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RESEARCH PAPER

Novel Mitochondria-Targeted Antioxidants: Plastoquinone Conjugated with Cationic Plant Alkaloids Berberine and Palmatine

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ABSTRACT

Purpose To develop effective mitochondria-targeted antioxidants composed entirely of natural constituents.

Methods Novel mitochondria-targeted antioxidants were synthesized containing plant electron carrier and antioxidant plastoquinone conjugated by nonyloxycarbonylmethyl residue with berberine or palmatine, penetrating cations of plant origin. These compounds, SkQBerb and SkQPalm, were tested in model planar phospholipid membranes and micelles, liposomes, isolated mitochondria and living cells.

Results SkQBerb and SkQPalm penetrated across planar bilayer phospholipid membrane in their cationic forms and accumulated in mitochondria isolated or in living human cells in culture. Reduced forms of SkQBerb and SkQPalm as well as C10Berb and C10Palm (SkQBerb and SkQPalm analogs lacking plastoquinol moiety) revealed radical scavenging activity in lipid micelles and liposomes, while oxidized forms were inactive. In isolated mitochondria and in living cells, berberine and palmatine moieties were not reduced, so antioxidant activity of C10Berb and C10Palm was not detected. SkQBerb and SkQPalm inhibited lipid peroxidation in isolated mitochondria at nanomolar concentrations; their prooxidant effect was observed at 1,000 times higher concentrations. In human cell cuture, nanomolar SkQBerb and SkQPalm prevented fragmentation of mitochondria and apoptosis induced by exogenous hydrogen peroxide.

Conclusion This is the first successful attempt to construct mitochondria-targeted antioxidants composed entirely of natural components, namely plastoquinone, nonyl, acetyl and berberine or palmatine residues.

KEY WORDS antioxidant · berberine · mitochondria · palmatine · plastoquinone · SkQ

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ABBREVIATIONS

Δψ	transmembrane electric potential difference
AAPH	2,2′-azobis(2-amidinopropane) dihydrochloride
AMVN	2,2′-azodi(2,4′-dimethylvaleronitrile)
BLM	bilayer planar phospholipid membrane
BSA	bovine serum albumin
C10Berb	13-(decyloxycarbonylmethyl)berberine
C10Palm	13-(decyloxycarbonylmethyl)palmatine
C12TPP	dodecyltriphenylphosphonium
CM-DCF-DA	5-(−6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate
FCCP	trifluoromethoxycarbonylcyanide phenylhydrazone
MDA	malondialdehyde
MitoQ	10-(6-ubiquinonyl) decyltriphenylphosphonium
ROS	reactive oxygen species
SkQ	cationic derivative of plastoquinone or methyl plastoquinone
SkQ1	10-(6-plastoquinonyl) decyltriphenylphosphonium
SkQBerb	13-[9- (6-plastoquinonyl) nonyloxycarbonyl-methyl] berberine
SkQPalm	13-[9-(6-plastoquinonyl) nonyloxycarbonyl-methyl] palmatine
SkQR1	10-(6-plastoquinonyl) decylrhodamine 19
TBARS	thiobarbituric acid-reactive substances

INTRODUCTION

Mitochondria are one of the major sources of ROS production in animal cells (1). Mitochondrial ROS and oxidative damage to mitochondria contributes to a wide range of pathologies, including diabetes, cardiovascular disorders and infarction, stroke, and neurodegenerative and other age-related diseases. This is why the mitochondrion is an important target for delivery of antioxidants. Several strategies were suggested to specifically address antioxidants to mitochondria (for review, see (2)). Historically, the first and the most popular approach was based on application of so-called penetrating ions, i.e. charged compounds that can easily penetrate across the mitochondrial membrane due to delocalization of the charge. The increased ionic radius of these compounds effectively lowers the enthalpy associated with desolvating charged species in a lipid environment (the Born energy) and penetration into the hydrophobic regions of the membrane. On entering the cell, penetrating cations will be selectively accumulated within mitochondria since their interior is the only cell compartment negatively charged relative to the cytosol. Since energized mitochondria maintain very high membrane potential across the inner membrane ($\Delta\psi$ about 180 mV), the concentration of penetrating monovalent cation in the mitochondrial matrix will, according to the Nernst equation, be 1,000 times higher than in the cytosol. Synthetic ions penetrating mitochondria were described in 1969 by E.A. Liberman, one of the present authors (V.P.S.), and coworkers (3). A phosphonium derivative was one of the first penetrating cations applied to detect membrane potential in mitochondria and to verify Mitchell's chemiosmotic hypothesis (4). Later we suggested that natural penetrating cations can be used by mitochondria as "molecular electric locomotives" to accumulate uncharged conjugated substances in their matrix (5,6).

In 2001, Murphy and coworkers designed the mitochondria-targeted synthetic antioxidant MitoQ where ubiquinol was conjugated with decyltriphenylphosphonium (7). In 2007, a new mitochondria-targeted antioxidant, SkQ1 (10-(6-plastoquinonyl)decyltriphenylphosphonium), was synthesized and studied by our group. It was shown that substitution of ubiquinol to plastoquinol significantly improved the antioxidant properties of the compound in model membranes as well as in isolated mitochondria, living cells, and organisms (8). SkQ1was found to be a very efficient agent at preventing a variety of degenerative processes and prolonging the lifespan of fungi, invertebrates, and vertebrates in nanomolar doses (9–15). We synthesized and tested several analogs of SkQ1, and in one of them (SkQR1) the phosphonium cation was substituted by the fluorescent cation rhodamine 19. Rhodamines were introduced as selective mitochondrial dyes by L.B. Chen and coworkers (16) and by our group (for review, see (6)) and were widely used for visualization of mitochondria and measurements of the membrane potential of mitochondria and bacteria *in vivo*. We showed that SkQR1 penetrated through lipid membranes (9,17) and selectively accumulated in mitochondria of various cell types *in vitro* (9) and in mitochondria of some tissues *in vivo* (10). The antioxidant and protective effects of SkQR1 were even stronger than those of SkQ1 in some cellular models *in vitro* (9) and especially in models of kidney and brain infarction *in vivo* (10,18).

Neither the alkyltriphenylphosphonium nor rhodamine cations are found in nature, so certain undesirable effects in long-term *in vivo* studies might be expected. This inspired us to search for natural penetrating cations that could be conjugated with plastoquinone. Our attention was attracted by berberine and palmatine, which are isoquinoline alkaloids derived from plants of the *Berberidaceae* family. Extracts containing berberine and palmatine have been used in traditional Chinese medicine for many centuries. Pharmacological studies of berberine revealed its antidiarrheic (19), antiinflammatory (20), antidiabetic (21), antiarrhythmic, and antihypertensive (22) effects. Anticancer activity of berberine mediated by its antiproliferative and antiangiogenic properties was also reported (23,24). Data on possible antioxidant properties of berberine and palma-

tine are controversial. Studies *in vivo* strongly indicated antioxidant effects of berberine (25) or its metabolites (26). Scavenging of radicals by berberine in the micellar model of lipid peroxidation was reported, and this effect was inherent only in its reduced form (27).

