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An Optimized Pyrimidinol Multifunctional Radical Quencher

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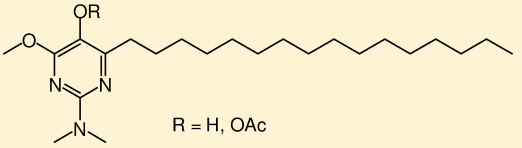
Supporting Information

ABSTRACT: A series of aza analogues (**4**–**9**) of the experimental neuroprotective drug idebenone (**1**) have been prepared and evaluated for their ability to attenuate oxidative stress induced by glutathione depletion and to compensate for the decrease in oxidative phosphorylation efficiency in cultured Friedreich's ataxia (FRDA) fibroblasts and lymphocytes and also coenzyme Q_{10}-deficient lymphocytes. Modification of the redox core of the previously reported **3** improved its antioxidant and cytoprotective properties. Compounds **4**–**9**, having the same redox core, exhibited a range of antioxidant activities, reflecting side chain differences. Compounds having side chains extending 14–16 atoms from the pyrimidinol ring (**6**, **7**, and **9**) were potent antioxidants. They were superior to idebenone and more active than **3**, **4**, **5**, and **8**. Optimized analogue **7** and its acetate (**7a**) are of interest in defining potential therapeutic agents capable of blocking oxidative stress, maintaining mitochondrial membrane integrity, and augmenting ATP levels. Compounds with such properties may find utility in treating mitochondrial and neurodegenerative diseases such as FRDA and Alzheimer's disease.



KEYWORDS: *Mitochondrial dysfunction, electron transport chain, lipid peroxidation, cytoprotection, adenosine triphosphate*

Mitochondrial dysfunction is linked to numerous neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, and Friedreich's ataxia.[1−6] Mitochondria are vitally important organelles involved in many essential cellular functions, notably energy metabolism. They produce 90% of our cellular ATP through oxidative phosphorylation within the mitochondrial respiratory chain.[7−11] Within mitochondria, the primary site of reactive oxygen species (ROS) generation is the electron transport chain;[12,13] normally, mitochondria have an extensive network of antioxidant and detoxification systems, ensuring that lipid peroxidation and levels of ROS are kept at physiologically acceptable levels.[14,15] Defects in the mitochondrial respiratory chain can undoubtedly lead to increased electron leakage and consequently to increased ROS production, causing progressive oxidative damage and ultimately cell death.[2−5,10,16]

The antioxidant and bioenergetic effects of coenzyme Q (CoQ_{10}) are well-known,[17,18] but its clinical utility is limited by its extreme hydrophobicity, which results in low bioavailability.[19,20] To facilitate the delivery of such molecules to the mitochondria of cells, we have designed antioxidants bearing smaller lipophilic side chains and having pyrimidinol[21] or pyridinol[22−26] redox cores, based on earlier studies.[27−30] We demonstrated that an aza analogue of idebenone (**3**, Figure 1), having the 1,4-benzoquinone core replaced with a pyrimidinol core, retained the ability to function within the mitochondria.[21] Because these aza analogues function at a number of levels to suppress damage that would otherwise be caused by electron leakage from the electron transport chain, we have denoted them multifunctional radical quenchers.[21] Recently, we described the importance of side chain optimization to improve antioxidant activity and the ability to maintain ATP levels in cellular mitochondrial disease models in comparison with **3**.[26] These encouraging results suggested that further optimization of the redox core and the side chain might afford multifunctional radical quenchers exhibiting improved potency and efficacy.

