized MV (3b–6b) (Fig. 1). Interestingly, several of these compounds proved to be capable of increasing frataxin levels and mitochondrial biogenesis, in addition to improving mitochondrial function and supporting ATP synthesis in FRDA lymphoblasts.

2. Results and discussion

2.1. Chemistry

The syntheses of MV analogues **1** and **2** having long alkyl substituents on the exocyclic nitrogen atom (Fig. 1) were carried out as described.²⁵ The syntheses of MB/MV analogues

3–6, having long alkyl substituents on the phenothiazine redox core position 2 (Fig. 1), employed a Wittig reaction as the key step.²⁶ Phenothiazines **3a–6a** were prepared as described before (Scheme 1).²⁶ Basic hydrolysis of MB has been shown to form a highly colored, somewhat more lipophilic species, namely methylene violet.²⁹ The MV analogues (**3b–6b**) (Fig. 1) were obtained by basic hydrolysis of the corresponding MB analogues **3a–6a** (Scheme 1).

They were characterized by ¹H and ¹³C NMR, and by high resolution mass spectrometry.

2.2. Biological activity

2.2.1. Cytotoxicity. MB is a unique pharmaceutical agent that has a hormetic dose-response with opposite effects at low and high doses; this has been attributed to its auto-oxidizing properties.³⁰ In this regard MB and MB analogues were tested for their cytotoxicity in FRDA lymphoblasts. MB analogues **3a–5a**, having 5–13 carbon atom alkyl side chains, exhibited significant cytotoxicity at 2.5 μM concentration as confirmed by FRDA cell culture grown in glucose-free medium

Fig. 1 Chemical structures of the synthesized methylene blue (MB) and methylene violet (MV) analogues.

Scheme 1 Synthetic routes employed for compounds 3–6.

containing galactose (Fig. 2). Under these conditions, cells are forced to rely predominantly on oxidative phosphorylation for ATP synthesis, given the low efficiency of this carbon source in supporting the glycolytic pathway; consequently, they are more sensitive to mitochondrial perturbation than cells grown in glucose media.^{31,32} In comparison, methylene blue analogue 6a having a 16-carbon side chain lacked cytotoxicity at 0.25 or 2.5 μM concentrations. The toxicity of the newly prepared methylene violet analogues (3b–6b) was also evaluated. MV itself and analogue 3b exhibited significant cytotoxicity toward FRDA lymphoblasts at 2.5 μM concentration after a 48 h incubation (Fig. 2). However, MV was less cytotoxic in comparison to MB, and 3b was significantly less cytotoxic than 3a. The remaining MV analogues with longer alkyl side chains (4b–6b) were not cytotoxic under any tested condition (Fig. 2). MV and all of its tested analogues were less cytotoxic than the corresponding MB analogues.

2.2.2. Preservation of mitochondrial inner membrane potential ($\Delta\psi_{\text{m}}$). The mitochondrial membrane potential ($\Delta\psi_{\text{m}}$) in cells has evolved as a key measure of mitochondrial func-

tional status and an indicator of cell fate. Accordingly, analogues 3–6 were tested for their effects on inner mitochondrial membrane potential in FRDA lymphoblasts, using the JC-1 probe as described previously.^{25,26} FACS analysis of $\Delta\psi_{\text{m}}$ showed that MV and analogue 3b (5-carbon side chains) exhibited some depolarization of $\Delta\psi_{\text{m}}$ at 2.5 μM concentration (Fig. 3) while compounds (4b–6b) having 11–16-carbon side chains exhibited no effect at 2.5 μM concentration. In comparison, MB analogues 3a–5a having 5-carbon to 13-carbon side chains significantly affected mitochondrial membrane potential, while the MB analogue with a 16-carbon side chain had no effect on $\Delta\psi_{\text{m}}$ even at 2.5 μM concentration. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a commonly used protonophore uncoupler of oxidative phosphorylation in mitochondria, was used as a negative control to dissipate the chemiosmotic proton gradient, resulting in depolarization of mitochondrial membrane potential.