Earlier, we analyzed the permeability of planar phospholipid bilayer membrane (BLM) and some natural membranes to berberine and palmatine and found that both compounds penetrate through BLM and bacterial plasma membrane as cations (28). It was suggested that the membrane potential-driven accumulation of these alkaloids in bacteria contributed to their antimicrobial activity. Membrane potential-linked uptake of berberine by isolated mitochondria and by mitochondria in living mammalian cells was demonstrated (24,29), but the possible role of mitochondrial targeting in therapeutic action of berberine remains to be clarified.

In the present work, we report the synthesis of novel mitochondria-targeted antioxidants where plastoquinone is conjugated with berberine or palmatine moieties (SkQBerb and SkQPalm) and also control compounds lacking quinone moieties C10Berb and C10Palm (Fig. 1a). SkQBerb and SkQPalm at nanomolar concentrations were found to inhibit lipid peroxidation in isolated mitochondria and to prevent cell death induced by exogenous hydrogen peroxide. The reduced forms of these compounds revealed radical scavenging activity in liposomes, while the oxidized forms were inactive. In isolated mitochondria and in living cells, berberine and palmatine moieties were not reduced, so the antioxidant activity is due to their plastoquinone moieties.

MATERIALS AND METHODS

The formulas of compounds used in this study and the scheme of their synthesis are shown in Fig.1. All berberine and palmatine analogs were synthesized from berberine or palmatine by the condensation of the cesium salt of 13-(carboxymethyl)tetrahydroberberine (or tetrahydropalmatine) with the appropriate bromide derivatives. SkQBerb was prepared from berberine bisulfate in six steps. Berberine (1) was reduced with sodium borohydride in pyridine (30 min, r.t.), yielding 91% of dihydroberberine (2), followed by enamine alkylation with methyl bromoacetate (1 h, 100°C) producing the 13-substituted iminium salt as an unstable product, which was reduced immediately with sodium borohydride in methanol (30 min, r.t.) to give the 13-substituted tetrahydroberberine (3) with 80% yield. Hydrolysis of (3) with 2% aqueous solution of lithium hydroxide in methanol (1.5 h, reflux) gave 61% of the tetrahydroberberine acid (4) (30). Coupling of the cesium salt of (4) with bromide derivative (5) (48 h, 60°C) then afforded the ester (6), followed by oxidation with N-bromosuccinimide (NBS) to yield 50% of the target compound, which was purified by HPLC. A brief description of the target compounds including NMR spectra are given below.

SkQBerb, 13-[9-(4,5-dimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)nonyloxycarbonyl-methyl]berberine: TLC: R_f (chloroform—methanol, 65:10)=0.10; R_f (chloroform—methanol, 4:1)=0.38, HPLC: τ=7.21 min (40–95% B during 11 min; A: 0.05% TFA, B: 0.05% TFA in MeCN; Luna C18(2)′ 0.46×15 cm, 5 μm, 1 ml/min). UV (ethanol): λmax 262 nm, 350 nm, 430 nm. ESI MS: m/z calculated for $C_{39}H_{44}NO_8$ 654.77; found 654.18. ^{1}H NMR ($CDCl_3$, AV-600, 293 k): 1.38 ppm {10H, m, (4′-8′)-5CH_2}; 1.49 ppm {2H, quint., J=7.44 Hz, 3′-CH_2}; 1.72 ppm {2H, quint., J=7.52 Hz, 2′-CH_2}; 2.02 ppm {3H, s, 3′-CH_3}; 2.03 ppm {3H, s, 4′-CH_3}; 2.40 ppm {2H, t, J=7.74 Hz, 9′-CH_2}; 3.16 ppm {2H, s, 5-CH_2}; 4.09 ppm {3H, s, 19-OCH_3}; 4.21 ppm {3H, s, 9-OCH_3}; 4.29 ppm {2H, t, J=6.77 Hz, 1′-CH_2}; 4.32 ppm {2H, s, 14-CH_2}; 4.92 ppm {2H, s, 6-CH_2}; 6.12 ppm {2H, s, OCH_2O}; 6.50 ppm {1H, s, 1′-CH}; 6.92 ppm {1H, s, 4-CH}; 7.26 ppm {1H, s, 1-CH}; 7.76 ppm {1H, d, J=9,26 Hz, 12-CH}; 7.89 ppm {1H, d, J=9.24 Hz, 11-CH}; 10.02 ppm {1H, s, 8-CH}. ^{13}C NMR ($CDCl_3$, AV-600, 293 k): 12.01 ppm {3″-CH_3}; 12.39 ppm {4″-CH_3}; 27.84 ppm {2′-CH_2}; 28.28 ppm {5-CH_2}; 28.53 ppm {2′-CH_3}; 29.07–29.73 ppm{4′,5′,6′,7′,8′-5CH_2}; 37.07 ppm {14-CH_2}; 56.88 ppm {10-OCH_3}; 57.50 ppm {6-CH_2}; 62.22 ppm {9-OCH_3}; 66.53 ppm {1′-CH_2}; 102.27 ppm {OCH_2O}; 108.58 ppm {4-CH}; 109.31 ppm (1-CH}; 119.31 ppm {12-CH}; 119.73 ppm {4-CH<u>C</u>CH_2-5}; 121.63 ppm {12-CH<u>C</u>CH-13}; 126.28 ppm{11-CH}; 126.39 ppm {13-C}; 132.15 ppm {1″-CH}; 133.56 ppm {1-CH<u>C</u>}; 133.79 ppm {8-CH<u>C</u>C-9}; 137.85 ppm {N<u>C</u>}; 140.57 ppm {4″-C}; 141.07 ppm {3″-C}; 146.00 ppm {9-C}; 146.08 ppm {8-CH}; 147.70 ppm {2-C); 150.36 ppm {10-C}; 150.38 ppm {3-C}; 170.70 ppm {15-C=O}; 187.65 ppm {2′-C=O}; 187.78 ppm {5″-C=O}.

