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Effects of cytoprotective antioxidants on lymphocytes from representative mitochondrial neurodegenerative diseases

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ABSTRACT

Two new aza analogues of the neuroprotective agent idebenone have been synthesized and characterized. Their antioxidant activity, and ability to augment ATP levels have been evaluated in several different cell lines having suboptimal mitochondrial function. Both compounds were found to be good ROS scavengers, and to protect the cells from oxidative stress induced by glutathione depletion. The compounds were more effective than idebenone in neurodegenerative disease cells. These novel pyrimidinol derivatives were also shown to augment ATP levels in coenzyme Q_{10}-deficient human lymphocytes. The more lipophilic side chains attached to the pyrimidinol redox core in these compounds resulted in less inhibition of the electron transport chain and improved antioxidant activity.

1. Introduction

Mitochondria perform numerous tasks in cells such as participating in controlling the cell cycle,[1] neuronal cell signaling,[2] apoptosis,[1,3] and monitoring cell differentiation,[4] growth and development.[5] Crucially, the mitochondria are the site of most cellular energy production through the conversion of adenosine 5′-diphosphate (ADP) to adenosine 5′-triphosphate (ATP).[5–7] ATP is produced as a consequence of electron flow through the electron transport chain (ETC), which is located in the inner mitochondrial membrane. The ETC consists of four protein complexes: NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II), cytochrome bc_1 complex (complex III), and cytochrome *c* oxidase (complex IV). The flow of electrons through these complexes results in the transport of protons from the inner mitochondrial membrane into the intermembrane space. These protons are used by a fifth complex, ATP synthase (complex V), to convert ADP to ATP.[8,9] Defects in any of the protein complexes can cause mitochondrial disorders, including hereditary mitochondrial cytopathies, and neurodegenerative diseases.[10,11] Such diseases may be caused either by mutations, acquired or inherited, in mitochondrial DNA (mtDNA) or in nuclear genes that code for proteins required for proper mitochondrial function.[12–14] Mitochondrial dysfunction can also result from the adverse effects of drugs, infections, or other environmental causes.[15,16]

Leigh's syndrome, for instance, is a neurometabolic disorder affecting the central nervous system. The most common causes of Leigh's syndrome are either mutation of the SURF1 gene in nuclear DNA,[17,18] or mutation of the MT-ATP6 gene in mitochondrial DNA.[19,20] SURF1 reduces the formation of the normal cytochrome *c* oxidase complex, while MT-ATP6 mutation damages the ATP synthase protein complex and blocks ATP generation, both of which impair mitochondrial energy production.[21,22] Defects in the ETC are believed to be accompanied by an impairment of electron flow through the different mitochondrial complexes, causing increased electron leakage, and increased production of reactive oxygen species (ROS).[23–25] Excessive amounts of ROS can damage DNA,[26] RNA,[27] lipid membranes[28] and proteins,[29] and are likely to contribute to disease progression.[27,30] Accordingly, agents able to augment ATP production and decrease redox stress should be highly beneficial.

Idebenone (Catena®), an analogue of coenzyme Q_{10}, is currently undergoing clinical evaluation for the treatment of mitochondrial and neurodegenerative diseases;[31–33] it is approved provisionally in Canada for the treatment of Friedreich's ataxia.[34] It has been proposed that its neuroprotective effects can be attributed, at least in part, to its ability to function as an antioxidant, involving redox cycling between hydroquinone and quinone.[35] However, biochemical studies have indicated that idebenone is a significant inhibitor of complex I of the mitochondrial respiratory chain,[36–38] and thus could potentially stimulate ROS production in mitochondria if used at high doses.[36–39]

Recently, we described an aza analogue of idebenone (compound **1**, Fig. 1), having the same side chain as idebenone

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but a different redox core.[40] Compound **1** was more effective than idebenone in suppressing ROS generation and lipid peroxidation, following the induction of oxidative stress in cultured cells. The compound was also effective in maintaining mitochondrial membrane potential and cell viability following glutathione depletion. Finally, the compound was found to be effective at rather low concentrations, consistent with the suggestion of catalytic function.

Presently, we describe the preparation and characterization of two new pyrimidinol derivatives in protecting several different cell lines from the effects of oxidative stress. These compounds, **2** and **3**, have the same redox core as **1**, but have modified side chains lacking the hydroxyl group present in **1** (Fig. 1). By the use of cultured cell lines derived from individuals with Leber's disease, Friedreich's ataxia, Alzheimers disease, Leigh's syndrome and Parkinson's disease, the effects of the new compounds on ROS suppression, cell viability and ATP levels have been studied. In addition, the potential as have the potential inhibitory effects of the compounds on ETC complexes I, III and IV have been evaluated.

2. Results

2.1. Design and synthesis of pyrimidinol analogues

Compounds **2** and **3** were synthesized in analogy with a previously reported procedure.[40] First, 2-amino-4,6-dimethylpyrimidine (**4**) was prepared in 95% yield by treating guanidine sulfate with 2,4-pentanedione (Scheme 1). The pyrimidine ring was brominated with *N*-bromosuccinimide in 83% yield. The exocyclic amine was then protected as the respective 2,5-dimethyl-1*H*-pyrrole derivative (**6**) by treatment with 2,5-hexanedione (81% yield). Bromide **6** was then converted to the corresponding pyrimidinol using KOH, tris(dibenzylideneacetone)palladium(0) ($Pd_2(dba)_3$) and di-*tert*-butylphosphino-2′,4′,6′-triisopropylbiphenyl (L_1), followed by O-benzylation with NaH and BnBr in 76% overall yield for the two steps. Intermediate **7** was then converted to the two desired products **2** and **3**. The fully protected pyrimidinol was monoalkylated on one of the methyl groups by generating the carbanion with *n*-BuLi followed by treatment with nonyl bromide and pentadecyl bromide to afford **8** and **9** in 53% and 24% yields, respectively. The exocyclic amines were then deprotected by treatment with hydroxylamine hydrochloride to afford **10** and **11** in 75% and 100% yields, respectively. The exocyclic amines were then dimethylated using formalin and sodium cyanoborohydride to afford **12** and **13** in 46% and 41% yields, respectively. Finally compounds **2** and **3** were obtained quantitatively by hydrogenolysis over $Pd(OH)_2/C$.

2.2. Biochemical and biological evaluation of the pyrimidinol analogues

2.2.1. Suppression of reactive oxygen species

The ability of the pyrimidinol analogues to suppress ROS induced by depletion of glutathione with diethyl maleate (DEM) was evaluated in three different cell lines (CEM leukemia cells, Alzheimer's lymphocytes and Leber's lymphocytes). ROS were measured in a quantitative FACS experiment, using dichlorodihydrofluorescein diacetate (DCFH-DA) as a substrate for determining intracellular oxidant production. DCFH-DA is hydrolyzed by esterases to afford dichlorodihydrofluorescein (DCFH), the latter of which is trapped within the cell. This non-fluorescent molecule is then oxidized to afford fluorescent dichlorofluorescein (DCF) by the action of cellular oxidants. The results obtained in CEM leukemia cells are presented in Figure 2, and show that analogues **1**, **2**, **3**, and decylubiquinone were all more effective than idebenone in suppressing ROS, and did so in a concentration dependent manner. Compounds **2** and **3** were the most potent, reducing ROS levels to the same as controls not treated with DEM, when employed at 0.25 and 2.5 μM concentrations. Compounds **1** and decylubiquinone suppressed ROS completely when used at 2.5 μM concentration, but were only partially effective at 0.25 μM concentration. Similar results were obtained using Alzheimer's and Leber's lymphocytes (Supplementary data Figs. 1 and 2), although the basal level of ROS in these cells was found to be elevated even in the absence of DEM treatment. Because the use of DCF fluorescence as an endpoint has been reported to lack specificity under certain circumstances,[41,42] controls were run to determine whether the results obtained were reliable. Accordingly, CEM leukemia cells that had been treated with DCF and DEM were additionally treated with superoxide dismutase + catalase, or with 5 mM *N*-acetylcysteine.[40,43] As shown in Figure 3 of the Supplementary data, these treatments reversed the increase in DCF fluorescence induced by 5 mM DEM treatment, suggesting that an increase in ROS was responsible for the observed increase in DCF fluorescence.