2.2.3. Cytoprotection. The ability of the prepared compounds (3–6) to confer cytoprotection was evaluated using a lymphoblast cell line derived from an individual with FRDA. Lymphoblasts were treated with diethyl maleate to induce oxidative stress through depletion of glutathione. The cytoprotection results obtained are shown in Fig. 4. MB and analogues (3a, 5a and possibly 4a) were less protective at 2.5 μM than at 0.5 μM concentration. This could relate to a hormetic dose-response behavior similar to that of MB. Increasing the side chain length (from 11 to 16 carbon atoms) in compounds 4a–6a afforded significantly improved cytoprotection relative to the parent compound MB itself and analogue 3a. The cytoprotective effects of all MV analogues (3b–6b) were dose dependent in the concentration range from 0.1 μM to 2.5 μM (Fig. 4). Increasing the side chain length (11 to 16 carbon atoms) in compounds 4b, 5b and 6b significantly improved cytoprotection relative to the parent compound MV itself and analogue 3b.

Fig. 3 Panel A: Effects of test compounds **3–6** on $\Delta\psi_m$ in FRDA lymphoblasts using the ratiometric fluorescent marker JC-1. 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 $\Delta\psi_m$. Panel B: The bar graph shows the percentage of cells with intact $\Delta\psi_m$, calculated using BD Accuri™ C6 software. For each analysis, 10 000 events were recorded. Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) is a potent mitochondrial oxidative phosphorylation uncoupler and served as positive control for depolarization of the mitochondrial membrane. Results obtained were verified by running duplicates and repeating each experiment in an independent run.

Fig. 4 Cytoprotective effect of compounds **3–6** in diethyl maleate (DEM)-treated FRDA lymphoblasts following preincubation with test compounds and subsequent treatment with DEM (5 mM) for 5 h. Results obtained were verified by running duplicates and repeating each experiment in an independent run.

2.2.4. Effect of MB/MV analogues on ATP levels. Methylene blue is a recyclable redox complex that can donate electrons to the electron transport chain in mitochondria, thereby promoting cytochrome *c* oxidase activity, oxygen consumption, and energy production. An important prerequisite for any CoQ₁₀ surrogate to be used therapeutically is its ability to augment ATP production by its ability to act as an electron carrier between the individual mitochondrial complexes without concomitant inhibition of these complexes, or producing cellular cytotoxicity. Thus, while such compounds must be able to associate with each of the mitochondrial complexes in the respiratory chain, the binding should not be excessively

tight, to avoid inhibiting any of the complexes that participate in electron transport. Accordingly, MB/MV analogues **3–6** were evaluated for their ability to support ATP production in FRDA lymphoblasts. A similar trend was noted as for the ability of the tested MB analogues (**3a–6a**) to maintain mitochondrial inner membrane potential (Fig. 5). MB strongly diminished ATP levels in a concentration dependent fashion (0.5 and 2.5 μM) in Friedreich's ataxia lymphoblasts, but had no effect at 0.1 μM concentration. Similar results were observed for analogues **3a–5a**. Compounds **6a** clearly increased the ATP level when employed at 0.1 and 0.5 μM concentrations, but suppressed the ATP level when used at 2.5 μM concentration.

Fig. 5 Effect of MB/MV analogues 3–6 on ATP level in FRDA lymphoblasts following incubation 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 each experiment in three independent runs.

On the other hand MV analogues (3b–6b) with similar substitution patterns of lipophilic alkyl side chain were also studied in this assay. Again, MV and compound 3b strongly diminished ATP levels in a concentration dependent fashion (0.5 and 2.5 μM) but had no effect at 0.1 μM concentration. The MV analogue with an 11-carbon atom alkyl side chain (4b) increased the ATP level when employed at 0.1 μM concentration, produced no change at 0.5 μM , but suppressed the ATP level when used at 2.5 μM concentration. The MV analogue with a 13-carbon atom alkyl side chain (5b) had no effect on ATP when employed at 0.1 and 0.5 μM concentrations, but suppressed the ATP level slightly when used at 2.5 μM concentration. Compound 6b with a 16-carbon atom alkyl side chain clearly increased the ATP level when employed at 0.1 and 0.5 μM concentrations, but suppressed the ATP level when used at 2.5 μM concentration. It is apparent that increasing hydrophobicity is essential to activity, presumably because interaction with mitochondrial respiratory complexes embedded in the inner mitochondrial membrane represent the locus of action and limits the undesired aqueous autoxidation of the phenothiazine redox core. This provides the likely explanation for the extreme hydrophobicity of endogenous CoQ₁₀. The hydrophobicity can fine-tune the relative rates of these reactions, allowing a

rational approach to the design of therapeutic mitochondria-targeted compounds which undergo redox cycling. Overall, MB analogues with short alkyl side chains (5–13 carbon atoms) exhibited stronger, dose dependent inhibition of ATP levels than their MV analogue counterparts.

