Supporting Information

for

A Strategy for Suppressing Redox Stress Within Mitochondria

Pablo M. Arce, Omar M. Khdour, Ruth Goldschmidt, Jeffrey S. Armstrong and

Sidney M. Hecht

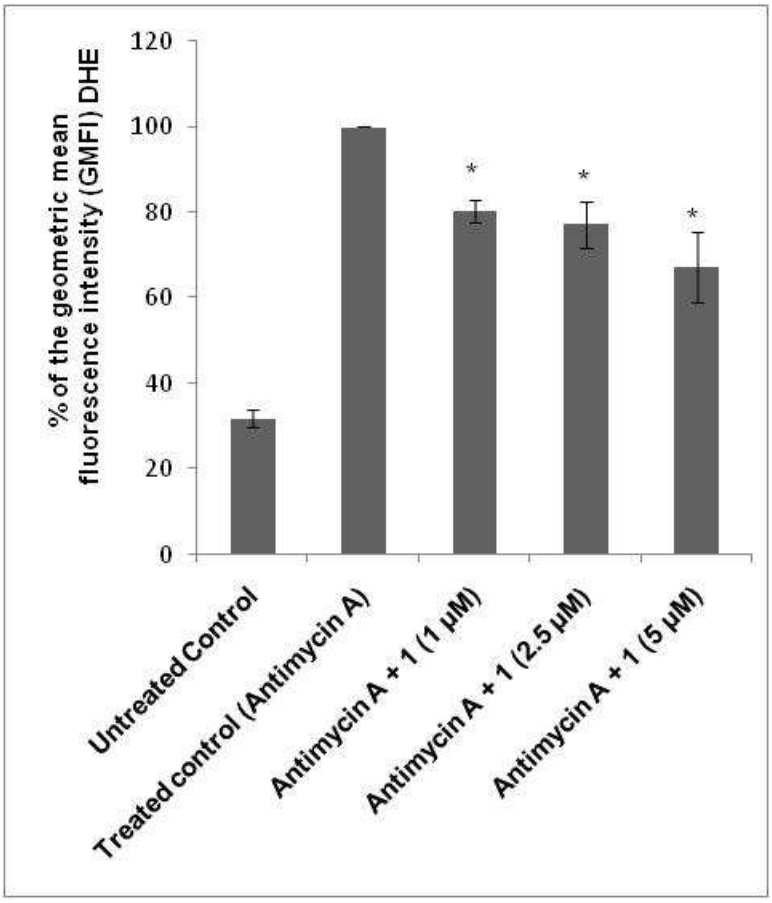


Figure S1. Flow cytometric analysis of CEM leukemia cells stained with dihydroethidium (DHE) for 20min, and pre-treated with compound **1** at 1, 2.5, and 5 μM for 15 hours, and then treated with antimycin A for 2 hours, to induce the production of superoxide. Data shown represent the mean ± SEM of five different experiments run as duplicates, $^{*}P < 0.005$, statistically significant when compared to treated control cells.