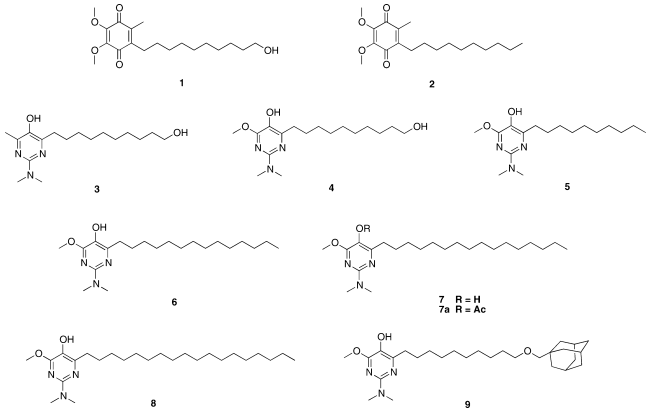


Figure 1. Structures of compounds synthesized and studied.

Presently, we describe the preparation and characterization of six new pyrimidinol derivatives (**4**–**9**). These compounds have redox cores different than that in previously reported **3**;[21] the methyl group ortho to the phenolic OH has been replaced with a methoxyl group. They also have modified side chains of varying lengths lacking the hydroxyl group present in

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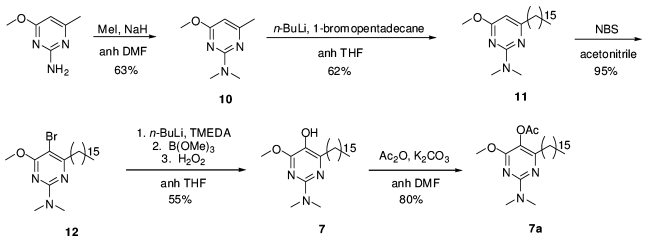
idebenone and **3** (Figure 1). Compounds **1**–**9** were evaluated for their ability to suppress lipid peroxidation, maintain mitochondrial membrane potential, confer cytoprotection to cultured cells under induced oxidative stress, and support electron transport through the respiratory chain as judged by an increase in ATP levels.

The preparation of **1** and **3** have been described previously.[21,31] Decylubiquinone (**2**) was commercially available. The syntheses of the 2-(*N*,*N*-dimethylamino)-4-alkyl-6-methoxypyrimidin-5-ols (**4**, **5**, and **7**) were accomplished using the strategy exemplified in Scheme 1 for **7** and its acetate **7a**.

Scheme 1. Route Employed for the Synthesis of 7 and Its Acetate (7a)



These were prepared in four and five steps, respectively. First, commercially available 2-amino-6-methoxy-4-methylpyrimidine was treated with methyl iodide in the presence of sodium hydride to afford **10** in 63% yield. Then, **10** was monoalkylated on the C-4 methyl group by treatment with *n*-BuLi in the presence of pentadecyl bromide, affording **11** in 62% yield. Aminopyrimidine **11** was then brominated at position 5 to obtain 5-bromo-2-(*N*,*N*-dimethylamino)-4-hexadecyl-6-methoxypyrimidine (**12**) in 95% yield. Finally, **7** was obtained in 55% yield by treating **12** successively with *n*-BuLi in the presence of *N*,*N*,*N*′,*N*′-tetramethylenediamine (TMEDA), then with trimethyl borate, and finally with hydrogen peroxide. Compound **7** was *O*-acetylated in 80% yield to obtain **7a**.

Compounds **4** and **5** were prepared in the same fashion, using the appropriate alkyl bromides for the alkylation of intermediate **10** (Scheme S1, Supporting Information). For the preparation of **6**, **8**, and **9**, a slightly different strategy was employed (Scheme S2, Supporting Information). Specifically, the alkylation of **10** was carried out with allyl bromide, affording 2-(*N*,*N*-dimethylamino)-4-(3-butenyl)-6-methoxypyrimidine in 50% yield. Following conversion of this compound to key intermediate 5-benzyloxy-2-(*N*,*N*-dimethylamino)-4-(3-butenyl)-6-methoxypyrimidine, the side chains of **6**, **8**, and **9** were introduced by cross-metathesis reactions using the appropriate terminal alkenes and Grubbs' second generation catalyst.[32] The characterization of all new compounds included high-resolution mass spectrometry.