2.2.5. Methylene blue/violet analogues induce FXN protein levels, increase mitochondrial biogenesis, and restore aconitase activity in FRDA patient cells. Since the lack of FXN is the underlying cause of mitochondrial dysfunction, increasing the amount of FXN should logically be an effective therapeutic approach. In this study, we demonstrate that MB/MV analogues not only act as effective antioxidants, but also, most interestingly, increase FXN levels and enhance mitochondrial biogenesis in FRDA patient-derived cells, when compared to the untreated control (Fig. 6 and 7). Both parent compounds (MB/MV) showed increased FXN levels when tested at 50 and 250 nM following 24 and 48 h treatment in FRDA affected cells (Fig. 6). Similar results were observed for their effect on mitochondrial biogenesis (Fig. 7). It should be noted that while most of the compounds that increased FXN levels and mitochondrial biogenesis did so at both 50 and 250 nM concentrations, MB analogues 4a and 6a were more effective at 50 nM and diminished FXN levels at 250 nM concentration. In comparison, MV analogue

Fig. 6 Effect of MB/MV analogues 3–6 on frataxin expression level in Friedreich ataxia cells. Frataxin levels were detected based on the principle of a solid-phase enzyme-linked immunosorbent assay following 24 and 48 hours of treatment. An In-Cell ELISA Kit was used to assess protein levels of frataxin. Data are representative of two independent experiments and values are expressed in means $\pm$ SEM. The effects of MB/MV and their analogues were compared using one way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test using GraphPad Software (La Jolla, CA). Dashed line represents 100% of untreated FRDA lymphoblasts; *p* values as depicted versus untreated control (FRDA). **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

Fig. 7 Effect of MB/MV analogues 3–6 on mitochondrial biogenesis in Friedreich's ataxia cells was evaluated using an ELISA kit (Abcam, Cambridge, UK) based on simultaneous detection of two mitochondrial proteins, succinate dehydrogenase (SDH-A), a subunit of a complex II (nDNA-encoded protein) and cytochrome c oxidase subunit 1 (COX1), a subunit of complex IV (mtDNA-encoded). The ratio between these proteins reflects the intensity of mitobiogenesis. All data are means $\pm$ SEM of two independent experiments. The effects of MB and its analogues were compared using one way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test using GraphPad Software (La Jolla, CA). Dashed line represents 100% of untreated FRDA lymphoblasts. Comparisons were made between control (FRDA) vs. drug treated groups. * p < 0.05, ** p < 0.01, and *** p < 0.001.

counterparts **4b** and **6b** were effective at both concentrations. Compound **5b** having an alkyl side chain length of one carbon atom less than **6b** and two carbon atoms more than **4b** was less effective in both increasing FXN levels and enhancing mitochondrial biogenesis in FRDA lymphoblasts. This underscores the ability of small structural differences to support real changes in potential therapeutic benefit.

The previously published MV analogues **1** and **2** (Fig. 1) having alkyl side chains attached to the exocyclic nitrogen of the phenothiazine redox core were evaluated for their effect on FXN levels and mitochondrial biogenesis in this study as shown in Table 1.²⁵ The results obtained to date have afforded several compounds (**1**, **2** and **6b**) having desirable properties in all of the assays that we employ, including those assays that we deem the most important. Deficiency in the mitochondrial iron-binding protein frataxin results in diminished activity of various mitochondrial iron-sulfur proteins including aconitases. To validate whether the increased FXN protein level was sufficient to improve Fe-S cluster biogenesis, we measured the cellular aconitase activity in FRDA cells upon treatment with MB/MV analogues. A significant increase in aconitase activity was observed in patient-derived

FRDA lymphoblast cell lines after treatment for 5 days (Fig. 8). MV analogue **6b** was more effective than its counterpart **6a** in increasing aconitase activity in a dose-dependent fashion following 5 day treatment in FRDA affected cells.