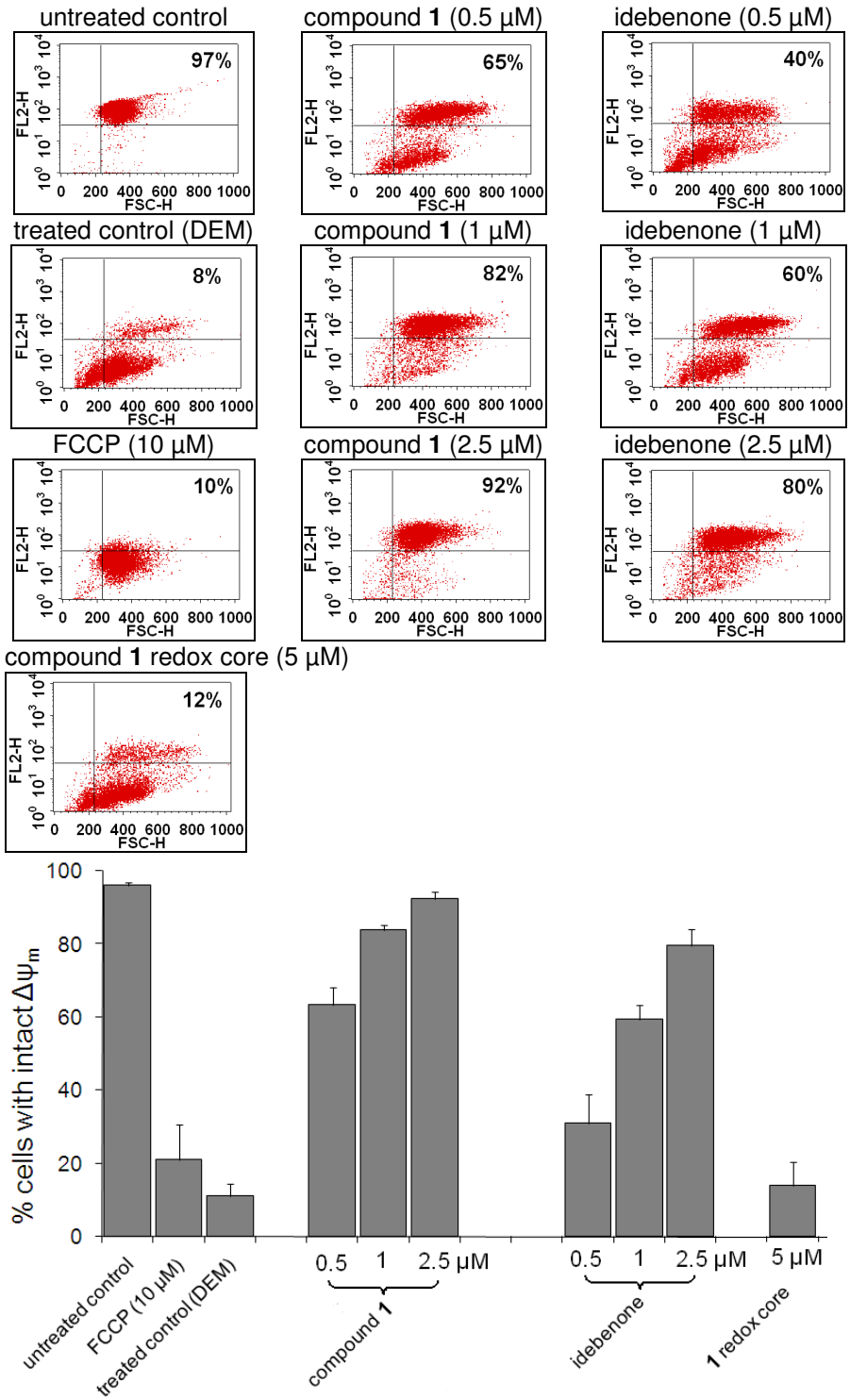

untreated control
97%
compound 1 (0.5 µM)
65%
idebenone (0.5 µM)
40%
treated control (DEM)
8%
compound 1 (1 µM)
82%
idebenone (1 µM)
60%
FCCP (10 µM)
10%
compound 1 (2.5 µM)
92%
idebenone (2.5 µM)
80%
compound 1 redox core (5 µM)
12%
FL2-H
FSC-H
% cells with intact Δψm
0.5 1 2.5 µM
0.5 1 2.5 µM 5 µM
untreated control
FCCP (10 µM)
treated control (DEM)
compound 1
idebenone
1 redox core

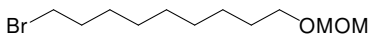
Figure S2. Representative flow cytometric two dimensional color density dot plot analyses of mitochondrial membrane potential ($\Delta\psi_m$) in CEM cells stained with 250 nM TMRM and analyzed using the FL2-H channel as described in Supporting Information. Following pretreatment with the indicated compounds (at 2.5, 1 or 0.5 μM concentration) for 12 hours, the cells were treated with 5 mM diethyl maleate (DEM) for 2 hours. The cells were washed in phosphate buffered saline, and suspended in Hanks' Balanced Salt Solution buffer (HSSB). Cells were loaded with 250 nM TMRM for 15 minutes, washed twice in phosphate buffered saline, and the red fluorescence was measured by flow cytometry using the FL2-H channel. The percentage of cells with intact $\Delta\psi_m$ is indicated in the top right quadrant of captions. Carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) depolarizes membrane potential. Each graph is a representative example from at least three independent experiments. In each analysis, 10,000 events were recorded. The bottom of the figure shows a bar graph of means the percentage of cells with intact $\Delta\psi_m$ recorded by FACS. Data are expressed as means ± SE (n = 3).

MATERIALS AND METHODS

Chemicals, including diethyl maleate (DEM), 2,2'-azobis-(2-amidinopropane dihydrochloride) (AAPH), L-buthionine-(*S*, *R*)-sulfoximine (BSO), (+)-alpha-tocopherol (α-TOH), (+)-alpha-tocopherol acetate, trolox, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), antimycin A and *N*-acetylcysteine were purchased from Sigma Aldrich (St. Louis, MO). 1-Stearoyl-2-oleoyl-phosphatidylcholine (SOPC) and 1, 2-dilinoleoylphosphatidylcholine (DLPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Fluorescent probes, including C_{11}-BODIPY$^{581/591}$ (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a, 4a-diaza-s-indacene-3-propionic acid), calcein acetoxymethyl (AM), tetramethylrhodamine methyl ester (TMRM), dihydroethidium (DHE) and dichlorodihydrofluorescein diacetate (DCFH-DA), were obtained from Molecular Probes (Eugene, OR). CEM leukemia cells (ATCC, catalogue number CRL-2264) were cultured in RPMI-1640 medium (Gibco, Grand Island, NY) with 10% fetal calf serum, 2 mM glutamine (HyClone, South Logan, UT) and 1% penicillin–streptomycin antibiotic supplement (Cellgro, Manassas, VA). Cells were passaged daily to maintain them in log phase growth and kept at a nominal concentration of 5-10 $\times 10^5$ mL. Primary fibroblasts were derived from a patient donor with a molecular diagnosis of FRDA. This cell line was obtained from Coriell Cell Repositories (Camden, NJ, USA; catalog number GM-04078). Fibroblasts were cultured in 64% (v/v) Eagle's minimal essential medium (MEM), lacking phenol red with Eagle's balanced salt (EBS) and 25% M199 with EBS (Gibco) supplemented with 15% (v/v) fetal bovine serum albumin (HyClone), 1% penicillin–streptomycin antibiotic mix (Cellgro), 10 μg/mL insulin (Sigma Aldrich), 10 ng/mL basic fibroblast growth factor (βFGF) (Lonza, Walkersville, MD) and 2 mM glutamine.