2.2.2. Cellular ATP levels

Total cellular ATP was measured in a CoQ_{10}-deficient human lymphocyte cell line (GM17932). The cells were grown on glucose-free media supplemented with galactose for two weeks to maximize ATP production via oxidative phosphorylation. As shown in Figure 4 of the Supplementary data, the CoQ_{10}-deficient lymphocytes had about 20% less ATP than a normal human lymphocyte cell line (GM15851). The CoQ_{10}-deficient human lymphocytes (GM17932) were verified to have a lower level of CoQ_{10} than

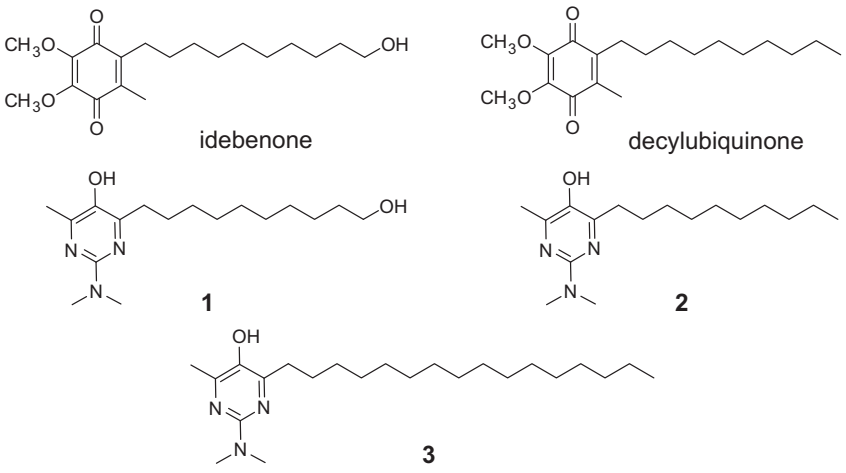
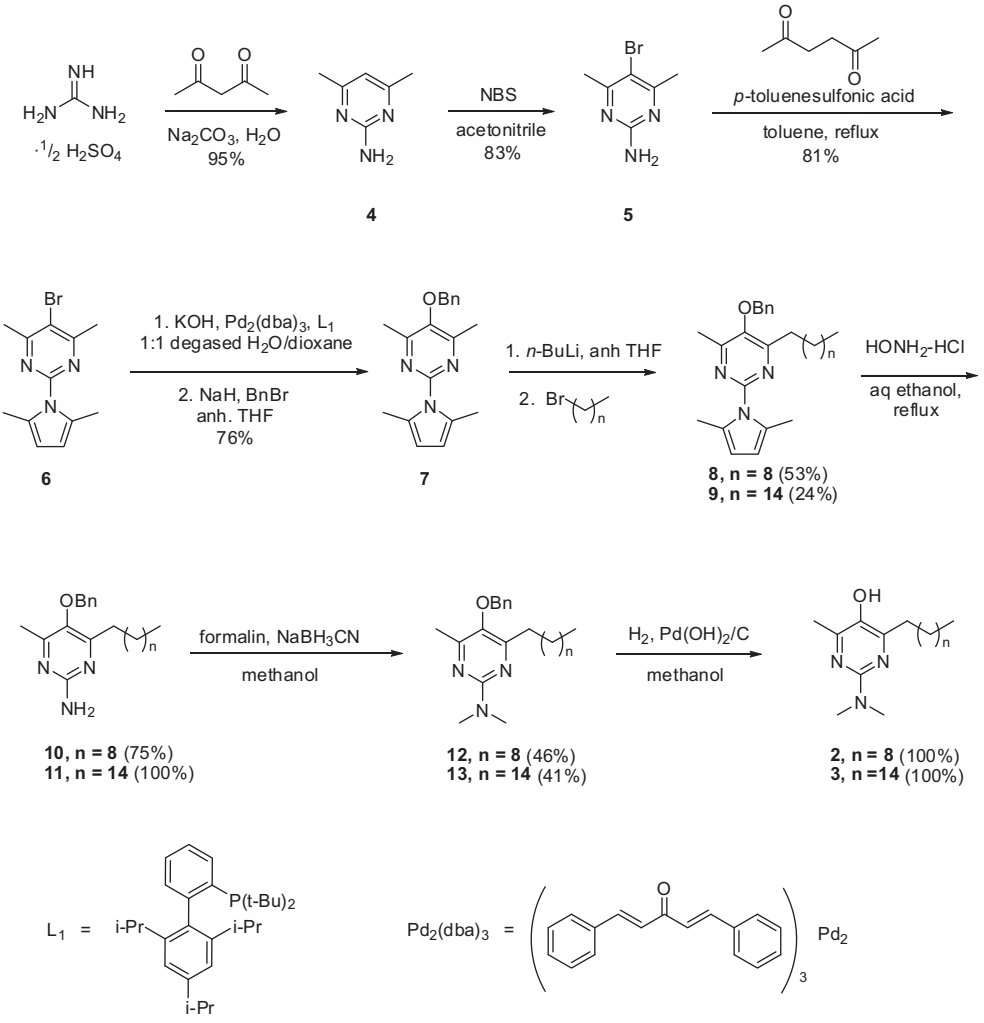


Figure 1. Chemical structures of compounds **1**, **2**, **3**, decylubiquinone and idebenone.



Scheme 1.

normal lymphocytes (GM15851) by 18% (80.4 ± 5 and 98.1 ± 7 pmol/mg of protein ($p = 0.02$ compared with normal)).

Also studied were the effects of idebenone, decylubiquinone and compounds **1**–**3** on cellular ATP levels. All the cells were adapted to growth on galactose for at least one week prior to measuring ATP levels. As shown in Table 1, idebenone strongly diminished ATP levels in a concentration dependent fashion in the CoQ_{10} deficient lymphocytes, as well as in Leber's lymphocytes and Friedreich's ataxia lymphocytes. In the presence of 25 μM idebenone, only residual ATP concentrations were detected. Decylubiquinone and compound **1** also produced somewhat lower ATP concentrations, especially at the highest concentration studied, although neither reduced ATP concentrations nearly as strongly as idebenone. When used at 5 μM concentration, compound **2** resulted in a slight increase in ATP concentrations in the CoQ_{10} deficient lymphocytes, and had essentially no effect on the ATP level in Leber's lymphocytes. This pyrimidinol analogue suppressed ATP levels strongly only when used at 25 μM concentration in Leber's and Friedreich's ataxia lymphocytes. Finally, compound **3** increased ATP levels in the CoQ_{10} deficient lymphocytes when used at 5 μM concentration, and lowered ATP levels minimally at any of the tested concentrations in the three cell lines studied.

2.2.3. Mitochondrial electron transport chain function

In order to understand the basis for the diminished levels of ATP noted in Table 1, the effects of the test compounds on ETC function was studied. The inhibitory effects of the test compounds on bovine heart mitochondrial complex I alone (NADH:ubiquinone oxidoreductase), or complexes I, III and IV together (NADH oxidase) were evaluated using submitochondrial particles (SMP). The results, presented in Tables 2 and 3, show that compounds **1** and **2** were as inhibitory to respiratory chain function as idebenone. Decylubiquinone was much less inhibitory to ETC function, as was compound **3**. Thus, the reduction in ATP levels in Table 1 likely reflects the ability of the test compounds to inhibit one or more of the complexes in the mitochondrial electron transport chain.