It is unclear if the upregulation stemmed from their antioxidant properties or their ability to act as alternative electron carriers within the mitochondrial electron transport chain. While not investigated here, the mechanism(s) by which MB/MV and their analogues increase frataxin protein levels remains to be elucidated, but may involve the transcriptional regulation of the FXN gene (mRNA) or post-transcriptional and/or post-translational modifications of frataxin or alterations in frataxin half-life and metabolism. Multiple mechanisms may be simultaneously activated by MB/MV and further investigations are needed to elucidate its protective role in FRDA. One possible mechanism could involve the nuclear factor erythroid-derived 2-like 2 (Nrf2). Nrf2 was found to be impaired and transcript levels were significantly correlated with frataxin expression in FRDA cell models.^{33–36} MB is known to be a Nrf2 inducer, thus influences mitochondrial biogenesis, particularly under stress conditions by activating proliferator-

Table 1 Effect of MV analogues **1**, **2** and **6b** on FXN levels, mitochondrial biogenesis, and aconitase activity in Friedreich's ataxia cells

Compound	FXN (%)		Cox-I/SDH-A (%)		Aconitase activity (%)
	24 h	48 h	24 h	48 h	
MV (50 nM)	111 $\pm$ 2	113 $\pm$ 2*	115 $\pm$ 2*	122 $\pm$ 3*	110 $\pm$ 1
MV (250 nM)	114 $\pm$ 3*	115 $\pm$ 2*	117 $\pm$ 5*	131 $\pm$ 2**	113 $\pm$ 2*
Cmpd 1 (50 nM)	114 $\pm$ 4*	120 $\pm$ 5**	120 $\pm$ 2*	128 $\pm$ 3**	116 $\pm$ 3*
Cmpd 1 (250 nM)	118 $\pm$ 4*	127 $\pm$ 3**	127 $\pm$ 2**	137 $\pm$ 3***	123 $\pm$ 3**
Cmpd 2 (50 nM)	116 $\pm$ 2*	121 $\pm$ 1**	117 $\pm$ 2*	123 $\pm$ 1**	115 $\pm$ 4*
Cmpd 2 (250 nM)	123 $\pm$ 4**	127 $\pm$ 2**	127 $\pm$ 2**	137 $\pm$ 4***	116 $\pm$ 3*
6b (50 nM)	120 $\pm$ 3**	124 $\pm$ 3**	127 $\pm$ 3**	132 $\pm$ 4***	121 $\pm$ 2**
6b (250 nM)	127 $\pm$ 2**	131 $\pm$ 2***	132 $\pm$ 2***	139 $\pm$ 2***	124 $\pm$ 2**

(%) relative to untreated FRDA lymphoblasts; p values as depicted versus untreated control (FRDA). * p < 0.05, ** p < 0.01, and *** p < 0.001.

Fig. 8 MB/MV analogues rescued the aconitase defect in patient-derived FRDA lymphocytes following 5 days of treatment with the test compounds. Total aconitase activities were measured and normalized as described in the experimental section. All data are the means $\pm$ SEM of two independent experiments. The effects of MB/MV and their analogues were compared using one way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test using GraphPad Software (La Jolla, CA). Comparison was made between control (FRDA) vs. drug treated groups. * $p < 0.05$, and ** $p < 0.01$.

activated receptor gamma coactivator 1 α (PGC-1 α).^{37,38} Interestingly, a recent report showed that Nrf2 protein binds to evolutionarily conserved Nrf2-binding sites upstream antioxidant response elements (ARE) that exist within the FXN gene promoter, and as a result rescues frataxin deficiency in animal models and buccal cells of patients with FRDA.³⁹ Nevertheless, the involvement of MB as an Nrf2 inducer could be acting by Nrf2-dependent induction of frataxin expression in FRDA patient derived lymphoblasts. It has been shown that FXN-deficiency causes a mitochondrial biogenesis defect in multiple FRDA models and that restoration of FXN expression rescues mitochondrial biogenesis.³⁹

A correlation was observed between frataxin expression and mitochondrial biogenesis following MB/MV analogue treatment in FRDA affected cells (Fig. 6 and 7), indicating that frataxin expression is essentially and positively performed in a synchronized manner with mitochondrial biogenesis processes, as recently reported.³⁹ Methylene blue is known to act as an effective NADH oxidation catalyst *via* its alternative electron transfer that strongly increased the NAD⁺/NADH ratio and stimulated the AMPK signaling pathway.^{38,40} Moreover, activated AMPK directly phosphorylates PGC-1 α and makes it more susceptible to deacetylation by SIRT1. Interestingly, PGC-1 α , the mitochondrial biogenesis master regulator, has been shown to be downregulated in FRDA patient fibroblasts, and the positive feedback loop between PGC-1 α and frataxin levels in FRDA is impaired.^{41,42} On the basis of these facts, MB could be involved in mitochondrial biogenesis *via* the SIRT1/PGC-1 α pathway.