SkQPalm, 13-[9-(4,5-dimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)nonyloxycarbonyl-methyl]palmatine: TLC: R_f (chloroform—methanol, 65:10)=0.10; R_f (chloroform—methanol, 4:1)=0.40. HPLC: τ=7.11 min (40–95% B during 11 min; A: 0.05% TFA, B: 0.05% TFA in MeCN; Luna C18(2)′ 0.46×15 cm, 5 μm, 1 ml/min). UV (ethanol): λmax 262 nm, 350 nm, 430 nm. ESI MS: m/z calculated for $C_{40}H_{48}NO_8$670.34; found 670.41. ^{1}H NMR ($CDCl_3$, AV-600, 293 k): 1.32 ppm {10H, m, (4′-8′)-5CH_2}; 1.49 ppm {2H, quint., J=7.36 Hz, 3′-CH_2}; 1.72 ppm {2H, quint., J=7.32 Hz, 2′-CH_2}; 2.03 ppm {3H, s, 3″-CH_3}; 2.04 ppm {3H, s, 4″-CH_3}; 2.41 ppm {2H, t, J=7.36 Hz, 9′-CH_2}; 3.21 ppm {2H, s, 5-CH_2}; 3.87 ppm

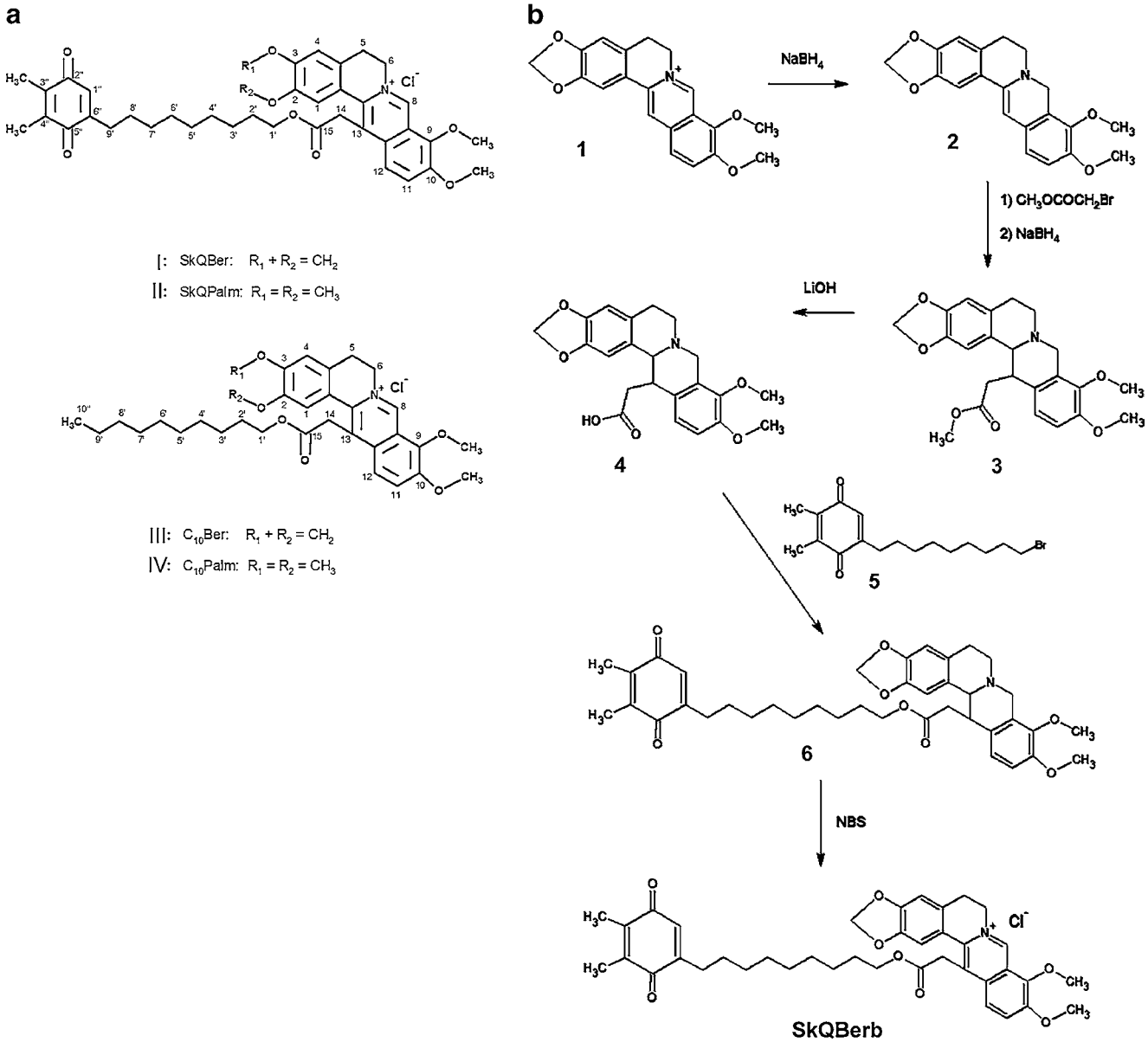

Fig. 1 Formulas of SkQBerb (I), SkQPalm (II), C10Berb (III), C10Palm (IV) (**a**) and the scheme of synthesis of SkQBerb (**b**). The same scheme was used for synthesis of C10Berb, SkQPalm, and C10Palm.

{3H, s, 2-OCH_3}; 4.02 ppm {3H, s, 3-OCH_3}; 4.09 ppm {3H, s, 10-OCH_3}; 4.24 ppm {3H, s, 9-OCH_3}; 4.27 ppm {2H, t, J=6.87 Hz, 1′-CH_2}; 4.36 ppm {2H, s, 14-CH_2}; 5.32 ppm {2H, s, 6-CH_2}; 6.51 ppm {1H, s, 1″-CH}; 6.94 ppm {1H, s, 4-CH}; 7.39 ppm {1H, s, 1-CH}; 7.75 ppm {1H, d, J=9.49 Hz, 12-CH}; 7.88 ppm {1H, d, J=9.49 Hz, 11-CH}; 10.10 ppm {1H, s, 8-CH}. ^{13}C ($CDCl_3$, AV-600, 293 k): 12.08 ppm {3″-CH_3}; 12.45 ppm {4″-CH_3}; 25.89 ppm {3′-CH_2}; 27.84 ppm {5-CH_2}; 28.58 ppm {2′-CH_3}; 29.06–29.75 ppm{4′,5′,6′,7′,8′-5CH_2}; 37.15 ppm {14-CH_2}; 56.24 ppm {2-OCH_3}; 56.33 ppm {3-OCH_3}; 57.02 ppm {10-OCH_3}; 57.59 ppm {6-CH_2}; 62.37 ppm {9-OCH_3}; 66.53 ppm {1′-CH_2}; 110.80 ppm {4-CH}; 112.40 ppm (1-CH}; 118.66 ppm {12-CH}; 119.13 ppm {4-CHCCH$_2$}; 121.70 ppm {12-CHCCH-13}; 125.71 ppm{11-CH}; 126.29 ppm {13-C}; 132.06 ppm {1″-CH}; 132.12 ppm {8-CHCC-9}; 133.67 ppm {1-CHC}; 138.19 ppm {NC}; 140.57 ppm {4″-C}; 141.07 ppm {3″-C}; 146.19 ppm {9-C}; 146.39 ppm {8-CH}; 149.04 ppm {2-C); 150.26 ppm {10-C}; 151.78 ppm {3-C}; 171.05 ppm {15-C=O}; 187.65 ppm {2″-C=O}; 187.77 ppm {5″-C=O}.