2.2.4. Cytoprotection

Cytoprotection was measured using six different lymphocyte cell lines, including five from individuals with mitochondrial or neurodegenerative diseases. The cultured cells were preincubated with the test compounds for 15 h, and treated with diethyl maleate to induce oxidative stress through depletion of glutathione. The extent of cytoprotection afforded by 0.5 μM idebenone, decylubiquinone, compound **2** and compound **3** is shown in Figure 3. It is clear from the data that compounds **2** and **3**, in addition to decylubiquinone, conferred very efficient cytoprotection in all of the cell lines, as has already been reported for compound **1**.[40] This was in sharp contrast to idebenone, which failed to confer complete protection to any of the cell lines. As might have been expected, idebenone was more effective when employed at 2.5 μM concentration (Supplementary data, Fig. 5).

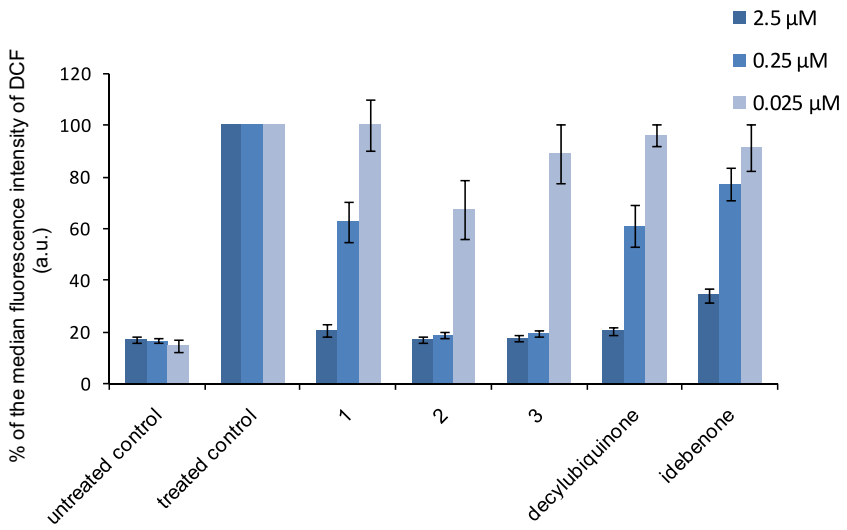


Figure 2. Flow cytometric analysis of CCRF CEM leukemia cells stained with dichlorofluorescein diacetate (DCFH-DA) for 25 min, following pretreatment with the test compounds at 2.5, 0.25 and 0.025 μM concentrations for 18 h, and subsequent treatment with diethyl maleate (DEM) for 1 h to induce the production of ROS. Data shown represent the mean ± S.E.M. of three different experiments run as duplicates.

Table 1
Total ATP concentration in CoQ_{10} deficient cells, Leber's disease and Friedreich's ataxia lymphocytes, upon incubation with the treated compounds for 48 h

Compound	Total ATP concentration (% of control)								
	CoQ_{10} deficient lymphocytes			Leber's lymphocytes			Friedreich's ataxia lymphocytes		
	5 μM	10 μM	25 μM	5 μM	10 μM	25 μM	5 μM	10 μM	25 μM
Untreated control	100	100	100	100	100	100	100	100	100
Idebenone	79.8 ± 2.9	45.7 ± 6.6	1.6 ± 0.5	55.7 ± 0.7	29.9 ± 5.9	3.0 ± 1.0	73.0 ± 6.6	45.6 ± 3.7	2.6 ± 0.8
Decylubiquinone	100.0 ± 5.6	86.2 ± 7.0	71.1 ± 6.9	75.1 ± 1.0	63.4 ± 3.4	47.0 ± 3.0	92.8 ± 3.5	76.4 ± 1.5	62.7 ± 6.4
1	98.8 ± 3.3	86.1 ± 1.6	46.4 ± 3.5	75.5 ± 4.2	45.6 ± 4.7	20.7 ± 1.5	77.2 ± 5.1	46.3 ± 4.2	27.7 ± 2.3
2	105.2 ± 1.4	97.1 ± 1.4	72.4 ± 2.6	95.6 ± 5.8	75.0 ± 6.4	40.6 ± 4.4	83.3 ± 6.8	74.4 ± 2.6	42.2 ± 3.5
3	108.2 ± 3.1	103.0 ± 3.4	95.0 ± 5.8	102.7 ± 4.3	95.6 ± 2.3	83.4 ± 4.1	95.3 ± 5.8	90.3 ± 3.4	74.7 ± 7.1

Table 2
The inhibitory effect of compounds **1**, **2**, **3**, idebenone, and decylubiquinone on bovine heart mitochondrial NADH oxidase activity (complexes I, III and IV)

Compound	NADH oxidase activity[a] (%)		
	1 μM	5 μM	10 μM
Idebenone	57.4 ± 4.6	19.3 ± 2.7	13.2 ± 1.1
Decylubiquinone	93.0 ± 6.9	82.4 ± 2.2	61.5 ± 6.6
1	50.0 ± 5.0	22.0 ± 4.7	15.0 ± 3.2
2	35.4 ± 1.4	17.7 ± 1.1	3.9 ± 0.3
3	96.2 ± 2.6	88.6 ± 5.0	76.9 ± 4.4

[a] Relative to an untreated control.

Table 3
The inhibitory effect of compounds **1, 2, 3**, idebenone, and decylubiquinone on bovine heart mitochondrial complex I

Compound	IC_{50} (μM)	I_{max}[a] (%)	K_i (μM)
Idebenone	6 ± 3	84 ± 2	4 ± 2
Decylubiquinone	>200	N/A	>200
1	1 ± 0	85 ± 3	0.7 ± 0
2	2 ± 1	88 ±2	1.4 ± 0.7
3	198 ± 12	84 ± 1	141 ± 9

[a] Maximal inhibition achievable.

3. Discussion

Idebenone has been studied extensively over a period of years, and has been found to be effective both as an antioxidant[37] and also in reducing complex III.[44] The latter property suggests the potential utility of such compounds in augmenting ATP production in cells having partially dysfunctional mitochondria. However, the reports that idebenone inhibits complex I at fairly modest concentrations[36–38] suggested the need for analogues lacking this unfavorable property.