3. Conclusions

In conclusion, a comparative analysis between MB analogues (3a–6a) and their MV counterparts (3b–6b) was conducted in this study by comparing their biological activities in several of the assays reported. The results show that introduction of lipophilic substituents significantly reduced the observed cytotoxicity of the parent compounds (*i.e.*, methylene blue and

methylene violet), although somewhat greater lipophilicity was required to do so for MB than MV. Overall, it is evident that MV analogues were more effective with less cytotoxicity even with shorter chain lengths in all of the assays that we employed. The results obtained to date have afforded several MV analogues (1, 2 and 6b) shown to have desirable properties in FXN and mitochondrial biogenesis assay analysis. The mechanism of the upregulation of FXN expression by MB/MV analogues requires further study. Since it has been shown recently that mitobiogenesis defect responds to frataxin increase, drugs that increase frataxin would be expected to increase mitochondrial biogenesis in FRDA patients. MB/MV analogues appear promising, which suggests that more detailed investigation may reveal yet more efficacious compounds.

4. Experimental section

4.1. Chemistry

All chemicals and solvents were of reagent grade and were used without further purification, except as noted below. Anhydrous tetrahydrofuran (THF) was distilled from sodium/benzophenone under dry argon. Dichloromethane (CH₂Cl₂) was distilled from calcium hydride under argon. Thin layer chromatography (TLC) plates (precoated glass plates with silica gel 60 F254, 0.25 mm thickness) were used for analytical TLC; the compounds were visualized by UV irradiation (254 nm). Column chromatography was carried out using Silicycle 200–400 mesh silica gel. ¹H and ¹³C 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 CHCl₃ (δ 7.26 ppm for ¹H NMR and δ 77.16 for ¹³C NMR) as the internal standard. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd doublet of doublets. High resolution mass spectra were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Facility. UV-vis spectra were recorded on an Agilent Technologies Cary 60 UV-vis spectrometer as a CH₂Cl₂ solution and a MeOH solution. Compounds 1–2 were prepared as described;²⁵ compounds 3a–6a were prepared as described.²⁶

4.1.1. 7-(*N,N*-Dimethylamino)-2-pentyl-3H-phenothiazin-3-one (3b). To a solution containing 0.65 g (1.85 mmol) of 3a (ref. 26) in 10 mL of 6:1 tetrahydrofuran–water was added 100 mg (1.85 mmol) of potassium hydroxide. The reaction mixture was stirred at 70 °C for 30 min at which time the solution had changed from green to red in color. The reaction mixture was then quenched with 20 mL of water. The product was extracted with two 30 mL portions of ethyl acetate. The organic phase was washed with 20 mL of brine, dried over Na₂SO₄ and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 $\times$ 1 cm) (100:0 $\rightarrow$ 0:100 hexane–EtOAc) to give 3b as a red solid: yield 20 mg (5%); silica gel TLC *R*_f 0.16 (4:1 hexane–ethyl acetate); ¹H NMR (400 MHz, CDCl₃) δ 0.91 (t, 3H, *J* = 7 Hz), 1.37 (m, 4H), 1.71 (m, 2H), 2.70 (t, 2H, *J* =

8 Hz), 2.87 (s, 6H), 6.71 (d, 1H, $J = 2$ Hz), 6.88 (dd, 1H, $J = 2.2$ Hz), 6.91 (s, 1H), 7.58 (d, 1H, $J = 10$ Hz) and 7.70 (s, 1H), ^{13}C NMR (100 MHz, CDCl_3) δ 14.1, 22.26, 29.7, 31.5, 31.9, 44.0, 112.8, 119.2, 123.3, 133.9, 134.5, 135.4, 136.3, 139.7, 143.2, 155.8 and 182.4; UV-vis (λ_{max}) 545 nm (CH_2Cl_2), 570 nm (MeOH); HRMS (APCI), m/z 327.1534 ($\text{M} + \text{H}^+$) ($\text{C}_{19}\text{H}_{23}\text{N}_2\text{OS}$ requires m/z 327.1531).