The antioxidant and bioenergetics properties of the newly prepared pyrimidinol analogues were tested and analyzed in selected biochemical and biological assays. The test compounds were dissolved in DMSO and added to the assay medium at final DMSO concentrations <0.5%. The ability of the pyrimidinol analogues to quench lipid peroxidation was studied in FRDA lymphocytes that had been depleted of glutathione by treatment with diethyl maleate (DEM). The fluorescent lipid peroxidation-sensitive fatty acid-conjugated dye (C_{11}-BODIPY$^{581/591}$) probe was used as described previously.[22,24,25] Figure 2 shows representative C_{11}-BODIPY$^{581/591}$-green (oxidized) FACS (fluorescence-activated cell sorting) histogram overlays

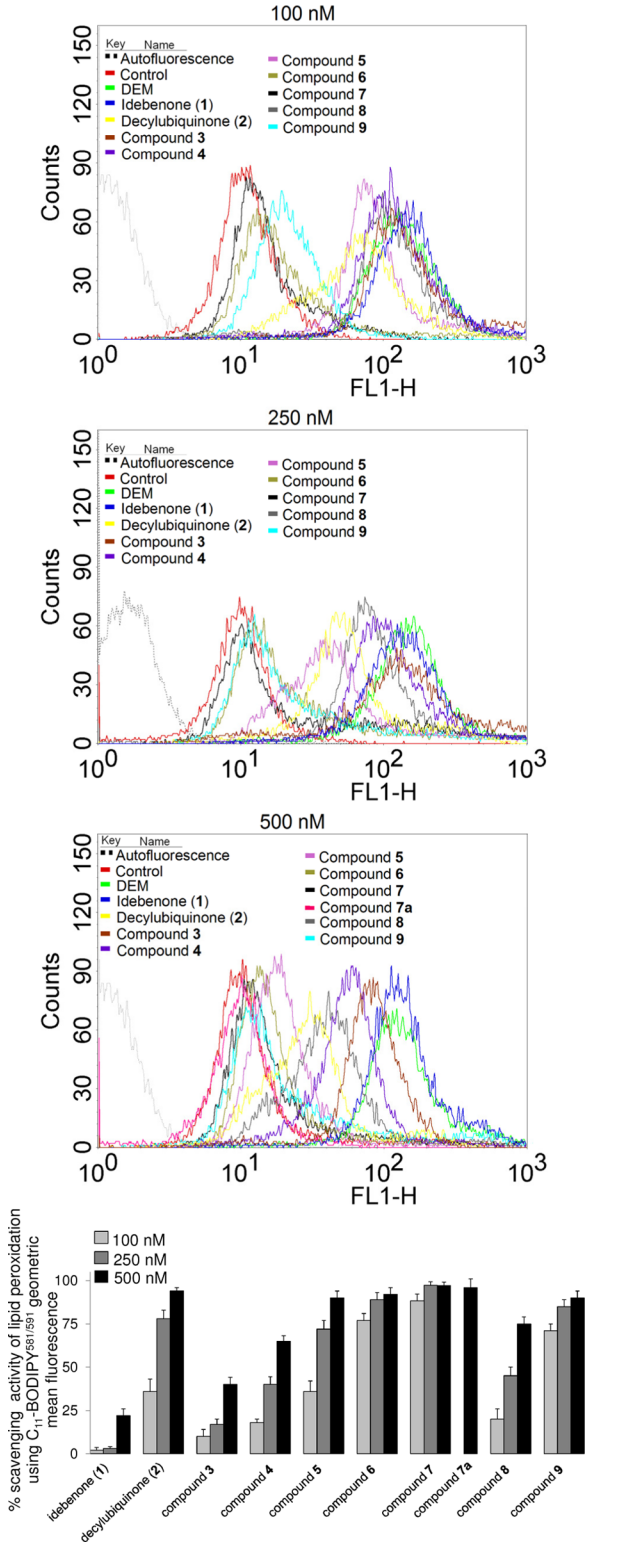


Figure 2. Lipid peroxidation in FRDA lymphocyte cells depleted of glutathione was detected by utilizing the oxidation-sensitive fatty acid probe C_{11}-BODIPY$^{581/591}$ (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3*a*,4*a*-diaza-s-indacene-3-propionic acid) using FACS. Increased C_{11}-BODIPY-green fluorescence (oxidized form), a measure of intracellular lipid peroxidation, was determined by increasing the geometric mean fluorescence intensity (GMFI) of C_{11}-BODIPY-green relative to the untreated control. A bar graph representing the percentage of lipid peroxidation scavenging activity is shown. Data are expressed as the mean ± SEM (n = 3).

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of FRDA lymphocyte cells stained with C_{11}-BODIPY$^{581/591}$-red (reduced) and analyzed using the FL1-H channel, as described in the Supporting Information. DEM treatment caused the C_{11}-BODIPY$^{581/591}$-green fluorescence to shift right on the *x*-axis of the FACS histogram, indicating increased membrane peroxidation as a result of glutathione depletion. Optimization of the redox core by replacing the methyl group ortho to the phenolic OH (**3**) with a better electron donating (methoxyl) group, as in **4**, significantly increased its ability to quench lipid peroxidation (Figure 2). These results are in agreement with the ability of more electron donating groups to lower the bond dissociation energy of the O−H bond, as described by Valgimigli et al.