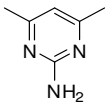
^{1}H NMR spectra were recorded on a Varian Inova 400 MHz, using chloroform-*d* or methanol-d_4. ^{1}H NMR chemical shifts were reported relative to residual chloroform at 7.24 ppm or to residual methanol at 3.31 ppm. ^{13}C NMR chemical shifts were reported relative to residual chloroform at 77.1 ppm or to residual methanol at 49.0 ppm. All solvents were analytical grade and were used without further purification. All chemicals were purchased from Aldrich Chemical Company and were used without further purification. The reactions were carried out under an argon atmosphere unless specified otherwise. Column chromatography was carried out using silica gel (Silicycle R10030B, 60 particle size, 230-240 mesh). Analytical thin layer chromatography separations were carried out on glass plates coated with silica gel (60, particle size F254, E. Merck 5608/7). The TLC chromatograms were developed using 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, followed by heating. Melting points were recorded on a MelTemp apparatus and are uncorrected.

Preparation of Compound 1.

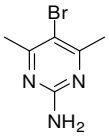


1-Bromo-9-(methoxymethoxy)nonane. To a stirred solution containing 5.00 g (22.4 mmol) of 9-bromo-1-nonanol in 60 mL of anh THF was added 5.10 mL (67.2 mmol) of MOMCl followed by 1.79 g (44.8 mmol) of a 60% suspension of NaH in mineral oil. The reaction mixture was stirred at 23 °C overnight. The reaction mixture was carefully quenched with satd aq sodium bicarbonate, poured into 200 mL of water and extracted with two 150-mL portions of ether. The combined organic solution was washed with 200 mL of brine, dried ($MgSO_4$) and then concentrated under diminished pressure. The residue was purified by chromatography on a silica

gel column (15 x 5 cm). Elution with 9:1 hexanes–ethyl acetate afforded the expected product as colorless oil: yield 4.21 g (70%); silica gel TLC R_f 0.45 (9:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.28-1.39 (br, 10H), 1.56 (quint, 2H, J = 7.2 Hz), 1.82 (quint, 2H, J = 7.2 Hz), 3.33 (s, 3H), 3.37 (t, 2H, J = 6.8 Hz), 3.48 (t, 2H, J = 6.8 Hz) and 4.59 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 26.1, 28.1, 28.7, 29.28, 29.33, 29.7, 32.8, 34.0, 55.0, 67.7 and 96.4; mass spectrum (APCI), *m/z* 267.0953 $(M+H)^+$ ($C_{11}H_{24}O_2Br$ requires 267.0960).

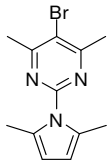


2-Amino-4,6-dimethylpyrimidine (2). To a stirred solution containing 4.00 g (37.0 mmol) of guanidine sulfate and 8.40 g (79.3 mmol) of sodium carbonate in 25 mL of water were added 6.00 mL (58.1 mmol) of 2,4-pentanedione. The reaction mixture was stirred at 100 ºC overnight. The reaction mixture was 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 **2** as a colorless solid: yield 4.31g (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).

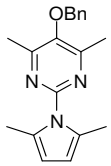


2-Amino-5-bromo-4,6-dimethylpyrimidine (3). To a stirred solution containing 4.31 g (34.8 mmol) of 2-amino-4,6-dimethylpyrimidine in 150 mL of acetonitrile was added 6.15 g (52.1 mmol) of *N*-bromosuccinimide. The reaction mixture was stirred at 23 °C for 3 h. The formed

precipitate was filtered and dried to afford **3** 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, 160.7 and 166.3; mass spectrum (APCI), *m/z* 201.9982 $(M+H)^+$ ($C_6H_9N_3Br$ requires 201.9980).

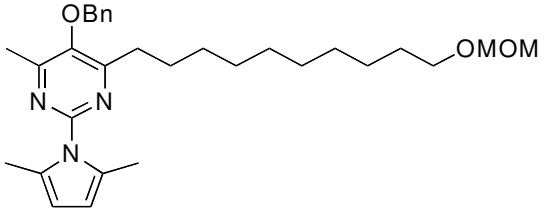


5-Bromo-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4,6-dimethylpyrimidine (4). To a stirred solution containing 2.00 g (9.89 mmol) of 2-amino-5-bromo-4,6-dimethylpyrimidine in 16 mL of anh toluene was added 1.36 mL (11.5 mmol) of 2,5-hexanedione followed by 96 mg (0.50 mmol) of *p*-toluenesulfonic acid. The reaction 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 x 5 cm). Elution with 5:1 hexanes–ethyl acetate afforded **4** 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).