4.1.2. 7-(*N,N*-Dimethylamino)-2-undecyl-3H-phenothiazin-3-one (4b). To a solution containing 80.0 mg (0.18 mmol) of 4a (ref. 26) in 4 mL of 6:1 tetrahydrofuran–water was added 10.0 mg (0.18 mmol) of potassium hydroxide. The reaction mixture was stirred at 25 °C for 30 min at which time the solution had changed from green to red in color. The reaction mixture was then quenched with 10 mL of water. The product was extracted with two 10 mL portions of ethyl acetate. The organic phase was washed with 10 mL of brine, dried over Na_2SO_4 and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 $\times$ 1 cm) (100:0 $\rightarrow$ 50:50 hexane–EtOAc) to give 4b as a red solid: yield 7 mg (13%); silica gel TLC R_f 0.85 (ethyl acetate); ^1H NMR (500 MHz, CDCl_3) δ 0.87 (t, 3H, $J = 7$ Hz), 1.26 (s, 16H), 1.70 (m, 2H), 2.70 (t, 2H, $J = 8$ Hz), 2.86 (s, 6H), 6.72 (d, 1H, $J = 2$ Hz), 6.88 (dd, 1H, $J = 2$ Hz), 6.92 (s, 1H), 7.58 (d, 1H, $J = 9.5$ Hz) and 7.70 (s, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 14.2, 22.8, 29.4, 29.6, 29.7, 29.8, 30.1, 31.6, 32.0, 44.0, 44.05, 112.8, 119.20, 119.27, 123.3, 133.9, 134.5, 136.4, 139.7, 143.2, 155.8 and 182.4; UV-vis (λ_{max}) 554.9 nm (CH_2Cl_2), 565 nm (MeOH); HRMS (APCI), m/z 411.2463 ($\text{M} + \text{H}^+$) ($\text{C}_{25}\text{H}_{35}\text{N}_2\text{OS}$ requires m/z 411.2470).

4.1.3. 7-(*N,N*-Dimethylamino)-2-tridecyl-3H-phenothiazin-3-one (5b). To a solution containing 780 mg (1.68 mmol) of 5a (ref. 26) in 12 mL of 6:1 tetrahydrofuran–water was added 90.0 mg (1.68 mmol) of potassium hydroxide. The reaction mixture was stirred at 25 °C for 30 min at which time the solution had changed from green to red in color. The reaction mixture was then quenched with 25 mL of water. The product was extracted with two 40 mL portions of ethyl acetate. The organic phase was washed with 25 mL of brine, dried over Na_2SO_4 and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 $\times$ 1 cm) (100:0 $\rightarrow$ 0:100 hexane–EtOAc) to afford 5b as a red solid: yield 40 mg (7%); silica gel TLC R_f 0.65 (1:1 hexane–ethyl acetate); ^1H NMR (500 MHz, CDCl_3) δ 0.87 (t, $J = 8.5$ Hz, 3H), 1.25 (s, 20H), 1.70 (m, 2H), 2.57 (s, 1H), 2.70 (t, 2H, $J = 7.5$ Hz), 2.87 (s, 4H), 3.13 (s, 1H), 6.71 (d, 1H, $J = 2$ Hz), 6.88 (dd, 1H, $J = 1.7$ Hz), 6.91 (s, 1H), 7.58 (d, 1H, $J = 10$ Hz) and 7.69 (s, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 14.2, 22.8, 29.5, 29.6, 29.71, 29.79, 29.82, 29.83, 30.1, 31.6, 32.0, 44.0, 112.8, 119.2, 123.3, 133.9, 134.5, 135.45, 135.46, 136.4, 139.7, 143.2, 155.8 and 182.4; UV-vis (λ_{max}) 544.5 nm (CH_2Cl_2), 565.5 nm (MeOH); HRMS (APCI), m/z 439.2777 ($\text{M} + \text{H}^+$) ($\text{C}_{27}\text{H}_{39}\text{N}_2\text{OS}$ requires m/z 439.2783).