[33] We have recently reported the importance of side chain length on the interaction of coenzyme Q analogues with the mitochondrial respiratory chain to achieve improved antioxidant activity.[26] In the light of these findings, we prepared analogues having different side chain lengths attached to the modified redox core. As shown (Figure 2), the optimal length for antioxidant activity was between 14 and 16 atoms. The analogue with an octadecyl side chain (**8**) had significantly reduced ability to quench lipid peroxidation in comparison to 14 and 16 atom side chains (**6**, **7**, and **9**).

The ability of the pyrimidinol analogues to preserve mitochondrial membrane integrity following treatment of FRDA lymphocytes with diethyl maleate (DEM) was assessed by FACS analysis of mitochondrial membrane potential ($\Delta\psi_m$) using the cationic fluorescent probe tetramethylrhodamine methyl ester (TMRM), as described previously.[24,25] Representative flow cytometric two-dimensional color density dot plot analyses of the mitochondrial membrane potential measurements in the presence and absence of selected compounds are shown (upper panel, Figure 3). The percentage of TMRM stained cells with intact mitochondrial membrane potential appears in the top right quadrant of individual treatments. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), a commonly used uncoupler of oxidative phosphorylation in mitochondria, was employed as positive control to dissipate the chemiosmotic proton gradient, which results in lowering of TMRM fluorescence as a result of the depolarization of mitochondrial inner membrane potential. The bar graph in the lower panel of Figure 3 summarizes the percentage of the cells with intact $\Delta\psi_m$ of the flow cytometric profiles. It shows clearly that **6**, **7**, and **9** had the greatest potency. Compound **4**, having the idebenone side chain, was slightly better than compound **3** in this assay. Idebenone was less active than **3** or **4** (Figure 3). Decylubiquinone (**2**) and **5** afforded significant protection of mitochondrial membrane integrity, as shown in Figure 3. Compound **8**, having an 18 carbon side chain, was as effective as **3** and **4**.