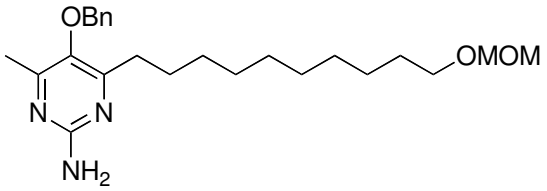
5-(Benzyloxy)-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4,6-dimethylpyrimidine (5). To a stirred solution containing 4.87 g (17.4 mmol) of 5-bromo-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4,6-dimethylpyrimidine (**4**) in 50 mL of 1:1 dioxane–degassed water was added 632 mg (0.69 mmol)

of Pd_2dba_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 anh THF and treated with 3.10 mL (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 and 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 x 6 cm). Elution with 9:1 hexanes–ethyl acetate afforded **5** 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, 128.6, 129.2, 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).



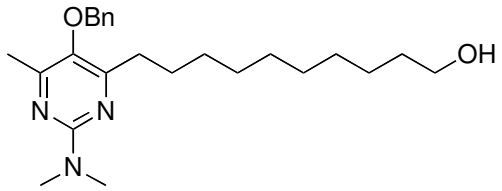
5-(Benzyloxy)-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4-(10-(methoxymethoxy)decyl)-6-methylpyrimidine (6). To a stirred solution at −78 °C containing 486 g (1.58 mmol) of 5-(benzyloxy)-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4,6-dimethylpyrimidine and 281 mg (1.05 mmol)

of 1-bromo-9-(methoxymethoxy)nonane in 10 mL of anh THF was added 987 μL (1.58 mmol) of a 1.6 M solution of *n*-BuLi in pentane. The reaction mixture was stirred under argon atmosphere at 23 °C for 30 min. The reaction was quenched with saturated aqueous ammonium chloride and then poured into 50 mL of water. The mixture was then extracted with two 50-mL portions of ethyl acetate. The combined organic layer was washed with 80 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 x 3 cm). Elution with 5:1 hexanes–ethyl acetate afforded **6** as a light yellow oil: yield 289 mg (56%); silica gel TLC R_f 0.55 (5:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 1.33 (m, 12H), 1.60 (m, 2H), 1.82 (m, 2H), 2.33 (s, 6H), 2.49 (s, 3H), 2.79 (dd, 2H, J = 7.6, 7.6 Hz), 3.36 (s, 3H), 3.52 (t, 2H, J = 6.4 Hz), 4.62 (s, 2H), 4.90 (s, 2H), 5.87 (s, 2H) and 7.37-7.48 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 14.4, 19.3, 26.2, 27.7, 29.4, 29.5, 29.6, 29.8, 31.6, 55.1, 67.9, 75.7, 96.4, 108.0, 128.0, 128.58, 128.62, 128.7, 129.3, 136.2, 147.4, 152.6, 161.6 and 165.1; mass spectrum (APCI), *m/z* 494.3395 $(M+H)^+$ ($C_{30}H_{44}N_3O_3$ requires 494.3383).



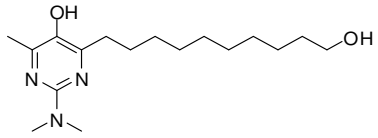
5-(Benzyloxy)-4-(10-(methoxymethoxy)decyl)-6-methylpyrimidin-2-ylamine (7). To a stirred solution containing 230 mg (0.47 mmol) of 5-(benzyloxy)-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)-4-(10-(methoxymethoxy)decyl)-6-methylpyrimidine (**3**) in 15 mL of 9:1 ethanol–water was added 327 mg (4.70 mmol) of hydroxylamine hydrochloride followed by 263 mg (4.70 mmol) of KOH. The reaction mixture was then heated and stirred at reflux for 5 h. A second portion of 327 mg (4.70 mmol) of hydroxylamine hydrochloride followed by 263 mg (4.70 mmol) of KOH was

added and the reaction mixture was heated and stirred at reflux for 12 h. The reaction mixture was poured into 70 mL of water and then treated with 1N NaOH until pH 9-10 was reached. The reaction 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 x 3 cm). Elution with 2:1 hexanes–ethyl acetate afforded **7** as a colorless oil: yield 133 mg (76%); silica gel TLC R_f 0.76 (9:1 dichloromethane–methanol); ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 12H), 1.53-1.63 (m, 4H), 2.26 (s, 3H), 2.54 (dd, 2H, J = 7.6, 7.6 Hz), 3.31 (s, 3H), 3.46 (t, 2H, J = 6.8 Hz), 4.57 (s, 2H), 4.69 (s, 2H), 5.19 (br s, 2H) and 7.35−7.37 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 18.9, 26.2, 28.4, 29.36, 29.40, 29.5, 29.67, 29.70, 31.9, 55.0, 67.8, 75.9, 96.3, 127.9, 128.3, 128.6, 136.8, 142.8, 158.9, 161.1 and 164.8; mass spectrum (APCI), *m/z* 416.2908 $(M+H)^+$ ($C_{24}H_{38}N_3O_3$ requires 416.2913).