Decylubiquinone is closely related to idebenone, differing in structure only in that it lacks the side chain OH group present in idebenone. The extensive use of decylubiquinone experimentally as a coenzyme Q surrogate suggested that it might be informative to characterize the properties of decylubiquinone in several assays that measure different facets of mitochondrial function. In fact, as shown in Tables 1–3, decylubiquinone was found to lack significant inhibition of the mitochondrial ETC, but to retain the ROS suppressive properties noted for idebenone (Fig. 2), and to have cytoprotective effects on several cultured cell lines superior to that of idebenone (Fig. 3). Accordingly, we prepared two pyrimidinol analogues of compound **1**[40] based on our results with decylubiquinone and idebenone. These new derivatives **2** and **3** lack the side chain OH group present in (idebenone and) **1**. Compound **2** has the same side chain as decylubiquinone while compound **3** has a longer (16-carbon) side chain. Both compounds were synthesized using a strategy similar to that used for the preparation of compound **1** (Scheme 1).[40] The biochemical effects of **2** and **3** on mitochondria and their ability to suppress ROS in cells have been tested and compared to those of idebenone and decylubiquinone. Surprisingly, the removal of the OH group from compound **1** did not diminish the inhibition of the ETC complexes by the new derivative. Compound **2** was as inhibitory to mitochondrial complex I

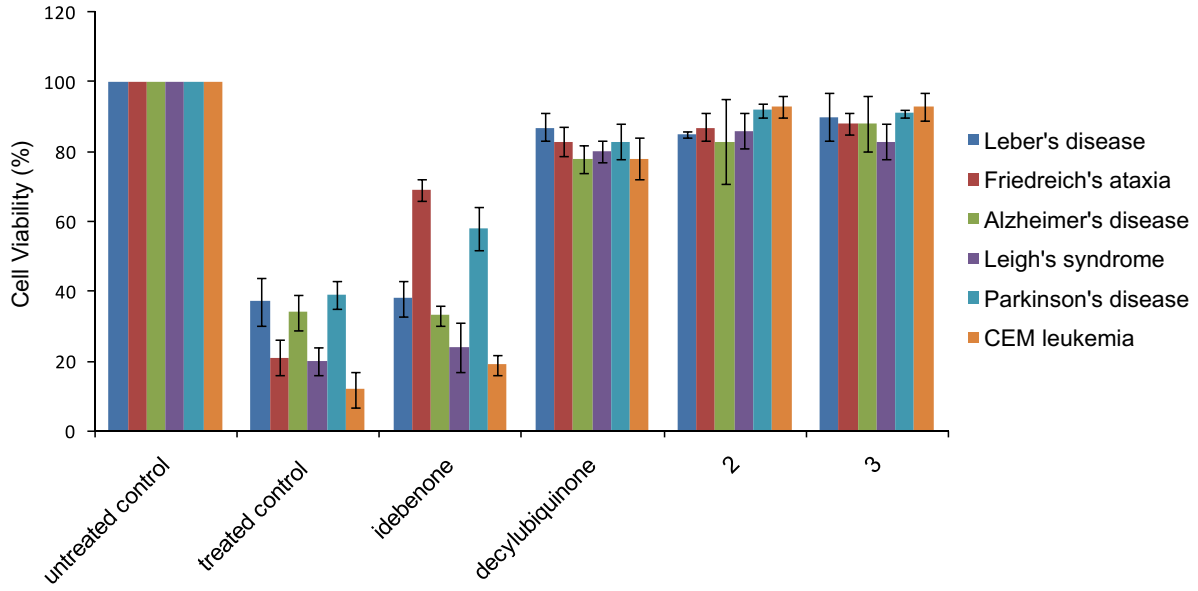


Figure 3. Cell viability of Leber's disease lymphocytes, Friedreich's ataxia lymphocytes, Alzheimer's disease lymphocytes, Leigh's syndrome lymphocytes, Parkinson's disease lymphocytes and CCRF CEM leukemia lymphocytes following pretreatment with compounds **1**, **2**, **3**, decylubiquinone and idebenone at 0.5 μM concentration for 15 h and then treatment with 5 mM DEM for 6 h. Data shown represent the average ± S.E.M. of three different experiments run as triplicates.

as compound **1** and slightly more inhibitory to NADH oxidase activity (Tables 2 and 3). However, compound **3**, having a longer side chain, was dramatically improved in this regard, having an IC_{50} value of about 200 μM for complex I inhibition, and exhibiting only modest inhibition of NADH oxidase activity. This was fully consistent with the observation that compound **3** was also better than **2** in preserving ATP levels at all tested concentrations in the three cell lines studied (Table 1). Nonetheless, compound **2** was superior both to idebenone and compound **1**, both in maintenance of ATP levels and ROS suppression (Table 1 and Fig. 2). Importantly, compound **3** actually increased total ATP above the basal level, when used at low doses in CoQ_{10}-deficient lymphocytes, and did not materially affect ATP levels in the other cell lines at 5 and 10 μM concentrations. This makes compound **3** the best analogue identified to date, exhibiting little inhibition of the ETC, and an ability to augment ATP production. The results in Table 1 for the effects of **3** on CoQ_{10} deficient lymphocytes are fully consistent with the belief that **3** can reduce complex III within the ETC. The basis for the lack of inhibitory effects noted for compound **3** on complex I function are currently not well understood. Nonetheless, the fact that it is possible to identify a CoQ_{10} analogue that functions in complex III without inhibiting complex I is significant and suggests the promise of the current strategy in identifying CoQ_{10} analogues of potential utility for therapeutic intervention.

As noted in Figure 2, as well as Figures 1 and 2 of Supplementary data, compounds **2** and **3** were more efficient in suppressing ROS than the other compounds tested, implying that the structural changes to the side chain of **1** did not decrease the antioxidant properties of these pyrimidinols.[40]

Finally, we have studied the ability of these compounds to protect a variety of mitochondrial disease cells from the cytotoxic effects of diethyl maleate (DEM), a depletor of glutathione that exposes the cells to oxidative stress. DEM was utilized because it induces oxidative stress at least as great as that likely to be encountered physiologically, permitting the identification of compounds anticipated to function well under pathophysiological conditions.

For this analysis, we used several cell lines derived from patients with mitochondrial neurodegeneratic diseases. These included Leber's hereditary optic neuropathy (LHON) lymphocytes bearing a mitochondrial DNA mutation in the NADH dehydrogenase subunit 4 gene (MTND4), in which the arginine at amino acid position 340 is replaced by a histidine (R340H). This mutation accounts for ~50% of LHON cases among Caucasians and >90% of the cases among Asians.[45] The second cell line was Alzheimer's disease (familial, type 3) lymphocytes. The cells carry a missense mutation [Ala246Glu (A246E)] in the AD3 [presenilin 1 (PSEN1)] gene. This mutation has been linked to early onset Alzheimer's disease by several investigators.[46–48] The third cell line involved the use of Parkinson's disease (familial, type 1) lymphocytes, identified with whole gene triplication, including amplification of SNCA exons. This was suggested as the cause of Parkinson's disease in a family from which the cells were derived, formerly classified as PARK.[49,50]

Another cell line employed was Leigh's syndrome lymphocytes, bearing an homoplasmic mutation of ATPase 6 (MTATP6). Holt et al. found a heteroplasmic T to G transversion at nucleotide pair 8993 in a maternal pedigree which resulted in the change of a hydrophobic leucine to a hydrophilic arginine at position 156 in subunit 6 of mitochondrial H(+)-ATPase.[51] The insertion of an arginine in the hydrophobic sequence of ATPase 6 probably interferes with the hydrogen ion channel formed by subunits 6 and 9 of the ATPase, thus causing failure of ATP synthesis. Tatuch et al. and Shoffner et al. demonstrated that the nucleotide 8993 mutation can cause Leigh's disease.[52,53] Also studied were Friedreich's ataxia lymphocytes having an identified mutation $(GAA)_n$ expansion. GAA triplet repeat expansions between 200 and 900 copies in the first intron of the frataxin gene occurred in 71 out of 74 FRDA patients studied by Campuzano et al.[54]

As is clear from Figure 3, compounds **2** and **3** both exhibited good cytoprotective activity in all of the cell lines, even when used at the low concentration of 0.5 μM. Their cytoprotective activity was clearly greater than that of idebenone, and slightly better than decylubiquinone in some of the disease cells (e.g., CEM leukemia and Parkinson's disease lymphocytes).