C10Berb, 13-(decyloxycarbonyl-methyl)berberine: TLC, R_f (chloroform—methanol, 9:1)=0.33; R_f (chloroform—methanol, 4:1)=0.62. HPLC: τ=6.56 min (40–95% B during 11 min; A: 0.05% TFA, B: 0.05% TFA in MeCN;

Luna C18(2)′ 0.46×15 cm, 5 μm, 1 ml/min). UV (ethanol): λmax 262 nm, 350 nm, 430 nm. ESI MS: m/z calculated for $C_{32}H_{40}NO_6$534.66; found 534.40. ^{1}H ($CDCl_3$, AV-600, 293 k): 0.88 ppm {3H, t, J=7.26 Hz, 10′-CH_3}; 1.27 ppm {14H, m, (2′-9′)-7CH_2}; 1.72 ppm {2H, quint., J=7.05 Hz, 2′-CH_2}; 3.25 ppm {2H, s, 5-CH_2}; 4.07 ppm {3H, s, 10-OCH_3}; 4.28 ppm {4H, m, (1′,14)-2CH_2}; 4.38 ppm {3H, s, 9-OMe}; 5.31 ppm {2H, s, 6-CH_2}; 6.10 ppm {2H, s, OCH_2O}; 6.91 ppm {1H, s, 4-CH}; 7.25 ppm {1H, s, 1-CH}; 7.73 ppm {1H, d, J=9.39 Hz, 12-CH}; 7.86 ppm {1H, d, J=9.39 Hz, 11-CH}; 10.62 ppm {1H, s, 8-CH}. ^{13}C ($CDCl_3$, AV-600, 293 k): 14.10 ppm {10′-CH_3}; 22.68 ppm {9′-CH_2}; 25.91 ppm {3′-CH_2}; 28.61–29.53 ppm{2′,4′,5′,6′,7′,5-6CH_2}; 31.88 ppm {8′-CH_2}; 37.18 ppm {14-CH_2}; 57.03 ppm {10-OCH_3}; 57.40 ppm {6-CH_2}; 63.17 ppm {9- OCH_3}; 66.50 ppm {1′-OCH_2}; 102.15 ppm {OCH_2O}; 108.70 ppm {4-CH}; 109.16 ppm (1-CH}; 119.38 ppm {12-CH}; 119.83 ppm {4-CHCCH$_2$-5}; 121.84 ppm {12-CHCC-13}; 125.69 ppm{13-C}; 125.89 ppm {11-CH}; 133.47 ppm {9-C}; 134.25 ppm {1-CHC}; 137.61 ppm {8-CHCC-9}; 146.70 ppm {3-C}; 147.43 ppm {8-CH}; 147.52 ppm {NC}; 150.22 ppm {2-C}; 150.75 ppm {10-C); 170.71 ppm {15-C=O}.

C10Palm, 13-(decyloxycarbonyl-methyl)palmatine: TLC, R_f (chloroform—methanol, 9:1)=0.30; R_f (chloroform—methanol, 4:1)=0.60. HPLC: τ=6.45 min (5–95% B during 11 min; A: 0.05% TFA, B: 0.05% TFA in MeCN; Luna C18(2)′ 0.46×15 cm, 5 μm, 1 ml/min). UV (ethanol): λmax 262 nm, 350 nm, 430 nm. ESI MS: m/z calculated for $C_{33}H_{44}NO_6$550.71; found 550.36. ^{1}H ($CDCl_3$, AV-600, 293 k): 0.88 ppm {3H, t, J=7.35 Hz, 10′-CH_3}; 1.27 ppm {14H, m, (2′-9′)-7CH_2}; 1.71 ppm {2H, quint., J=7.80 Hz, 2′-CH_2}; 3.29 ppm {2H, s, 5-CH_2}; 3.85 ppm {3H, s, 2-OCH_3};4.01 ppm {3H, s, 3-OCH_3}; 4.07 ppm {3H, s, 10-OCH_3}; 4.27 ppm {2H, t, J=6.92 Hz 1′-CH_2}; 4.33 ppm {2H, s, 14-CH_2} 4.38 ppm {3H, s, 9- OCH_3}; 5.31 ppm {2H, s, 6-CH_2}; 6.94 ppm {1H, s, 4-CH}; 7.38 ppm {1H, s, 1-CH}; 7.73 ppm {1H, d, J=9.44 Hz, 12-CH}; 7.86 ppm {1H, d, J=9.42 Hz, 11-CH}; 10.62 ppm {1H, s, 8-CH}. ^{13}C ($CDCl_3$, AV-600, 293 k): 14.10 ppm {10′-CH_3}; 22.68 ppm {9′-CH_2}; 25.89 ppm {3′-CH_2}; 28.07–29.51 ppm{2′,4′,5′,6′,7′,5-6CH_2}; 31.86 ppm {8′-CH_2}; 37.19 ppm {14-CH_2}; 56.22 ppm {2-OCH_3}; 56.35 ppm {3-OCH_3}; 57.02 ppm {10-OCH_3}; 57.50 ppm {6-CH_2}; 63.17 ppm {9- OCH_3}; 66.53 ppm {1′-OCH_2}; 110.84 ppm {4-CH}; 112.39 ppm (1-CH}; 118.79 ppm {4-CHCCH$_2$-5}; 119.12 ppm {12-CH}; 121.86 ppm {12-CHCC-13}; 125.15 ppm{13-C}; 125.84 ppm {11-CH}; 132.36 ppm {9-C}; 133.55 ppm {1-CHC}; 138.00 ppm {8-CHCC-9}; 146.71 ppm {3-C}; 147.41 ppm {8-CH}; 148.26 ppm {NC}; 150.60 ppm {2-C}; 151.67 ppm {10-C); 171.06 ppm {15-C=O}.