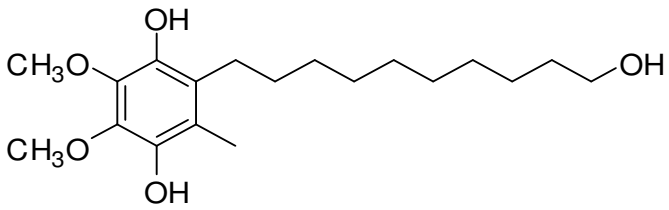


10-(5-(Benzyloxy)-2-(dimethylamino)-6-methylpyrimidin-4-yl)decan-1-ol (8). A stirred solution containing 180 mg (0.43 mmol) of 5-(benzyloxy)-4-(10-(methoxymethoxy)decyl)-6-methylpyrimidin-2-ylamine in 10 mL of 35% aq 1:1 formaldehyde−formic acid was heated and stirred at reflux for 16 h. The reaction mixture was 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 then 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 x 3 cm). Elution with 1:2 acetone–hexanes afforded **8** as a colorless oil: yield 44

mg (44%); silica gel TLC R_f 0.58 (1:2 acetone–hexanes); ^{1}H NMR (methanol-d_4) δ 1.27 (m, 12H), 1.53-1.73 (m, 4H), 2.26 (s, 3H), 2.55 (dd, 2H, J = 7.6, 7.6 Hz), 3.09 (s, 6H), 3.50 (t, 2H, J = 6.8 Hz), 4.71 (s, 2H) and 7.35-7.37 (m, 5H); ^{13}C NMR (methanol-d_4) δ 14.0, 17.8, 25.5, 27.6, 29.1, 29.2, 29.3, 31.3, 32.3, 36.3, 61.6, 75.5, 127.9, 128.3, 128.6, 137.1, 141.0, 158.4, 160.5, 161.5 and 163.7; mass spectrum (APCI), *m/z* 400.2969 $(M+H)^+$ ($C_{24}H_{38}N_3O_2$ requires 400.2964).



2-(Dimethylamino)-4-(10-hydroxydecyl)-6-methylpyrimidin-5-ol (1). To a stirred solution containing 44.0 mg (0.11 mmol) of 10-(5-(benzyloxy)-2-(dimethylamino)-6-methylpyrimidin-4-yl)decan-1-ol in 3 mL of methanol was added 3 mg of 20% palladium hydroxide on carbon (Degussa type E101 NE/N). 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 **1** as a colorless oil: yield 33 mg (100%). An analytical sample was obtained by chromatography on a silica gel column (10 x 1 cm). Elution with 2:1 toluene–ethyl acetate afforded the purified product as a colorless oil; silica gel TLC R_f 0.25 (2:1 toluene–ethyl acetate); ^{1}H NMR (methanol-d_4) δ 1.29-1.35 (m, 12H), 1.49 (m, 2H), 1.65 (m, 2H), 2.26 (s, 3H), 2.62 (dd, 2H, J = 7.2, 7.2 Hz), 3.06 (s, 6H) and 3.51 (t, 2H, J = 6.4 Hz); ^{13}C NMR (methanol-d_4) δ 17.8, 25.5, 27.3, 29.13, 29.15, 29.18, 29.3, 31.2, 32.2, 36.5, 61.6, 138.0, 155.6, 157.3 and 159.4; mass spectrum (APCI), *m/z* 310.2490 $(M+H)^+$ ($C_{17}H_{32}N_3O_2$ requires 310.2495).



Reduction of Idebenone to Idebenol

Idebenol (ε_{290} = 3.94 $mM^{-1}cm^{-1}$)[1] was prepared by the reduction of idebenone (ε_{275} = 14 $mM^{-1}cm^{-1}$ in absolute ethanol)[1] in analogy with the reduction of decylubiquinone, as described by Trounce et al.[2] Briefly, 5 mg of idebenone was dissolved in 2 mL of ethanol and treated with excess sodium borohydride (color change from yellow to colorless). The resulting solution was acidified to pH 2 with 1 M HCl. The idebenol was recovered by partition into 2:1 diethyl ether–cyclohexane and the solution was evaporated to dryness under a stream of nitrogen, then dissolved in 1 mL of ethanol acidified to pH 2 with 0.1 N HCl and stored at –80 ºC.

BIOASSAY PROTOCOLS

Assay of Cellular Reactive Oxygen Species. The intracellular levels of reactive oxygen species (ROS) were measured using the fluorescent probe, 2,7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes) that detects cellular ROS levels.[3] DCFH-DA is a membrane permeable and oxidant-sensitive fluorescent dye. Once inside the cell, it is cleaved by endogenous esterases and can no longer pass out of the cell. The de-esterified product becomes highly fluorescent dichlorofluorescein (DCF). Intracellular DCF fluorescence is used as an index of cellular ROS production, mainly hydrogen peroxide.

CEM leukemia cells were placed under oxidative stress by pharmacological depletion of glutathione (GSH) using diethyl maleate (DEM).[4,5] Briefly, CEM leukemia cells were cultured in RPMI medium with 10% FBS, 2 mM glutamine and 1% penicillin–streptomycin. CEM cells (5 x

10^5 cell/ mL) were plated (1 mL in 12-well plates) and treated with the test compounds at final concentrations of 1 and 5 μM, and incubated at 37 °C for 3 h in a humidified atmosphere containing 5% CO_2 in air. Cells were treated with 5 mM DEM for 40 minutes, collected by centrifugation at 300 x g for 3 min, and then washed with phosphate buffered saline. Cells were resuspended in phosphate buffered saline with 20 mM glucose and incubated at 37 °C in the dark for 20 min with 10 μM DCFH-DA. Cells were collected by centrifugation at 300 x g for 3 min, and then washed with phosphate buffered saline. The samples were analyzed immediately by flow cytometry (Becton-Dickinson FACS Caliber instrument equipped with Cell Quest software, BD Biosciences) using 488 nm excitation laser and FL1-H channel 538 nm emission filter. In each analysis, 10,000 events were recorded. Results obtained were verified by repeating the experiments in triplicate.