4. Conclusions

Two new pyrimidinol derivatives, **2** and **3**, have been synthesized and characterized biochemically. They both exhibited improved antioxidant activity in comparison with **1**, and the ability

to maintain ATP levels in cellular mitochondrial disease models. In addition, compound **3** was shown to have a much lower inhibitory effect on ETC function than the other compounds tested. These findings suggest the importance of side chain optimization to achieve the best compounds suitable for potential therapeutic use. This is in agreement with the work of Fato et al.[36] who concluded that hydrophilic quinones may enhance oxidative stress by interaction with a different site on complex I than the physiological quinone reducing site used by more hydrophobic quinones.

It is interesting that side chains optimal for CoQ_{10} analogues having a benzoquinone redox core were not optimal for the pyrimidinol analogues of primary interest in the current study. This observation indicates that interaction with the mitochondrial complexes of the respiratory chain must involve recognition both of the redox core and the side chain of individual analogues, and suggests that recognition of individual types of redox cores may differ qualitatively.

5. Experimental section

5.1. Chemistry

^{1}H NMR spectra were recorded on a Varian Inova 400 MHz, using chloroform-*d* or methanol-d_4 as solvents. ^{1}H NMR chemical shifts were reported relative to residual chloroform at 7.24 ppm or to residual methanol at 3.31 ppm. ^{13}C NMR chemical shifts were reported relative to residual chloroform at 77.1 ppm or to residual methanol at 49.0 ppm. All solvents were of analytical grade and were used without further purification. All chemicals were purchased from Sigma–Aldrich Chemical Co. and were used without further purification. Reactions were carried out under an argon atmosphere. Column chromatography was carried out using silica gel (60 Å particle size, 230–240 mesh, Silicycle). Analytical thin layer chromatography separations were carried out on glass plates coated with silica gel (60 Å particle size, F-254, E. Merck). The TLC chromatograms were visualized by UV irradiation or by immersing the plates in 2.5% potassium permanganate in ethanol, or 2% anisaldehyde + 5% sulfuric acid + 1.5% glacial acetic acid in ethanol, both applications being followed by heating of the TLC plates. Melting points were recorded on a MelTemp apparatus and are uncorrected. High resolution mass spectra were obtained in the Arizona State University CLAS High Resolution Mass Spectrometry Laboratory.

5.1.1. 2-Amino-4,6-dimethylpyrimidine (4)

To a stirred solution containing 4.00 g (37.0 mmol) of guanidine sulfate and 8.40 g (79.3 mmol) of sodium carbonate in 25 mL of water was added 6.00 mL (5.88 g; 58.1 mmol) of 2,4-pentanedione. The reaction mixture was stirred at 100 °C for 24 h. The cooled reaction mixture was poured into 150 mL of water and then extracted with two 150-mL portions of dichloromethane. The combined organic phase was washed with 150 mL of brine, dried ($MgSO_4$) and then concentrated under diminished pressure to afford **4** as a colorless solid: yield 4.31 g (95%); mp 152–153 °C; silica gel TLC R_f 0.50 (9:1 dichloromethane/methanol); ^{1}H NMR ($CDCl_3$) δ 2.24 (s, 6H), 5.39 (br s, 2H) and 6.33 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 23.7, 110.5, 162.9 and 167.7; mass spectrum (APCI), *m/z* 124.0869 $(M+H)^+$ ($C_6H_{10}N_3$ requires 124.0875).

5.1.2. 2-Amino-5-bromo-4,6-dimethylpyrimidine (5)

To a stirred solution containing 4.31 g (34.8 mmol) of 2-amino-4,6-dimethylpyrimidine (**4**) in 150 mL of acetonitrile was added 6.15 g (52.1 mmol) of *N*-bromosuccinimide. The reaction mixture was stirred at 23 °C for 3 h. The formed precipitate was filtered and dried to afford **5** as a colorless solid: yield 5.93 g (83%); mp 183–185 °C; silica gel TLC R_f 0.15 (2:1 ethyl acetate/hexanes); ^{1}H NMR ($CDCl_3$) δ 2.44 (s, 6H) and 5.19 (br s, 2H); ^{13}C NMR ($CDCl_3$) δ 24.7, 109.6, 160.7 and 166.3; mass spectrum (APCI), *m/z* 201.9982 $(M+H)^+$ ($C_6H_9N_3Br$ requires 201.9980).

5.1.3. 5-Bromo-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4,6-dimethylpyrimidine (6)

To a stirred solution containing 2.00 g (9.89 mmol) of 2-amino-5-bromo-4,6-dimethylpyrimidine (**5**) in 16 mL of toluene was added 1.36 mL (1.32 g; 11.5 mmol) of 2,5-hexanedione followed by 96 mg (0.50 mmol) of *p*-toluenesulfonic acid. The reaction mixture was heated and stirred at reflux for 12 h. The reaction mixture was poured into 150 mL of water and then extracted with 200 mL of ethyl acetate. The organic solution was washed with 150 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 5 cm). Elution with 5:1 hexanes/ethyl acetate afforded **6** as light yellow crystals: yield 2.23 g (81%); mp 64–65 °C; silica gel TLC R_f 0.65 (6:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.34 (s, 6H), 2.67 (s, 6H) and 5.89 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 14.5, 24.9, 108.7, 118.6, 129.5, 155.3 and 166.9; mass spectrum (APCI), *m/z* 280.0458 $(M+H)^+$ ($C_{12}H_{15}N_3Br$ requires 280.0449).

5.1.4. 5-Benzyloxy-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4,6-dimethylpyrimidine (7)

To a stirred solution containing 4.87 g (17.4 mmol) of 5-bromo-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4,6-dimethylpyrimidine (**6**) in 50 mL of 1:1 dioxane/degassed water was added 632 mg (0.69 mmol) of $Pd_2(dba)_3$ followed by 293 mg (0.69 mmol) of 2-di-*tert*-butylphosphino-2′,4′,6′-triisopropylbiphenyl (L_1) and 2.92 g (52.1 mmol) of KOH. The reaction mixture was stirred at 100 °C for 3 h. The cooled reaction mixture was poured into 200 mL of water and extracted with 100 mL of ethyl acetate. The aqueous layer was acidified with HCl (pH 2–3) and then extracted with two 150-mL portions of ethyl acetate. The combined organic layer was washed with 150 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was dissolved in 50 mL of anhyd THF and treated with 3.10 mL (4.46 g; 26.0 mmol) of benzyl bromide followed by 1.40 g (34.8 mmol) of a 60% suspension of NaH in mineral oil. The reaction mixture was stirred at 23 °C for 48 h. The reaction mixture was quenched with satd aq sodium bicarbonate, poured into 150 mL of water and extracted with two 150-mL portions of ether. The combined organic layer was washed with 150 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 6 cm). Elution with 9:1 hexanes/ethyl acetate afforded **7** as a light yellow oil: yield 4.09 g (76%); silica gel TLC R_f 0.6 (6:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 2.29 (s, 6H), 2.47 (s, 6H), 4.92 (s, 2H), 5.86 (s, 2H) and 7.42 m, 5H); ^{13}C NMR ($CDCl_3$) δ 14.1, 19.1, 75.3, 107.9, 128.3, 128.6, 129.0, 129.2, 136.0, 147.7, 152.3 and 161.6; mass spectrum (APCI), *m/z* 307.1675 $(M)^+$ ($C_{19}H_{21}N_3O$ requires 307.1685).

5.1.5. 5-Benzyloxy-4-decyl-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-6-methylpyrimidine (8)

To a stirred solution at −78 °C containing 1.00 g (3.25 mmol) of 5-benzyloxy-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4,6-dimethylpyrimidine (**7**) and 415 μL (452 mg; 2.16 mmol) of 1-bromononane in 30 mL of anhyd THF was added 2.70 mL (4.32 mmol) of a 1.60 M solution of *n*-BuLi in pentane. The reaction mixture was stirred at 23 °C for 30 min. The reaction was quenched with satd aq ammonium chloride and then poured into 70 mL of water. The mixture was extracted with two 70-mL portions of ether. The combined organic layer was then washed with 100 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 5 cm).