The length of aliphatic linker in these structures was chosen when their octanol:water distribution coefficient was taken into account. A series of analogs with -$(CH_2)_n$- aliphatic chains, where *n* varied from 4 to 10, was synthesized. Their distribution coefficients were estimated using a method based on correlation of this parameter with retention time in a reversed-phase high performance liquid chromatography (HPLC) column (31). It was previously shown for related compounds (SkQ1 and MitoQ) that this method estimates the coefficient with satisfactory accuracy (32). The distribution coefficient for analogs with -$(CH_2)_9$- chain (SkQBerb and SkQPalm) and the control compounds lacking quinone moiety with -$(CH_2)_{10}$- chain (C10Berb and C10Palm) were of the same order of magnitude as for SkQ1 according to HPLC method. They were equivalent to octanol:water distribution ratio within the range 2,000–3,000:1. Stability of SkQBerb and SkQPalm (as well as SkQ1) in aqueous medium was high in contrast to SkQR1. The similar rates of biotransformation in HeLa cells were observed for all members of SkQ family. These data indicated that the ester bond in SkQBerb and SkQPalm was not attacked in living cell, probably due to high lipophillicity of the compounds. The similar ester bond in SkQR1 also was not cleaved in the cells, and no rhodamine-containing products were detected.

All reagents for synthesis and biochemical studies (if not indicated otherwise) were from Sigma-Aldrich. Phospholipids were from Avanti Polar Lipids. MitoTracker Green, MitoTracker Red, CM-DCF-DA (5-(−6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate), and BODIPY 581/591 C11 were from Molecular Probes. Reagents for cell cultures were from Gibco.

Permeability of Planar Bilayer Phospholipid Membrane (BLM)

A planar bilayer phospholipid membrane was formed on a 0.6-mm aperture in a Teflon septum separating the experimental chamber into two compartments. *Escherichia coli* phospholipids (57% phosphatidylethanolamine, 15% phosphatidylglycerol, 10% cardiolipin, 18% others) were used to form the BLM. The phospholipids were dissolved in decane (final concentration, 2%). The two compartments initially contained equal concentrations of the studied cation (10^{-7} M), and a transmembrane electric potential difference was measured after addition of increasing concentration of the same cation into one of the compartments. Electric parameters were measured with AgCl electrodes and a VA-J-5 electrometer (for details, see (33)).

Oxidation of methyl ester of linoleate in micelles of Triton X-100 was induced by azo-initiator 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH, Polysciences) and followed by measuring O_2 consumption with a

computerized 5300 Oxygen Biological Monitor (Yellow Springs Instruments), using a Clark electrode as an O_2 sensor as described (34,35). Experiments were performed at 37°C in 50 mM phosphate buffer (pH 7.4) containing 20 mM methyl linoleate, 50 mM Triton X-100, and 3 mM AAPH. The tested compounds were reduced by $NaBH_4$ in acidic phosphate buffer (pH 5.0).

Liposomes were prepared by evaporation under a stream of nitrogen of a solution of egg yolk phosphatidylcholine and heart cardiolipin (80%:20%, w:w) in chloroform, followed by hydration with buffer solution containing 100 mM KCl, 10 mM Tris, pH 7.4. The mixture was vortexed, passed through a cycle of freezing and thawing, and extruded through a 0.1-μm pore size Nucleopore polycarbonate membrane, using an Avanti Mini-Extruder. Lipid oxidation was induced by 2,2′-azodi(2,4′-dimethylvaleronitrile) (AMVN, Wako Pure Chemical Industries) and measured via fluorescence of BODIPY 581/591 C_{11} as described previously (36,37). The fluorescence at 594 nm (excitation at 540 nm) was monitored with a Panorama Fluorat 02 spectrofluorimeter (Lumex, Russia). Experiments were carried out using 0.1 mg/ml liposomes, 0.4 μM BODIPY 581/591C_{11}, and 0.25 mM AMVN at 60°C in 100 mM KCl, 10 mM Tris, pH 7.4. The tested compounds were reduced by $NaBH_4$ in aqueous buffer solution at neutral pH.

Mitochondria from rat heart were isolated as in (38) and suspended in medium containing 250 mM sucrose, 10 mM Mops-KOH, pH 7.4, 1 mM EGTA, and 0.1% BSA. Protein concentration of mitochondria was measured with bicinchoninic acid according to the reagent producer's instruction (Pierce), using BSA as a standard. Uptake of SkQBerb and SkQPalm by mitochondria was measured in medium containing 250 mM sucrose, 10 mM HEPES, 1 mM EGTA, 2 mM $MgCl_2$, 2 mM KH_2PO_4, BSA 0,1%, pH 7,4, ("Medium A"), 4 mM glutamate, 1 mM malate, and SkQBerb or SkQPalm (5 μM in both cases) and mitochondria (0.1 mg protein/ml) at 25°C. After incubation, the mitochondria were sedimented, lysed in medium containing 150 mM NaCl, 50 mM Tris (pH 7,4), 0.1% SDS, 0.25% sodium deoxycholate, and 1% Triton X 100 ("RIPA"), and fluorescence (excitation/emission maxima, 350/530 nm) was measured using a FluoroMax-3 spectrofluorimeter (Jobin Yvon Horiba).

Reduction of SkQBerb and SkQPalm (5 μM in both cases) in mitochondria was measured after 30 min incubation in "Medium A" containing 5 mM succinate and 1 μM rotenone. In some assays, 5 mM KCN was added. After incubation, the mitochondria were sedimented and lysed in medium "RIPA," and fluorescence spectra (excitation at 350 nm) were measured using the FluoroMax-3 spectrofluorimeter. The spectra of the reduced compounds were measured after addition of $NaBH_4$ in "RIPA" lysates.

Lipid peroxidation was evaluated by measuring thiobarbituric acid (TBA)-reactive substances (TBARS) as described in (39). Amounts of TBARS (adducts of MDA and TBA) are expressed as nanomoles of malondialdehyde per mg protein. Light absorption was read at 535 and 572 nm with an Aminco DW2000 dual wavelength spectrophotometer. MDA formation was initiated by addition of 10 mM ascorbate and 100 μM $FeSO_4$ to mitochondria oxidizing 4 mM glutamate and 1 mM malate in medium containing 250 mM sucrose, 10 mM Mops-KOH, 0.1 mM EGTA, pH 7.4, and mitochondria (0.05 mg protein/ml).

Production of hydrogen peroxide in mitochondria was measured using Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine; Invitrogen). Resorufin, the fluorescent product of hydrogen peroxide-induced Amplex Red oxidation, was measured by its fluorescence (excitation/emission maxima, 550/595 nm) in the same medium containing 1 U/ml horseradish peroxidase, 4 μM Amplex Red, and mitochondria (0.25 mg protein/ml). The fluorescence was recorded with a Hitachi MPF4 spectrofluorimeter.