Assay of Cellular Superoxide. In a 24-well cell culture plate, were added 1mL of a suspension containing $5x10^5$ CCRF-CEM leukemia cells. These were treated with the test compounds at final concentrations of 1, 2.5 or 5 μM for 15 h. Cells were then treated with 50 μM of antimycin A at 37 °C for 2 h in a humidified atmosphere of 5% CO_2 in air. Then, 6 μM of dihydroethidium (DHE) was added to the cells, and incubated for an additional 20 min at 37 °C in the dark. Cells were then collected in Falcon tubes and analyzed immediately by flow cytometry using the FACS Calibur (BD Biosciences) at 488 nm excitation laser and FL2-H channel emission filter. In each analysis, 10,000 events were recorded. Duplicates were run for each experiment.

Evaluation of Lipid Oxidation. The oxidation-sensitive fluorescent probe C11-BODIPY$^{581/591}$ has been used to detect lipid oxidation at a cellular level and model membranes.[6–8] The dye is

relatively non-fluorescent in solution and it possesses two conjugated double bonds that are susceptible to oxidation. Upon oxidation of these bonds, the fluorescence properties of the BODIPY probe shift from a red-emitting form (595 nm) to a green-emitting form (520 nm).[9] Changes in fluorescence indirectly reflect the oxidation of unsaturated fatty acids.[10]

Preparation of Liposomes. Polyunsaturated fatty acid liposomes were prepared from phosphatidylcholines as described previously.[11] Briefly, 25 mg of DLPC and 25 mg of SOPC were dissolved in chloroform and the solvent was concentrated to dryness under a stream of nitrogen to give a thin film of phosphatidylcholine in a round bottomed flask. Cold 10 mM Tris-HCl, pH 7.4, containing 100 mM KCl, was added in a volume such that the lipid concentration was 1 mg/mL. The mixture was then shaken and sonicated on ice for 15 sec to give a milky suspension. The liposomes so obtained were filtered through a 0.2 μM membrane filter.

Measurement of C_{11}-BODIPY$^{581/591}$ Oxidation in Membrane Model System. The fatty acid indicator C_{11} BODIPY$^{581/591}$ was incorporated into liposomes and oxidized by peroxyl radicals derived from the thermal decomposition of AAPH in presence and absence of the test compounds. Briefly, liposomes (1 mg/mL), suspended in 10 mM Tris-HCl, pH 7.4, containing 100 mM KCl, were transferred to a 1-mL quartz cuvette and placed in a Varian Cary Eclipse fluorometer (Varian, Cary, NC) equipped with a thermostatted cuvette holder at 40 °C. Liposomes were pre-incubated at 40 °C for 10 min with 200 nM C_{11} BODIPY$^{581/591}$ to allow their incorporation into the lipid phase of the liposomes. After the addition of 10 mM AAPH, the decay of red fluorescence emission was followed at λ_{ex} 570 nm, λ_{em} 600 nm. Relative

fluorescence units were normalized to 100% intensity. The results obtained were verified in three independent experiments.

Assay for Thiobarbituric Acid Reactive Species (TBARS). Lipid peroxidation was monitored by measuring malondialdehyde (MDA), an end product of lipid peroxidation cascades[12] using the thiobarbituric acid reactive species (TBARS) assay as described previously.[8,13] In this assay MDA reacts with thiobarbituric acid under acidic condition and heat, and the product of this reaction can be detected fluorometrically. Briefly, intact mouse liver mitochondria were prepared by homogenization followed by differential centrifugation in 200 mM Tris-MOPS, 1 mM EGTA, pH 7.4.[14] To measure thiobarbituric acid reactive species (TBARS), mouse liver mitochondria (2 mg of protein/mL) were suspended at 37 °C in 100 mM KCl, 10 mM Tris-HCl, pH 7.6. Aliquots (0.8 mL) were preincubated with the tested compounds for 5 min at 37 °C in presence of 5 mM succinate and 10 μM rotenone for some incubations, whereas for the other incubations 20 mM malonate was present. Ascorbic acid (300 μM) and 100 μM ammonium ferric sulfate were then added to induce lipid peroxidation for 40 min. The samples from these incubations were then mixed with 0.4 mL of 0.5% thiobarbituric acids in 35% $HClO_4$, heated at 100 ºC for 15 min, and then diluted with 3 mL of water. The product of the reaction was extracted into 3 mL of *n*-butanol. Triplicate aliquots of the n-butanol phase were used to determined TBARS fluorometrically (λ_{ex} 535 nm; λ_{em} 550 nm). Emission fluorescence units were converted to MDA equivalents from a standard curve constructed using 1, 1,3,3-tetraethoxypropane. Lipid peroxidation was measured in MDA equivalents concentration expressed as nmol/mg protein. Measurements were carried out on a Varian Cary Eclipse fluorimeter (Varian, Cary, NC) set at high sensitivity and excitation/emission bandwidth of 5 nm.