The ability of the compounds to protect FRDA skin fibroblasts from oxidative damage-induced death was measured as described previously with some modification.[34,35] The cells were subjected to L-buthionine (*S*,*R*)-sulfoximine (BSO), an inhibitor of de novo glutathione (GSH) biosynthesis.[36] The half-maximal effective concentrations (EC_{50}) were determined (Table 1). Oxidative damage-induced death of FRDA fibroblasts was blocked by exogenous antioxidants.[34,35] Compound **6**, **7**, and **9** were by far the most efficient, having EC_{50} values of 22 ± 7, 25 ± 4, and 32 ± 4 nM, respectively (Table 1). Again, in this assay the optimal side chain length was between 14 and 16 atoms. Compounds having a side chain with less than 14 or more than 16 atoms were less protective in this assay. Compounds **3** and **4**, having the idebenone side chain,

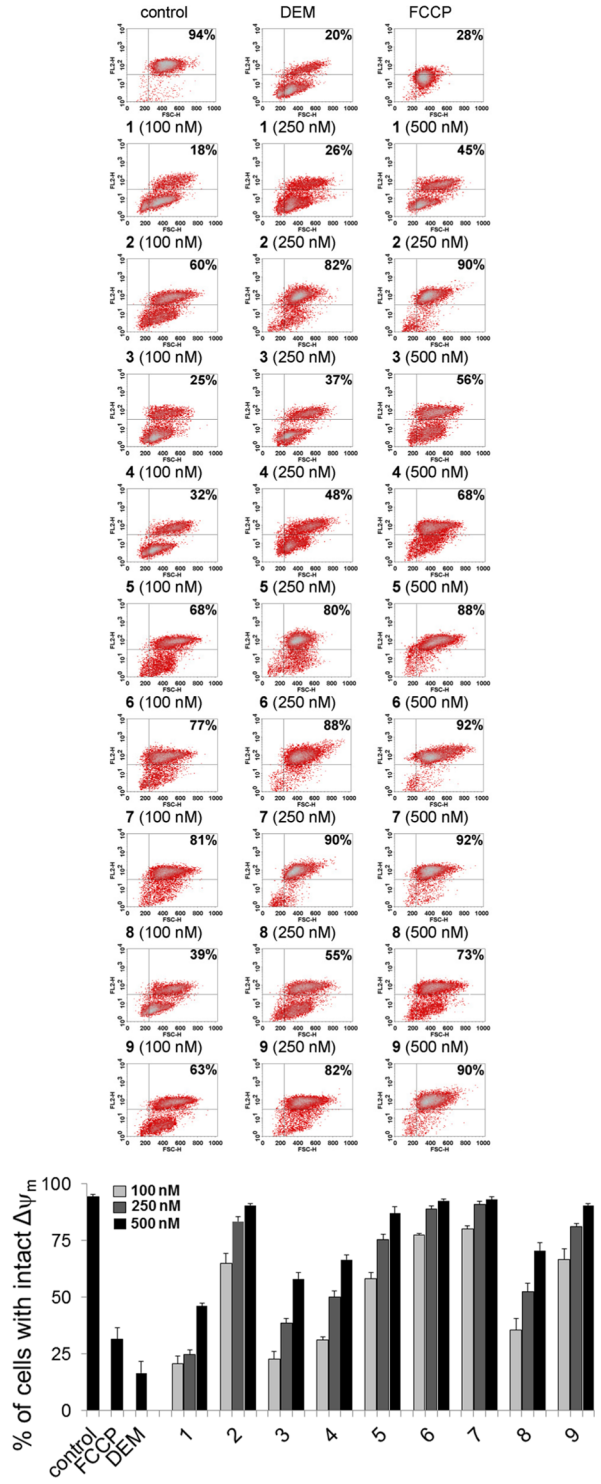

Figure 3. Representative flow cytometric two-dimensional color density dot plot analyses of the ability of **1**−**9** to maintain mitochondrial membrane potential ($\Delta\psi_m$) in DEM-treated FRDA lymphocyte cells stained with 250 nM TMRM and analyzed using the FL2-H channel as described in the Supporting Information. The percentage of cells with intact $\Delta\psi_m$ is indicated in the top right quadrant of captions. Representative examples from at least three independent experiments are shown. A total of 10,000 events were recorded for each sample and analyzed with the CellQuest software (BD Biosciences). A bar graph representing the percentage of the cells with intact $\Delta\psi_m$ is shown. Data are expressed as means ± SEM of three independent experiments run in duplicate.