Respiration was measured using an Oroboros Oxygraph-2 k in medium containing 250 mM sucrose, 10 mM HEPES, 1 mM EGTA, 2 mM $MgCl_2$, 2 mM KH_2PO_4, BSA 0.1%, pH 7.4, and mitochondria (0.1 mg protein/ml) at 25°C. Additions of 2.5 mM ADP, 4 mM glutamate, and 1 mM malate or 5 mM succinate were made where indicated in the figures.

Cell culture HeLa cells and human subcutaneous diploid fibroblasts (Cell Culture Collection, Institute of Medical Genetics, Russian Academy of Medical Sciences, Moscow) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, streptomycin (100 U/ml), and penicillin (100 U/ml). Fibroblasts between 5 and 10 passages were used for experiments.

Accumulation of the antioxidants was analyzed after incubation of substrate-attached HeLa cells with 5 μM SkQBerb or SkQPalm. Fifty μM verapamil was added to the cells 15 min before SkQBerb or SkQPalm. Cells were detached with trypsin and EDTA, washed, and lysed in the "RIPA" medium, and fluorescence (excitation/emission maxima, ~350/530 nm) was measured using the FluoroMax-3 spectrofluorimeter.

For microscopy, HeLa cells were incubated with 0.5 μM SkQBerb or SkQPalm for 60 min. Two hundred nM MitoTracker Red was added 15 min before the observation. In experiments on fragmentation of mitochondria, the organelles were visualized with MitoTracker Green (200 nM, 15 min) and analyzed with an Axiovert microscope (Carl Zeiss) or with an LSM 510 confocal microscope (Carl Zeiss). A total of 300 cells was counted in each sample.

Cell viability was determined with CellTiter-Blue reagent (Promega). Intensity of fluorescence was measured with a Fluoscan Ascent fluorimeter (Thermo).

RESULTS AND DISCUSSION

Model Lipid Membranes

In the first series of experiments, the novel compounds were tested for their ability to penetrate through planar phospholipid bilayer membrane (BLM). If a cation penetrates through a membrane separating two compartments differing in concentrations of this cation, a 60 mV transmembrane electric potential difference ($\Delta\psi$) should be generated for ten-fold cation gradient according to the Nernst equation. SkQBerb and SkQPalm were shown to generate $\Delta\psi$ of proper direction (compartment with lower cation positive). Within the concentration range from 10^{-7} to 10^{-6} M, SkQBerb and SkQPalm generated almost the same $\Delta\psi$ as SkQ1 (Fig. 2), indicating that permeabilities of BLM for these three compounds were similar. Theoretical (Nernstian) potential was not reached at such low concentrations of the cations. Further increase in the concentrations was limited by poor solubility of SkQBerb and SkQPalm in aqueous solutions.

Antioxidant activity of SkQBerb and SkQPalm was analyzed using lipid micelles as described earlier (40). Peroxidation of the methyl ester of linoleate in micelles of Triton X-100 was induced by the azo-initiator AAPH and

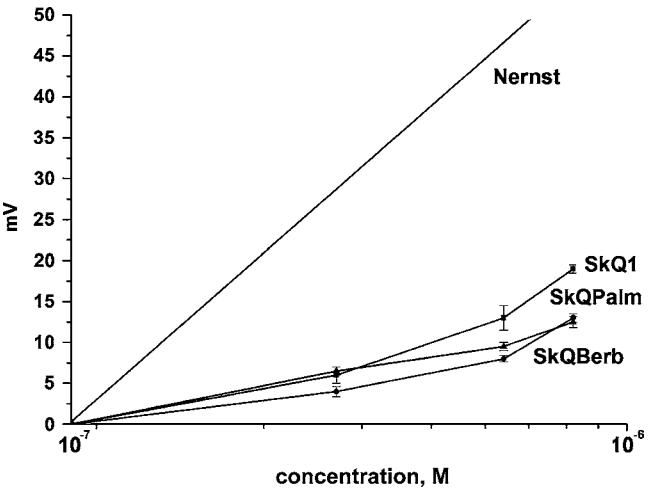

Fig. 2 Generation of transmembrane electric potential difference ($\Delta\psi$) across a BLM. Two compartments of the experimental chamber separated by a BLM (formed on an aperture in a Teflon septum) initially contained equal concentrations (10^{-7} M) of the studied cation: SkQBerb (−●-), SkQPalm (−▲-) and SkQ1 (−■-).$\Delta\psi$ was measured after addition of increasing cation concentration into one compartment. The theoretical Nernstian dependence of $\Delta\psi$ on the gradient of a monovalent cation is shown for comparison.

followed by measuring O_2 consumption. Antioxidant chain-breaking activity of the quinol conjugates was characterized by the rate constant k_I for the reaction between the peroxyl radical of oxygenated linoleate residue ($LO_2^{\bullet}$) and the quinol (QH_2):

$$LO_2^{\bullet} + QH_2 \rightarrow LOOH + QH^{\bullet},$$

which competes with the reaction of chain propagation:

$$LO_2^{\bullet} + LH \rightarrow LOOH + L^{\bullet}.$$

SkQ1, SkQBerb, and SkQPalm were reduced by $NaBH_4$ before the experiment. The addition of the reduced compounds resulted in a strong decrease in the rate of oxygen consumption, which then increased progressively with consumption of the antioxidant. Importantly, the oxidized forms of the compounds were inactive in this system. The absolute values of k_I calculated as described in (34,35) were $1.3 \cdot 10^5$ $M^{-1}s^{-1}$ for SkQPalm and $1.9 \cdot 10^5$ $M^{-1}s^{-1}$ for SkQBerb, while the value for SkQ1 measured earlier was $2.2 \cdot 10^5$ $M^{-1}s^{-1}$. Although the rate constants for the chain-breaking reaction were similar for all three compounds, the periods of inhibition were significantly longer with SkQBerb and SkQPalm, indicating some additional antioxidant activity of these compounds compared with SkQ1. We analyzed reduced C10Berb and C10Palm (lacking the quinol residue) in the same assay and found some chain-breaking activity. Again the oxidized forms of the compounds were almost inactive. These data are in line with the reports on antioxidant activity of berberine and palmatine in various models (25–27,41).