Preparation of the MDA standard curve. The breakdown product of 1,1,3,3-tetraethoxypropane (TEP) was used as standard. TEP undergoes hydrolysis to liberate stoichiometric amounts of MDA. A stock standard solution was obtained by dissolving 25 µL 1,1,3,3 tetraethoxypropane (TEP) in 100 ml of water to give a 1 mM working standard; it was prepared by hydrolysis of 1 mL TEP stock solution in 50 mL 1% sulfuric acid and incubation for 2 h at room temperature. The resulting MDA standard of 20 nmol/mL was further diluted with 1% sulfuric acid to yield the final concentrations of 0.5, 1, 1.5, 2, 4, 6 and 10 nmol/mL. These solutions were employed to produce the standard curve for the estimation of total MDA.

Measurement of Mitochondrial Membrane Potential ($\Delta\psi_m$) (FACS). The mitochondrial membrane potential was evaluated by staining the cells with TMRM, a potentiometric membrane dyes redistribute across cell membranes according to the Nernst equation in a voltage dependent manner.[15] In normally functioning TMRM stained cells, the intensity of the fluorescent signal is high and it is localized mainly within the mitochondria and not in the cytoplasm. Mitochondrial depolarization then causes the redistribution of dye from mitochondria into the cytosol, causing a change in signal intensity.

For the determination of $\Delta\psi_m$, CEM leukemia cells were pre-treated with the test compounds overnight. The cells were treated with 5 mM DEM for 2 h, collected by centrifugation at 300 x g for 3 min and then washed with phosphate buffered saline. The cells were resuspended in phosphate buffered saline containing 20 mM glucose and incubated at 37 °C in the dark for 15 min with 250 nM TMRM. Cells were collected by centrifugation at 300 x g for 3 min and then washed twice with phosphate buffered saline. The samples were analyzed immediately by flow cytometry using 488 nm excitation laser and the FL2-H channel. The

results obtained were verified in three independent experiments. The protonophore FCCP (10 μM) was used to dissipate the chemiosmotic proton gradient ($\Delta\mu H^+$) and served as a control.

LIVE/DEAD Viability/Cytotoxicity Assay. Cell death was measured by an ethidium homodimer-1 (EthD-1) /calcein-AM combined fluorescent assay, which determines intracellular esterase activity and plasma membrane integrity.[16,17] This assay employs calcein-AM, a polyanionic dye, which is retained within live cells after de-esterification and produces a green fluorescence. It also employs the ethidium bromide homodimer dye (red fluorescence), which can enter the cells through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membrane of live cells. This assay was used to determine the cytoprotective effect of the test compounds on cell death induced by GSH depletion in primary FRDA patient-derived fibroblasts. FRDA fibroblasts were treated with L-buthionine (*S*, *R*)-sulfoximine (BSO) to inhibit the *de novo* synthesis of GSH[18] resulting in oxidative stress, and cell death.

The test compounds were screened according to a published protocol.[19] This experiment was performed according to the manufacturer's fluorescence microplate reader protocol provided with the kit (Molecular Probes, Eugene, OR). Briefly, fibroblasts were seeded in 96-well microtiter black-walled cell culture plates (Costar, Corning, NY) at a density of 3000 cells per well (100 μL). The plates were incubated at 37 °C overnight in a humidified atmosphere of 5% CO_2 in air to allow attachment of the cells to the culture plate. Serial dilutions of the test compounds were made and added in each well. Plates were incubated at 37 °C overnight in a humidified atmosphere containing 5% CO_2 in air. The following day BSO solution (in culture media) was added to each well to achieve a final BSO concentration of 1 mM. Cell viability was assessed after the first signs of toxicity appeared in BSO-treated cells (typically after 24 h) by

examining cultures under a phase-contrast microscope. The cell culture medium was discarded by and washed with pre-warmed HSSB to remove any traces of serum esterase activity. Cells were stained with the Live/Dead® reagent (4 μM ethidium bromide homodimer, 1.2 μM calcein-AM) and then incubated at 37 °C for 60 min in the dark to allow the dye to enter the cell and be hydrolyzed by esterases. Fluorescence intensities were measured with a Spectramax M5 spectrofluorometer (Molecular Devices, Sunnyvale, CA) using excitation and emission wavelengths of 485 nm and 525 nm, respectively. The test compounds were assayed in triplicate. The viability of non-BSO treated fibroblasts was set as 100%, and the viability of the BSO-treated and sample-treated cells was calculated relative to this value. Cell viability was expressed as the percentage of control. Data are expressed as means ± S.E.M. (n = 3).