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Table 1. Effect of Pyrimidinol Antioxidants on the Cellular Viability of FRDA Fibroblasts Treated with BSO

compd	EC_{50} (nM)
idebenone (**1**)	551 ± 23
decylubiquinone (**2**)	56 ± 4
3	456 ± 22
4	345 ± 21
5	68 ± 8
6	22 ± 7
7	25 ± 4
8	266 ± 19
9	32 ± 4
pyrimidinol redox core	>50000

were both more efficient than idebenone, but less effective than decylubiquinone and **5** (Table 1).

In this study, we used a nutrient-sensitized screening strategy to identify CoQ_{10} analogues that function within the mitochondrial respiratory chain by measuring the total ATP level in galactose-containing media.[37] Total cellular ATP was measured in cultured FRDA and CoQ_{10}-deficient cell lines as described previously.[26] The cells were grown on glucose-free media supplemented with galactose. Since cells grown in galactose rely mostly on oxidative phosphorylation (OXPHOS) to produce their ATP, they become more sensitive to mitochondria respiratory chain inhibitors than cells grown in glucose medium.[37,38] Compound **7** and its acetate (**7a**) increased ATP levels in the CoQ_{10}-deficient lymphocytes above the basal level when used at 5 and 10 μM concentrations and slightly lowered ATP levels in both cell lines at 25 μM (Table 2). Idebenone strongly diminished ATP levels in a concentration-dependent fashion in the CoQ_{10} deficient lymphocytes, as well as in Friedreich's ataxia lymphocytes (Table 2). In the presence of 25 μM idebenone, ATP concentrations were essentially completely depleted. Decylubiquinone (**2**), which differs from idebenone only by the absence of the side chain OH group, showed an ATP level reduced by 37% at 25 μM concentration compared to the control. There was no increase in ATP levels at the lower concentrations (5 and 10 μM) beyond the basal level. The reduction in ATP levels in idebenone-treated cells likely reflects the ability of idebenone to inhibit complex I activity in the mitochondrial electron transport chain, as we have shown previously in bovine heart mitochondria.[26] Our earlier study suggested that compounds having lipophilic side chains exhibit lesser inhibition of mitochondrial respiratory chain complexes and correspondingly permit more effective maintenance of cellular ATP levels.[26] In that study, two pyrimidinol analogues having lipophilic side chains were shown to be able to increase ATP levels in CoQ_{10}-deficient lymphocytes, albeit not in Friedreich's ataxia lymphocytes, when used at low concentration.

In the present study, **1**–**9** were evaluated for their ability to enhance ATP levels in CoQ_{10} deficient lymphocytes and in FRDA lymphocytes. As noted above, idebenone (**1**) and decylubiquinone (**2**) simply diminished ATP levels in a concentration-dependent fashion. Compound **3**, which was included in the earlier study,[26] had the same effect as did **4**. Compound **5**, having a 6-methoxyl substituent on the redox core and a 4-decyl substituent noted to be suboptimal for the assays described above, also failed to increase ATP levels. Compound **8**, whose side chain was found to be suboptimal in the assays described above, was found to be without effect on ATP levels in either of the lymphocyte cell lines studied. In comparison, **6**, **7**, **7a**, and **9** all effected a statistically significant increase in ATP levels when tested at 5 μM concentration in both CoQ_{10}-deficient lymphocytes and FRDA lymphocytes. The most favorable response was noted for **7** in CoQ_{10}-deficient lymphocytes, namely, 118 ± 2% of control. The cell line used for this experiment (GM17932, Coriell Cell Repository) has been shown previously to have an ATP level that is about 20% lower than normal human lymphocytes,[26] so **7** effected essentially full restoration of ATP levels.