In experiments on isolated mitochondria, it was shown that cardiolipin was critical for initiation of phospholipid peroxidation and that SkQ1 effectively protected mitochondrial cardiolipin against oxidation (42). To compare the antioxidant activity of the novel compounds in cardiolipin-containing membranes, we used liposomes prepared from egg phosphatidylcholine and heart cardiolipin (80%:20%, w:w). Peroxidation was initiated by azo-initiator AMVN and measured using the fluorescent lipophilic probe BODIPY 581/591C11 as described (40). The addition of reduced SkQBerb and SkQPalm prior to AMVN led to concentration-dependent inhibition of BODIPY 581/591C11 oxidation, while the oxidized forms of the compounds were substantially less active (Fig. 3a). It was found that reduced forms of SkQBerb and SkQPalm exhibited stronger antioxidant activity compared to SkQ1 at the same concentration. This suggests that the palmatine and berberine moieties in the molecules possess additional antioxidant activity in this model. In line with this suggestion, it was found that C10Palm reduced by $NaBH_4$ also protected BODIPY $581/591C_{11}$ from oxidation initiated by AMVN (Fig. 3b). Similar data were obtained

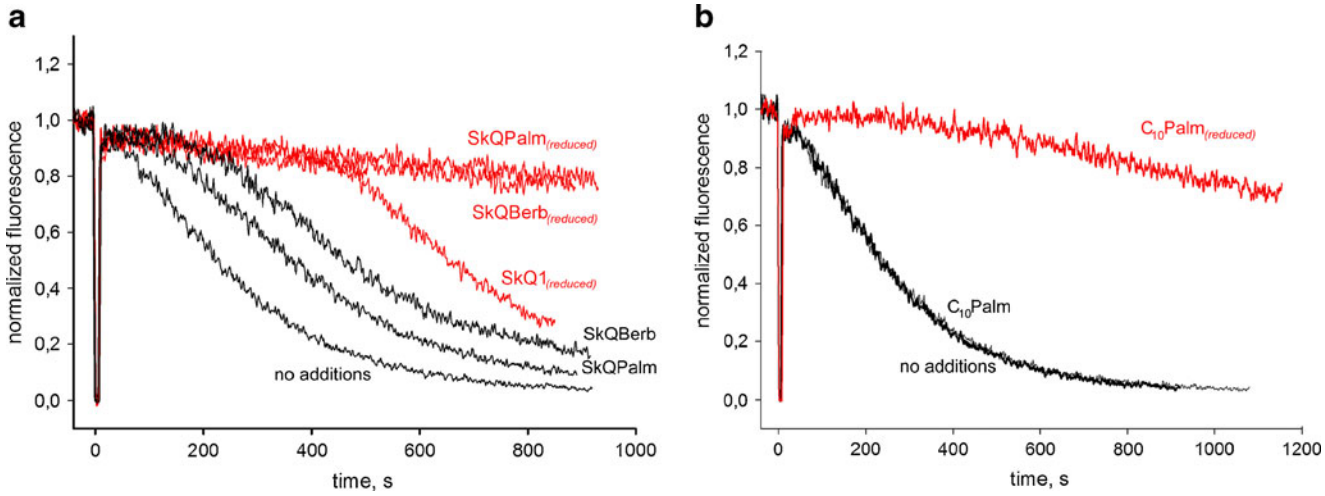

Fig. 3 Antioxidant activity of SkQBerb and SkQPalm (**a**) and C10Palm (**b**) in phosphatidylcholine/cardiolipin liposomes. All the tested compounds were added at 2 μM concentration . Lipid oxidation was induced by azo-initiator AMVN added at zero time and monitored as a decrease in fluorescence of BODIPY 581/591 C_{11}. In certain cases, the tested compounds were pretreated by $NaBH_4$ which reduced the plastoquinone as well as the berberine and palmatine moieties of the studied compounds (*red curves*).

with C10Berb (data not shown). Oxidized forms of C10Berb and C10Palm were ineffective. These results together with the data of experiments with lipid peroxidation in micelles demonstrated high antioxidant activity of SkQBerb and SkQPalm and indicated a contribution of the cationic residues (in reduced form) to this activity.

Isolated Mitochondria

Uptake of SkQBerb and SkQPalm by isolated mitochondria was measured using their fluorescence. It was shown that accumulation of both compounds was completed in 15–20 min. Addition of uncoupler FCCP discharging $\Delta\psi$ inhibited accumulation only partially, consistent with high distribution coefficient of SkQBerb and SkQPalm in membrane:water systems (data not shown). The similar time course of accumulation and the effect of uncoupler were observed earlier for SkQ1 using a delocalized cation-sensitive electrode (9).

It was shown earlier that the plastoquinone residue of SkQ1 is reduced by the mitochondrial respiratory chain (9). The same parameters of plastoquinone reduction were observed for SkQBerb and SkQPalm based on measurements of absorbance at 274 nm (not shown). However, no reduction of the berberine or palmatine residues was observed either with NAD-linked substrates or with succinate. Inhibition of respiration with rotenone (Fig.4) or with KCN (not shown), which shifted redox balance of mitochondria to reduced state, was not able to induce reduction of the berberine or palmatine residues.

To estimate antioxidant activity of SkQBerb and SkQPalm in isolated mitochondria, we measured prevention by these compounds of malondialdehyde (MDA) formation initiated by Fe^{2+} and ascorbate. It was shown earlier that under these conditions cardiolipin was rapidly oxidized, and this effect was strongly lowered by 100 nM SkQ1 (9). The data shown in Fig. 5 demonstrate that SkQBerb and SkQPalm are almost as effective as SkQ1 in prevention of MDA accumulation. Uncoupler significantly decreased the efficiency of SkQBerb, consistent with the data on $\Delta\psi$-dependent accumulation of SkQBerb in mitochondria (Fig. 5). C10Berb and C10Palm, the analogs lacking the quinol residue, were inactive, confirming the inability of the mitochondrial electron transport chain to reduce the palmatine and berberine residues.