Trypan Blue Exclusion Assay. Trypan blue exclusion, a common colorimetric method for assessment of plasma membrane integrity as indication of cell viability, where viable cells exclude trypan blue and non-viable cells take up the dye and stain blue. This technique was used to assess the cytoprotective effects of the tested compounds in cultured leukemia CEM cells treated with DEM to induce cell death by GSH depletion. Briefly, CEM cells were seeded at a density of 5 x10^5 cells/mL and treated with different concentrations of the indicated compounds. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 3 h. After pre-incubation, the cells were treated with 5 mM DEM. Cell viability was determined by staining cells with 0.4% trypan blue using a hemacytometer. At the time of assay, >90% of DEM-treated cells were trypan blue positive; whereas, in non-DEM treated control cell cultures >95% cells were viable. Cell viability was expressed as the percentage of control. Data are expressed as means ± S.E.M. (n = 3).

References

(1) Mordente, A.; Martorana, G. E.; Minotti, G.; Giardina, B. *Chem. Res. Toxicol.* **1998**, *11*, 54–63.

(2) Trounce, I. A.; Kim, L. Y.; Jun, S. A.; Wallace, C. D. *Methods Enzymol*. **1996**, *264*, 484–509.

(3) LeBel, C. P.; Ishiropoulos, H.; and Bondy, S. C. Evaluation of the probe 2′,7′ dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.* **1992**, *5*, 227–231.

(4) Armstrong, J. P.; Yang, H.; Duan, W.; Whiteman, M. Cytochrome bc_1regulates the mitochondrial permeability transition by two distinct pathways. *J. Biol. Chem.* **2004**, *279,* 50420–50428.

(5) Tirmenstein, M. A.; Nicholls-Grzemski, F. A.; Zhang, J. G.; Farriss, M. W. Glutathione depletion and the production of reactive oxygen species in isolated hepatocyte suspensions. *Chem. Biol. Interact.* **2000**, *127,* 201–217.

(6) Bapat, S.; Post, J. A.; Braam, B.; Goldschmeding, R.; Koomans, H. A.; Verkleij, A. J.; Joles, J. A. Visualizing tubular lipid peroxidation in intact renal tissue in hypertensive rats. *J. Am. Soc. Nephrol.* **2002,** *12*, 2990–2996.

(7) Drummen, G. C. P.; van Liebergen, L. C. M.; Op den Kamp, J. A. F.; Post, J. A. C11-BODIPY$^{581/591}$, an oxidation-sensitive fluorescent lipid peroxidation probe: (Micro) spectroscopic characterization and validation of methodology. *Free Rad. Biol. Med.* **2002**, *33*, 473–490.

(8) Trnka, J.; Blaikie, F. H.; Logan, A.; Smith, R. A. J.; Murphy, M. P. Antioxidant

properties of MitoTEMPOL and its hydroxylamine. *Free Rad. Res.* **2009,** *43*, 4–12.

(9) Drummen, G. P. C.; Gadella, B. M.; Post, J. A.; Brouwers, J. F. Mass spectroscopic characterization of the fluorescent lipid peroxidation reporter molecule. *Free Rad. Biol. Med.* **2004**, *36*, 1635–1644.

(10) Borst, J. W.; Visser, N. V.; Kouptsova, O.; Visser, A. J. Oxidation of unsaturated phospholipids in membrane bilayer mixtures is accompanied by membrane fluidity changes. *Biochim. Biophys. Acta.* **2000**, *1487*, 61–73.

(11) Wu, G.-S.; Stein, R. A.; Mead, F. J. Autoxidation of phosphatidylcholine liposomes. *Lipids* **1982**, *17*, 403–413

(12) Mukai, F. H.; Goldstein, B. D. Mutagenicity of malonaldehyde, a decomposition product of peroxidized polyunsaturated fatty acids. *Science* **1976,** *191*, 868–869.

(13) Kelso, G. F; Proteous, C. M; Coulter, C. V; Hughes, G; Proteous, W. K; Ledgerwood, E. C; Smith, R. A. J; Murphy, M. P. Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. *J. Biol. Chem.* **2001***, 276*, 4588–4596.

(14) Frezza, C.; Cipolat, S.; Scorrano, L.Organelle isolation: functional mitochondria from mouse liver, muscle and cultured filroblasts. *Nat. Prot.* **2007**, 2, 287–295.

(15) Ehrenberg, B.; Montana, V.; Wei, M.; Wuskell, J. P.; Loew, L. M. Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. *Biophys. J.* **1988**, *53*, 785–794.

(16) Moore, P. L.; MacCoubrey, I. C.; Haugland, R. P. A rapid pH insensitive, two color fluorescence viability (cytotoxicity) assay. *J. Cell Biol.* **1990**, *111***,** 58a [Abstract No. 304].

(17) MacCoubrey, I. C.; Moore, P. L.; Haugland, R. P. Quantitative fluorescence measurements of cell viability (cytotoxicity) with a multi-well plate scanner. *J. Cell Biol.* **1990**, *111*, 58a [Abstract No. 303].

(18) Griffith, O. W.; Meister, A. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (*S*-n-butyl homocysteine sulfoximine). *J. Biol. Chem.* **1979**, *254*, 7558–7560.

(19) Jauslin, M. L.; Wirth, T.; Meier, T.; Schoumacher, F. A cellular model for Friedreich Ataxia reveals small-molecule glutathione peroxidase mimetics as novel treatment strategy. *Hum. Mol. Genet.* **2002**, *11*, 3055–3063.