4.1.4. 7-(*N,N*-Dimethylamino)-2-hexadecyl-3H-phenothiazin-3-one (6b). To a solution containing 310 mg (0.61 mmol) of 6a (ref. 26) in 8 mL of 6:1 tetrahydrofuran–water was added 40.0 mg (0.61 mmol) of potassium hydrox-

ide. The reaction mixture was stirred at 25 °C for 30 min at which time the solution had changed from green to red in color. The reaction mixture was then quenched with 15 mL of water. The product was extracted with two 20 mL portions of ethyl acetate. The organic phase was washed with 15 mL of brine, dried over Na_2SO_4 and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 $\times$ 1 cm) (100:0 $\rightarrow$ 0:100 hexane/EtOAc) to afford 6b as a violet solid: yield 10 mg (5%); silica gel TLC R_f 0.65 (1:1 hexane–ethyl acetate); ^1H NMR (500 MHz, CDCl_3) δ 0.87 (t, 3H, $J = 7.5$ Hz), 1.25 (s, 26H), 1.59 (s, 1H), 1.70 (m, 2H), 2.70 (t, 2H, $J = 7.5$ Hz), 2.88 (s, 5H), 6.72 (d, 1H, $J = 2$ Hz), 6.88 (dd, 1H, $J = 5$ Hz), 6.92 (s, 1H), 7.58 (d, 1H, $J = 10$ Hz) and 7.70 (s, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 14.2, 22.8, 29.5, 29.6, 29.7, 29.81, 29.85, 30.1, 31.6, 32.0, 44.0, 112.8, 119.2, 123.3, 134.0, 134.5, 136.4, 139.7, 143.3, 155.8 and 182.4; UV-vis (λ_{max}) 545 nm (CH_2Cl_2), 566 nm (MeOH); HRMS (APCI), m/z 481.3250 ($\text{M} + \text{H}^+$) ($\text{C}_{30}\text{H}_{45}\text{N}_2\text{OS}$ requires m/z 481.3253).

4.2. Biochemical and biological evaluation

4.2.1. Cell culture. Friedreich's ataxia lymphoblasts (GM16216 and GM15850) and clinically unaffected lymphoblast cell lines (GM16213 and GM15851) were obtained from Coriell Cell Repositories (Camden, NJ). Lymphoblasts were cultured routinely 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 of $5\text{--}10 \times 10^5$ cell per mL.

4.2.2. Cytotoxicity assay. Measurement of the *in vitro* cytotoxicity of the prepared MB/MV derivatives was carried out by incubation of the compounds (3–6) for 24 or 48 h with FRDA lymphoblasts. Cell viability and cytotoxicity were determined by simultaneous staining of live and dead cells using a two-color fluorescence assay, the Live/Dead® Viability/Cytotoxicity Kit (Molecular Probes) as described before.^{25,26} Briefly, one mL of FRDA lymphoblast cells (5×10^5 cells) was plated in a 24-well plate in glucose free media (25 mM galactose), treated with the test compounds and incubated at 37 °C for 24 or 48 h in a humidified atmosphere containing 5% CO_2 in air. Cells were collected by centrifugation at $300 \times g$ for 3 min and washed with phosphate buffered saline. The cells were resuspended in phosphate buffered saline containing 25 mM galactose. The 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 min. The cells were collected by centrifugation at $300 \times g$ for 3 min and then washed with phosphate buffered saline (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 and the FL2-H channel 585 ± 15 nm emission filter. For each analysis, 10 000 events were recorded and analyzed using C6 Accuri software (BD

Biosciences). The results obtained were verified by running duplicates and repeating each experiment as a second independent experiment.

4.2.3. Cytoprotection. To confirm the cytoprotective effect of MB/MV analogues, the Live/Dead® Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes) was used, following the manufacturer's instructions as described before.²⁵ Briefly, FRDA lymphoblast cells were seeded at a density of 5×10^5 cell per mL and treated with different concentrations of the test compound. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air for 16 h. Oxidative stress was then induced by incubation with 5 mM DEM (diethyl maleate) for 5 h, followed by evaluation of cytoprotection. Cells were collected by centrifugation at $300 \times g$ for 3 min and washed with phosphate buffered saline. The cells were resuspended in phosphate buffered saline containing 25 mM galactose. The cell suspension was then stained with 0.1 μ M calcein AM and 0.1 μ M EthD-1 and incubated in the dark at 37 °C for 15 min. Cells were collected by centrifugation at $300 \times g$ 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 and the FL2-H channel 585 ± 15 nm emission filter. For each analysis, 10 000 events were recorded and analyzed using C6 Accuri software (BD Biosciences). Cytoprotection by the test compounds was assessed with respect to the untreated controls. Cells not treated with DEM had >90% cell viability whereas DEM treatment reduced cell viability to <25%. The cell viability was expressed relative to the vehicle control (DMSO only) group ($n = 3$).