Elution with 4:1 hexanes/ether afforded **8** as a colorless oil: yield 502 mg (53%); silica gel TLC R_f 0.60 (4:1 hexanes/ether); ^{1}H NMR ($CDCl_3$) δ 0.89 (t, 3H, J = 7.2 Hz), 1.20–1.30 (m, 14H), 1.75 (m, 2H), 2.31 (s, 6H), 2.48 (s, 3H), 2.77 (dd, 2H, J = 7.6, 7.6 Hz), 4.90 (s, 2H), 5.86 (s, 2H) and 7.40–7.44 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 14.1, 14.3, 19.3, 22.7 27.7, 29.3, 29.4, 29.51, 29.57, 31.62, 31.9, 75.7, 107.9, 128.0, 128.6, 128.7, 129.3, 136.2, 147.4, 152.6, 161.6 and 165.1; mass spectrum (APCI), m/z 434.3172 $(M+H)^+$ ($C_{28}H_{40}N_3O$ requires 434.3171).

5.1.6. 5-Benzyloxy-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4-hexadecyl-6-methylpyrimidine (9)

To a stirred solution at −78 °C containing 1.00 g (3.25 mmol) of 5-benzyloxy-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4,6-dimethylpyrimidine (**7**) and 630 μL (630 mg; 2.16 mmol) of 1-bromopentadecane in 20 mL of anhyd THF was added 2.70 mL (4.32 mmol) of a 1.60 M solution of *n*-BuLi in pentane. The reaction mixture was stirred at 23 °C for 30 min. The reaction was quenched with satd aq ammonium chloride and then poured into 70 mL of water. The mixture was then extracted with two 70-mL portions of ether. The combined organic layer was washed with 100 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 5 cm). Elution with 4:1 hexanes/ether afforded **9** as a colorless oil: yield 269 mg (24%); silica gel TLC R_f 0.5 (4:1 hexanes/ether); ^{1}H NMR ($CDCl_3$) δ 0.89 (t, 3H, J = 7.2 Hz), 1.20–1.30 (m, 26H), 1.75 (quint, 2H, J = 7.2 Hz), 2.29 (s, 6H), 2.47 (s, 3H), 2.76 (dd, 2H, J = 8.0, 8.0 Hz), 4.89 (s, 2H), 5.85 (s, 2H) and 7.30–7.42 m, 5H); ^{13}C NMR ($CDCl_3$) δ 14.1, 14.30, 14.30, 19.3, 22.7, 27.7, 29.3, 29.4, 29.50, 29.55, 29.63, 29.67, 31.6, 31.9, 75.7, 107.9, 128.0, 128.5, 128.7, 129.3, 136.2, 147.4, 152.6, 161.6 and 165.1; mass spectrum (APCI), m/z 518.4113 $(M+H)^+$ ($C_{34}H_{52}N_3O$ requires 518.4110).

5.1.7. 2-Amino-5-benzyloxy-4-decyl-6-methylpyrimidine (10)

To a stirred solution containing 502 mg (1.15 mmol) of 5-benzyloxy-4-decyl-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-6-methylpyrimidine (**8**) in 10 mL of 9:1 ethanol/water was added 800 mg (11.5 mmol) of hydroxylamine hydrochloride. The reaction mixture was stirred at reflux for 5 h. A second portion of 800 mg (11.5 mmol) of hydroxylamine hydrochloride was added and the reaction mixture was stirred at reflux for 16 h. The cooled reaction mixture was then poured into 70 mL of water and then adjusted to pH 9–10 with 1 N aq NaOH. The mixture was extracted with two 70-mL portions of ethyl acetate. The combined organic layer was washed with 70 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 3 cm). Elution with 3:2 hexanes/ethyl acetate afforded **10** as a colorless oil: yield 305 mg (75%); silica gel TLC R_f 0.25 (3:2 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 3H, J = 6.4 Hz), 1.26 (m, 14H), 1.61 (m, 2H), 2.27 (s, 3H), 2.56 (dd, 2H, J = 8.0, 8.0 Hz), 4.71 (s, 2H), 5.24 (br s, 2H) and 7.35–7.40 m, 5H); ^{13}C NMR ($CDCl_3$) δ 13.9, 18.9, 22.6, 28.4, 29.3, 29.4, 29.5, 29.6, 29.7, 31.87, 31.88, 75.9, 127.9, 128.2, 128.6, 136.8, 142.8, 158.9, 161.1 and 162.8; mass spectrum (APCI), m/z 356.2704 $(M+H)^+$ ($C_{22}H_{34}N_3O$ requires 356.2702).

5.1.8. 2-Amino-5-benzyloxy-4-hexadecyl-6-methylpyrimidine (11)

To a stirred solution containing 269 mg (0.52 mmol) of 5-benzyloxy-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4-hexadecyl-6-methylpyrimidine (**9**) in 10 mL of ethanol was added 723 mg (10.4 mmol) of hydroxylamine hydrochloride. The reaction mixture was stirred at reflux for 5 h. A second portion of 723 mg (10.4 mmol) of hydroxylamine hydrochloride was added and the reaction mixture was stirred at reflux for 16 h. The reaction mixture was then poured into 70 mL of water and then adjusted to pH 9–10 with 1 N aq NaOH. The mixture was extracted with two 70 mL portions of ethyl acetate. The combined organic layer was washed with 70 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 3 cm). Elution with 3:2 hexanes/ethyl acetate afforded **11** as a colorless oil: yield 231 mg (100%); silica gel TLC R_f 0.25 (1:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 3H, J = 6.8 Hz), 1.26 (m, 26H), 1.61 (quint, 2H, J = 7.2 Hz), 2.28 (s, 3H), 2.56 (dd, 2H, J = 7.6, 7.6 Hz), 4.72 (s, 2H), 5.03 (br s, 2H) and 7.35–7.40 m, 5H); ^{13}C NMR ($CDCl_3$) δ 14.1, 18.9, 22.7, 28.4, 29.3, 29.4, 29.5, 29.62, 29.63, 29.67, 29.73, 31.9, 75.9, 127.9, 128.2, 128.6, 136.8, 142.9, 158.8, 161.1 and 164.9; mass spectrum (APCI), m/z 440.3646 $(M+H)^+$ ($C_{28}H_{46}N_3O$ requires 440.3641).

5.1.9. 5-Benzyloxy-4-decyl-2-(*N*,*N*-dimethylamino)-6-methylpyrimidine (12)

To a stirred solution containing 305 mg (0.86 mmol) of 2-amino-5-benzyloxy-4-decyl-6-methylpyrimidine (**10**) in 3 mL of methanol was added 3 mL of 35% aq formaldehyde, followed by 271 mg (4.30 mmol) of $NaCNBH_3$. The reaction mixture was stirred at 23 °C for 3 h. The reaction was quenched with acetic acid until bubbling ceased, then poured into 20 mL of water and extracted with two 40-mL portions of ethyl acetate. The combined organic layer was washed with 40 mL of satd aq $NaHCO_3$ and 40 mL of brine. The organic solution was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (8 × 3 cm). Elution with 2:1 hexanes/ethyl acetate afforded **12** as a colorless oil: yield 154 mg (46%); silica gel TLC R_f 0.8 (2:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, J = 6.4 Hz), 1.33 (m, 14H), 1.69 (quint, 2H, J = 7.2 Hz), 2.32 (s, 3H), 2.61 (dd, 2H, J = 7.6, 7.6 Hz), 3.14 (s, 6H), 4.70 (s, 2H) and 7.33–7.44 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 14.1, 19.3, 22.7, 27.9, 29.3, 29.50, 29.55, 29.59, 29.6, 31.7, 31.9, 37.2, 75.7, 127.9, 128.1, 128.5, 137.2, 141.3, 158.6, 159.0 and 163.5; mass spectrum (APCI), m/z 384.3003 $(M+H)^+$ ($C_{24}H_{38}N_3O$ requires 384.3015).