Given the important roles of CoQ_{10} in mitochondria,[17,18] we assessed the effects of idebenone (**1**), **4**, and **7** on FRDA fibroblasts in which CoQ_{10} had been depleted pharmacologically. Treatment of cultured mammalian cells with 4-nitrobenzoate, which competitively inhibits 4-hydroxybenzoate:polyprenyltransferase (COQ2),[39] was reported to decrease CoQ_{10} biosynthesis compared to control.[39−41] Analogous treatment of FRDA fibroblasts with 1 mM 4-nitrobenzoate depleted CoQ_{10} levels by 29% compared to control (Figure S1a, Supporting Information) and reduced ATP levels by 20% compared to control (Figure S1b, Supporting Information). Co-treatment of FRDA fibroblasts with 1 mM 4-nitrobenzoate and 5 μM compound **7** restored ATP, essentially to normal levels (Figure S2, Supporting Information), while **4** having the idebenone side chain was less effective at 5 μM concentration and ineffective at

Table 2. Total ATP Concentration in CoQ_{10} Deficient Lymphocytes and FRDA Lymphocytes Following Incubation with Compounds 1−9 for 48 h

	total ATP level (% of control)					
	CoQ_{10} deficient lymphocytes			Friedreich's ataxia lymphocytes		
compd	5 μM	10 μM	25 μM	5 μM	10 μM	25 μM
untreated control	100	100	100	100	100	100
idebenone (**1**)	80 ± 3	46 ± 7	2 ± 1	73 ± 7	46 ± 4	3 ± 1
decylubiquinone (**2**)	100 ± 6	86 ± 7	63 ± 4	93 ± 4	76 ± 2	63 ± 6
3	99 ± 3	86 ± 2	46 ± 4	77 ± 5	46 ± 4	28 ± 2
4	102 ± 2	95 ± 2	54 ± 4	80 ± 4	66 ± 5	46 ± 5
5	96 ± 5	83 ± 6	58 ± 6	94 ± 7	87 ± 2	58 ± 5
6	105 ± 3	97 ± 3	65 ± 5	109 ± 4	97 ± 4	55 ± 3
7	118 ± 2	106 ± 9	90 ± 8	108 ± 5	96 ± 4	80 ± 4
7a	116 ± 4	109 ± 4	96 ± 6	109 ± 5	101 ± 4	92 ± 4
8	98 ± 3	98 ± 3	96 ± 4	101 ± 2	102 ± 3	102 ± 3
9	105 ± 2	97 ± 5	64 ± 5	107 ± 3	103 ± 3	72 ± 3

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10 μM concentration. Idebenone itself diminished ATP levels with or without 4-nitrobenzoate coadministration. It has been reported that addition of decylubiquinone (**2**) to 4-nitrobenzoate treated cells significantly increased respiration beyond that of the treated control.[39]

In summary, new pyrimidinol derivatives, have been synthesized and characterized biochemically. The results indicate that careful optimization of the redox core and side chain can afford multifunctional radical quenchers capable of potently suppressing ROS and lipid peroxidation, preserving mitochondrial membrane potential, affording cytoprotection against induced oxidative stress, and maintaining normal ATP levels. The greatly improved cytoprotective activities of the present compounds capable of enhancing ATP production, in comparison with prototype analogue **3**, suggests that optimal activity results from multiple properties of the compounds in suppressing lipid peroxidation and ROS, in addition to enhancing energy production, consistent with our earlier mechanistic hypothesis.[21] While the limited number of analogues studied here does not completely define the side chain and redox core optimal for improving antioxidant and bioenergetic properties, it does suggest that further structural optimization may afford compounds suitable for therapeutic intervention in mitochondrial and neurodegenerative diseases associated with increased levels of ROS and reduced energy production.

■ ASSOCIATED CONTENT

Supporting Information

Procedures and characterization for all new compounds, procedures for biochemical assays, and effects of **1**, **4**, and **7** on ATP levels in 4-nitrobenzoate-treated human fibroblasts. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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