4.2.4. JC-1 mitochondrial membrane potential ($\Delta\psi_m$) assay. The uptake and retention of the cationic fluorescent dye JC-1 was used to evaluate mitochondrial membrane potential ($\Delta\psi_m$). JC-1 is a ratiometric dye which exhibits potential-dependent accumulation in mitochondria, which can identify high membrane potential through J-aggregates (red fluorescence) and low membrane potential through J-monomers (green fluorescence). Changes in $\Delta\psi_m$ were assessed by flow cytometry as described before.^{25,26} Briefly, FRDA lymphoblast cells (5×10^5 cells) were pretreated with or without the test compounds for 16 h. The cells were incubated at 37 °C in the dark for 20 min with 1 μ M JC-1. Cells were collected by centrifugation at $300 \times g$ for 3 min and washed with PBS. Cells were resuspended in PBS supplemented with 20 mM glucose and were analyzed immediately by flow cytometry using a 488 nm excitation laser and the FL1-H channel 530 ± 15 nm emission filter and the FL2-H channel 585 ± 15 nm emission filter. For each analysis, 10 000 events were recorded and analyzed using C6 Accuri software. FCCP (carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone), a mitochondrial uncoupler, 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 each experiment as a second independent experiment.

4.2.5. Measurement of cellular ATP concentration. The total cellular ATP levels in FRDA lymphoblasts were measured. Briefly, lymphoblasts (2×10^5 cell per 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 compound concentrations of 0.1, 0.5 and 5 μ M, and then incubated at 37 °C for 24 h in a humidified atmosphere containing 5% CO₂ in air. Cells in each well were mixed and transferred (100 μ L) 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.

4.2.6. Frataxin ELISA assay. Frataxin levels were measured using the frataxin human SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit (Abcam, UK) as described in a previous study.²⁶ Briefly, FRDA cells were seeded at a density of 5×10^5 cell per mL in 24-well plates and treated with different concentrations of the test compounds. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air for 24 and 48 h. After incubation, cells were lysed in the ELISA lysis buffer then the lysate was cleared of insoluble material by centrifugation and assayed for protein content using the BCA™ Protein Assay Kit (Thermo Scientific). Twenty-five μ g of protein was added to immunoaffinity anti-tag antibody coated 96-well with affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocaptured the sample analyte in solution. After a 1 h immunocapture incubation period, a signal was generated using a TMB-HRP reaction (3,3',5,5'-tetramethylbenzidine: horseradish peroxidase) solution that produced a colorimetric signal proportional to the amount of bound analyte (frataxin); the signal was measured at 600 nm. An rFXN standard curve was prepared and added to the microplate according to the manufacturer's instructions. Frataxin levels were normalized to the protein content in each sample.

4.2.7. Mitochondrial biogenesis assay. Mitochondrial biogenesis was analyzed using a Mitobiogenesis™ In-Cell ELISA Kit (Abcam, UK) according to the manufacturer's protocol. Briefly, FRDA lymphoblasts were plated in 24-well plates (5×10^5 cell per mL), treated with the test compounds and incubated at 37 °C for 24 and 48 h in a humidified atmosphere containing 5% CO₂ in air. After incubation, a suspension of lymphoblast cells were transferred (200 μ L) to the amine assay plate (Corning, USA), followed immediately by the fixation step using 4% paraformaldehyde. After the fixation step, the cells were incubated with an antibody solution for an 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 the protocol described in the In-Cell

mitochondrial biogenesis ELISA Kit (Abcam, UK). The experiment was run in triplicate and at a minimum of two repetitions per group. The ratio of COX-I/SDH-A was calculated to determine mitochondrial biogenesis.

4.2.8. Aconitase activity assay. Total aconitase activity was performed using a colorimetric assay kit (K716-100; BioVision Incorporated, Mountain View, CA) according to the manufacturer's instructions. Briefly, FRDA lymphoblasts were plated in a 24-well plate (5×10^5 cell per mL), treated with the test compounds and incubated at 37 °C for 5 days in a humidified atmosphere containing 5% CO₂ in air. After incubation, the suspended lymphoblast cells were collected by centrifugation and then the cells were lysed using 100 µL of the lysis buffer on ice, centrifuged at $800 \times g$ for 10 min at 4 °C, and then the supernatant was collected and used to measure total aconitase activity.

Conflicts of interest

The authors declare no competing interest.

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