5.1.10. 5-Benzyloxy-2-(*N*,*N*-dimethylamino)-4-hexadecyl-6-methylpyrimidine (13)

To a stirred solution containing 230 mg (0.52 mmol) of 2-amino-5-benzyloxy-4-hexadecyl-6-methylpyrimidine (**11**) in 4 mL of methanol was added 4 mL of 35% aq formaldehyde, followed by 263 mg (4.18 mmol) of $NaCNBH_3$. The reaction mixture was stirred at 23 °C for 3 h. The reaction was quenched with acetic acid until bubbling ceased, then poured into 20 mL of water and extracted with two 40-mL portions of ethyl acetate. The combined organic layer was washed with 40 mL of satd aq $NaHCO_3$ and 40 mL of brine. The organic solution was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (8 × 3 cm). Elution with 4:1 hexanes/ethyl acetate afforded **13** as a colorless oil: yield 100 mg (41%); silica gel TLC R_f 0.75 (4:1 hexanes/ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 3H, J = 6.8 Hz), 1.23–1.28 (br m, 26H), 1.68 (quint, 2H, J = 6.8 Hz), 2.31 (s, 3H), 2.61 (dd, 2H, J = 7.6, 7.6 Hz), 3.14 (s, 6H), 4.69 (s, 2H) and 7.40–7.46 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 14.1, 19.3, 22.6, 27.8, 29.3, 29.50, 29.55, 29.62, 29.64, 29.66, 31.7, 31.9, 37.2, 75.7, 127.9, 128.2, 128.5, 137.2, 141.3, 158.6, 159.0 and 163.6; mass spectrum (APCI), m/z 468.3950 $(M+H)^+$ ($C_{30}H_{50}N_3O$ requires 468.3954).

5.1.11. 4-Decyl-2-(*N*,*N*-dimethylamino)-6-methylpyrimidin-5-ol (2)

To a stirred solution containing 150 mg (0.48 mmol) of 5-benzyloxy-4-decyl-(2-*N*,*N*-dimethylamino)-6-methylpyrimidine (**12**) in 5 mL of methanol was added 5 mg of 20% palladium hydroxide

on carbon (Degussa type E101 NE/E). The reaction mixture was stirred at 23 °C under a hydrogen atmosphere for 15 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure to afford **2** as a colorless solid: yield 140 mg (100%). An analytical sample was obtained by chromatography on a silica gel column (8 × 1 cm). Elution with 4:1 hexanes/ethyl acetate afforded **2** as a colorless solid; mp 71–72 °C; silica gel TLC R_f 0.25 (4:1 hexanes/ethyl acetate); ^{1}H NMR (methanol-d_4) δ 0.87 (t, 3H, J = 6.8 Hz), 1.21–1.31 (m, 14H), 1.65 (quint, 2H, J = 7.2 Hz), 2.26 (s, 3H), 2.62 (dd, 2H, J = 7.2, 7.2 Hz) and 3.05 (s, 6H); ^{13}C NMR (methanol-d_4) δ 13.0, 17.6, 22.3, 27.3, 29.0, 29.15, 29.17, 29.26, 29.29, 31.2, 31.6, 36.52, 36.52, 138.0, 155.6, 157.3 and 159.4; mass spectrum (APCI), m/z 294.2554 $(M+H)^+$ ($C_{17}H_{32}N_3O$ requires 294.2545).

5.1.12. 2-(*N*,*N*-Dimethylamino)-4-hexadecyl-6-methylpyrimidin-5-ol (3)

To a stirred solution containing 100 mg (0.21 mmol) 5-benzyloxy-2-(*N*,*N*-dimethylamino)-4-hexadecyl-6-methylpyrimidine (**13**) in 5 mL of methanol was added 5 mg of 20% palladium hydroxide on carbon (Degussa type E101 NE/E). The reaction mixture was stirred at 23 °C under hydrogen atmosphere for 15 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure to afford **3** as a colorless solid: yield 79 mg (100%). An analytical sample was obtained by chromatography on a silica gel column (8 × 1 cm). Elution with 4:1 hexanes/ethyl acetate afforded **3** as a colorless solid; mp 86–87 °C; silica gel TLC R_f 0.45 (4:1 hexanes/ethyl acetate); ^{1}H NMR (methanol-d_4) δ 0.89 (t, 3H, J = 6.8 Hz), 1.28–1.34 (m, 26H), 1.67 (quint, 2H, J = 7.2 Hz), 2.28 (s, 3H), 2.63 (dd, 2H, J = 7.6, 7.6 Hz) and 3.07 (s, 6H); ^{13}C NMR (methanol-d_4) δ 13.0, 17.5, 22.3, 27.2, 29.0, 29.10, 29.12, 29.20, 29.29, 29.30, 31.2, 31.6, 36.50, 138.0, 155.6, 157.3 and 159.4; mass spectrum (APCI), m/z 378.3491 $(M+H)^+$ ($C_{23}H_{44}N_3O$ requires 378.3484).

5.2. Biochemical and biological evaluation of the pyrimidinol analogues

5.2.1. Cell culture

A CoQ_{10}-deficient lymphocyte cell line (GM17932) was obtained from Coriell Cell Repositories (Camden, NJ). The lymphocytes were cultured in glucose-free media supplemented with galactose for two weeks to force energy production predominantly through oxidative phosphorylation rather than glycolysis.[55,56] The lymphocytes were cultured in RPMI 1640 glucose-free medium (Gibco, Grand Island, NY) supplemented with 25 mM galactose, 2 mM glutamine and 1% penicillin–streptomycin (Cellgro, Manassas, VA), and 10% dialyzed fetal bovine serum (FBS) (<0.5 μg/mL) (Gemini Bio-Product, West Sacramento, CA).

Friedreich's ataxia lymphocytes (GM15850), normal lymphocytes (GM15851), Leber's optic atrophy lymphocytes (GM10744), Alzheimers disease lymphocytes (AG06849), Parkinson's disease lymphocytes (GM15010), and Leigh's syndrome lymphocytes (GM13740) were obtained from Coriell, and were cultured in RPMI medium with 15% FBS (Fisher Scientific, TX), 2 mM glutamine (HyClone, South Logan, UT) and 1% penicillin–streptomycin mix antibiotics. Cells were maintained in log phase at a concentration between 1×10^5 and 1×10^6 cells/mL.

CEM leukemia cells (ATCC, CRL-2264) were cultured in RPMI medium with 10% FBS (Fisher Scientific), 2 mM glutamine and 1% penicillin–streptomycin mix antibiotics. Cells were maintained in log phase at a concentration between 1×10^5 and 1×10^6 cells/mL.

5.2.2. Suppression of reactive oxygen species (ROS)

Intracellular ROS production was measured using the oxidant sensitive fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR). One milliliter of cells (2.5×10^5 cells) were plated in a 24-well plate, treated with the test compounds and incubated at 37 °C for 18 h in a humidified atmosphere containing 5% CO_2 in air. Cells were treated with 5 mM diethyl maleate (DEM) (97%, Sigma–Aldrich, St. Louis, MO) for 1 h, collected by centrifugation at 300×g for 3 min and then washed twice with phosphate buffered saline (PBS) (Invitrogen, Grand Island, NY). Cells were resuspended in PBS containing 10 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 twice 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. The generation of ROS, mainly peroxides, was detected as a result of the oxidation of DCFH (λ_{ex} 488 nm; λ_{em} 515–540 nm). In each analysis, 10,000 events were recorded after cell debris were electronically gated out. The results obtained were verified by running duplicates and repeating experiments in three independent experiments. Hydrogen peroxide was used to produce a positive control. Data are reported as means ± S.E.M. (n = 3).

5.2.3. Cell viability analysis

A hemocytometer-based assay was used to determine the number of viable cells present in a cell suspension. The assay is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. This technique was used to assess the cytoprotective effects of the test compounds in cultured CEM leukemia cells, Friedreich's ataxia lymphocytes, Leber's optic atrophy lymphocytes, Alzheimers disease lymphocytes, Parkinson's disease lymphocytes, or Leigh's syndrome lymphocytes treated with DEM to induce oxidative cell stress by glutathione depletion. Briefly, cells were seeded at a density of 5×10^5 cells/mL and incubated with 0.5 μM or 2.5 μM of the indicated compounds at 37 °C for 16 h under a 5% CO_2 in air atmosphere. After preincubation, the cells were treated with 5 mM DEM for 6 h. Cells not treated with DEM exhibited >90% cell viability, whereas DEM treatment reduced cell viability to ~30%. The ability of the test compounds to protect the cells against the effects of DEM was determined. Cell viability was expressed as the percentage of untreated control, normalized to 100%. The results obtained were run in three independent experiments as triplicates. Data are expressed as means ± S.E.M. (n = 3).

5.2.4. Mitochondria isolation and preparation of submitochondrial particles

Mitochondria were prepared as described.[57,58] Briefly, one beef heart was ground and blended in sucrose buffer (0.25 M sucrose, 10 mM Tris–HCl, pH 7.8, containing 0.2 mM EDTA) at 4 °C. Cell debris were removed by centrifugation at 1200×g for 20 min. The supernatant was filtered through two layers of cheesecloth. Mitochondria were harvested by centrifugation at 26,000×g for 15 min and then homogenized in the same buffer with a Dounce homogenizer. Mitochondria were harvested by centrifugation at 12,000×g for 30 min, and stored at −80 °C in sucrose buffer. Submitochondrial particles (SMPs) were prepared as described by Linnane and Titchener.[59] Briefly, mitochondria were sonicated with a sonic dismembrator (Fisher Scientific) in 10 mM Tris–HCl, pH 7.8, containing 0.25 M sucrose, 5 mM $MgCl_2$,1 mM ATP, 10 mM $MnCl_2$, and 1 mM sodium succinate at 4 °C. Intact mitochondria were pelleted by centrifugation at 20,000×g at 4 °C for 7 min. SMPs were harvested by centrifugation at 152,000×g at 4 °C for 30 min and stored at −80 °C in 10 mM Tris–HCl, pH 7.5, containing 0.25 M sucrose, 5 mM $MgCl_2$, 2 mM ATP, 2 mM glutathione and 1 mM sodium succinate. The protein concentration was determined by BCA

titration (Pierce, Rockford, IL) and the sample was diluted as described below.

5.2.5. NADH oxidase activity

The inhibitory effects of the test compounds on bovine heart mitochondrial complexes I, III and IV were evaluated. The compounds were dissolved in dimethylsulfoxide (DMSO), and then used to make serial dilutions. Maximal DMSO concentrations never exceeded 2% and were shown to have no influence on the enzymatic activity. Bovine heart SMPs were diluted to 0.5 mg/mL. Activity was assayed at 30 °C and monitored spectrophotometrically using a Beckman Coulter DU-530 (340 nm, ε 6.22 mM^{-1} cm^{-1}). NADH oxidase activity was determined in a reaction medium (2.5 mL total volume) containing 50 mM Hepes, pH 7.5, and 5 mM $MgCl_2$. The final mitochondrial protein concentration was 30 μg. After the pre-equilibration of SMP with inhibitor for 5 min, the initial rates were calculated from the linear portion of the traces. Data are reported as the mean of three independent experiments each run in triplicate.

5.2.6. NADH:ubiquinone oxidoreductase activity

The inhibition of NADH:ubiquinone oxidoreductase activity was determined using the same experimental conditions described previously.[60,61] Twelve micrograms of SMPs were incubated at 39 °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 50 μM NADH and 50 μM of coenzyme Q_1. Enzymatic activity, measured by the loss of NADH absorbance, was monitored at 340 nm. Data are reported as the mean of three independent experiments each run in triplicate. The K_I values were determined as described.[62]

5.2.7. Cellular ATP concentration

Briefly, lymphocytes (2×10^5 cell/mL) were plated (1 mL in 12-well plates) and treated with the test compounds at final concentrations of 5, 10 and 25 μM, and then incubated at 37 °C for 48 h in a humidified atmosphere containing 5% CO_2 in air. Cells were 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, Walkersville, MD) following the manufacturer's protocol. The total ATP level was expressed as a percentage of untreated control. To compare the ATP content between normal and CoQ_{10} deficient lymphocytes, a fixed number of lymphocytes (1×10^6 cell mL) were incubated for 2 h under the above culture conditions. Cells were washed in PBS, harvested and lysed immediately in 0.5% Triton X-100, 10 mM Tris–HCl, pH 7.5, containing 1 mM EDTA, and incubated for 10 min on ice. After removal of cell debris by centrifugation (12,000×*g*, 15 min, 4 °C), the ATP level in the resulting supernatant was determined by luciferase bioluminescent assay, using appropriate ATP standards. ATP content measured in total cell lysates was expressed as nmoles/mg cellular protein. The protein content was determined by the bicinchoninic acid assay method (micro-BCA kit, Pierce) using bovine serum albumin (BSA) as a reference.

5.2.7.1. Cellular CoQ_{10} levels. CoQ_{10} from normal (GM15851) and CoQ_{10} deficient (GM17932) lymphocytes was extracted in 5:1 *n*-propanol–water mixture. Briefly, cells were grown in glucose free medium for one week. The cells were collected by centrifugation (400×*g*, 3 min), then washed with phosphate buffered saline (PBS) before the extraction procedure. A mixture of 500 μL of *n*-propanol–water was added, followed by 2 min of agitation using a mechanical vortex-type mixer and centrifugation for 10 min at (12,000×*g*, 4 °C). The resulting supernatant was separated from the precipitate and transferred to a glass vial. The supernatant layer was concentrated following the lipid extraction. Prior to injection on HPLC, lipids were dissolved in 100 μL of *n*-propanol and then 25 μL of a 2 mg/mL solution of 1,4-benzoquinone was added, vortexed and left to stand for 10 min. The addition of 1,4-benzoquinone oxidizes the CoQ_{10}, allowing for determination of total CoQ_{10} in each sample.[63] Samples were analyzed by HPLC on a reversed phase Zorbax SB-Phenyl (150 mm × 4.6 mm, 5 μm), using a mobile phase consisting of 50% aq methanol → 100% methanol from 0 to 15 min, 100% aq methanol from 15 to 23 min, and 100% methanol → 50% aq methanol from 23 to 28 min at a flow rate of 1.5 mL/min with UV detection at 275 nm. Prior to injection on HPLC, the CoQ_{10} concentration was estimated by comparison of the peak area with those of a standard solution of known concentration and normalized to protein concentration. The protein content was determined by the bicinchoninic acid assay method (micro-BCA kit, Pierce) using bovine serum albumin (BSA) as a reference. Data are reported as the mean of at least three independent experiments.

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Supplementary data

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