We also estimated the prooxidant action of SkQBerb and SkQPalm by measuring H_2O_2 production in mitochondria oxidizing glutamate and malate in State 4. Figure 6 shows that the prooxidant activity became pronounced at micromolar concentration of these compounds (cf. antioxidant effects developing at nanomolar concentrations, see above Fig. 5). Prooxidant activity of SkQBerb and SkQPalm proved to be lower than in the case of SkQ1. To analyze this difference, we addressed the effects of SkQBerb and SkQPalm on respiration of mitochondria. We found that these compounds at 10^{-6} – 10^{-5} M severely inhibited respiration in State 3 when glutamate and malate were used as substrates (Fig. 7a). Oxidation of succinate was much less sensitive. Addition of succinate after almost complete inhibition of glutamate + malate oxidation strongly stimulated oxygen consumption (Fig. 7b), indicating that the inhibitory effect was not related to inhibition of ATP synthase or ATP/ADP transporter. These data are consistent with earlier observations on inhibition of Complex I by berberine (29,43). Taking into account the slow development of the inhibition, we preincubated mitochondria for 20 min with

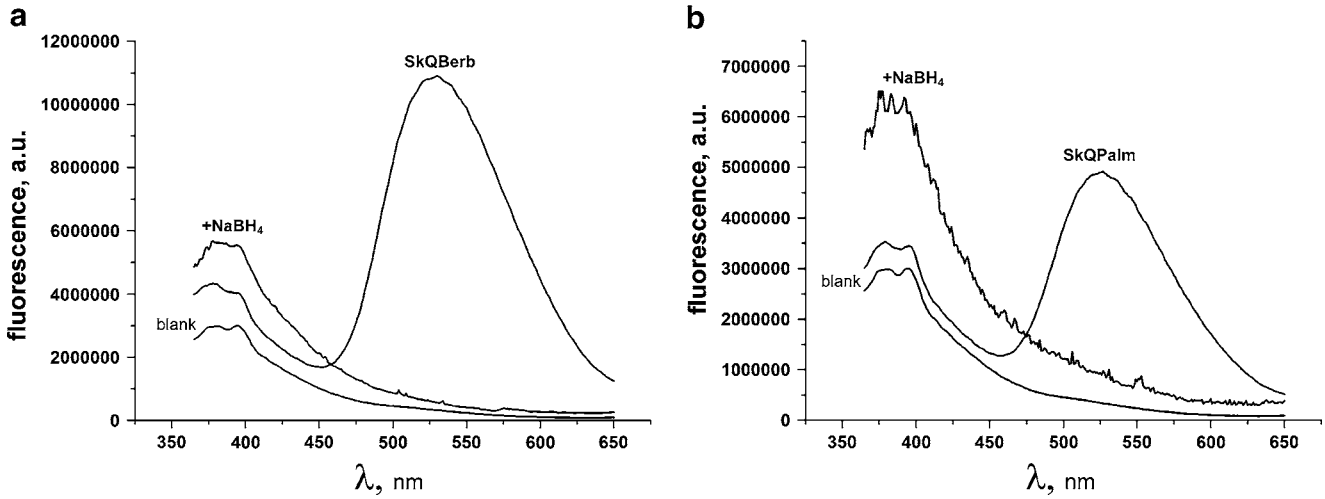

Fig. 4 Alkaloid moieties of SkQBerb (**a**) and SkQPalm (**b**) were not reduced in isolated mitochondria. Mitochondria were incubated for 30 min with 5 μM SkQBerb or SkQPalm in medium containing 5 mM succinate and 1 μM rotenone. Fluorescence spectra (excitation at 350 nm) were measured after solubilization of mitochondria with detergent mixture (curves SkQBerb and SkQPalm). The spectrum of mitochondria without SkQBerb or SkQPalm ("*blank*") and the spectra of the reduced SkQBerb and SkQPalm measured after addition of $NaBH_4$ to solubilized mitochondria are shown.

the tested compounds and measured constant oxygen consumption rates after addition of glutamate + malate and ADP. Half-maximal inhibitory concentrations ($C_{1/2}$) are presented in Table I.

We found that SkQBerb and C10Berb inhibited respiration with similar efficiency, while the palmatine conjugates were slightly less efficient (Table I). The effects of SkQBerb and C10Berb were observed at 5–10 times lower concentrations than that of berberine, probably due to greater hydrophobicity of the former compounds. This conclusion is consistent with the analysis of Complex I inhibition by aliphatic conjugates of berberine (43). It should be mentioned that the inhibitory (as well as the prooxidant) action of SkQBerb and SkQPalm was observed at 1,000 times higher concentrations than their antioxidant effects. The window between anti- and prooxidant concentrations of SkQBerb and SkQPalm was practically the same as for the other members of SkQ family (9) confirming that plastoquinone is the only redox active component of these compounds.

Cell Culture

We studied the interaction of SkQBerb and SkQPalm with HeLa carcinoma cells and human subcutaneous fibroblasts

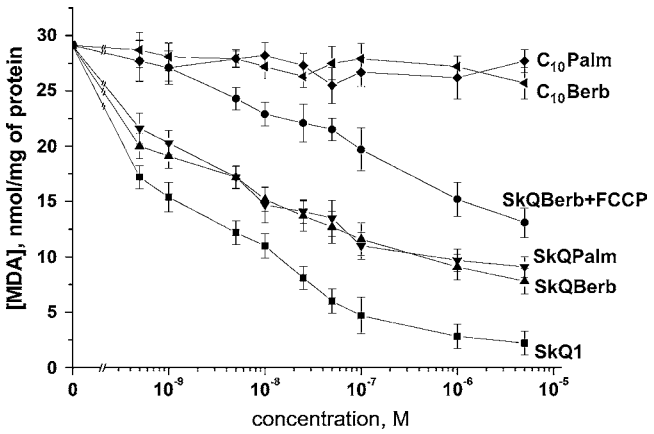

Fig. 5 Antioxidant activity of SkQBerb and SkQPalm in isolated mitochondria. Mitochondria were incubated with antioxidants for 20 min. Then lipid peroxidation was initiated by addition of 10 mM ascorbate and 100 μM $FeSO_4$. Accumulation of TBARS was measured and expressed as nanomoles of malondialdehyde per mg protein. 1 μM FCCP was added where indicated.

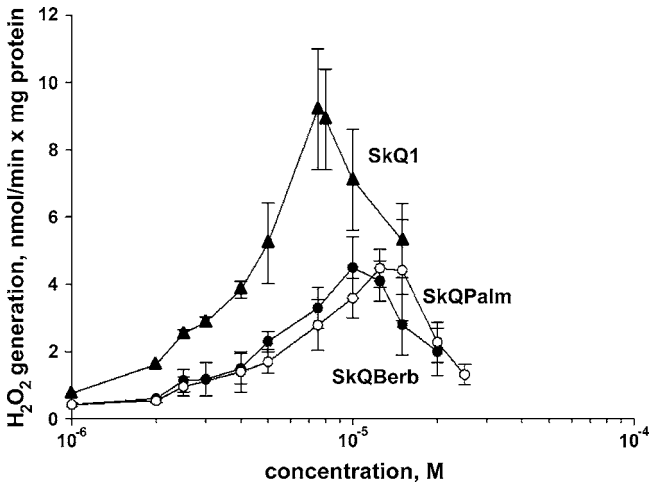

Fig. 6 Prooxidant action of SkQBerb (−●-) and SkQPalm (−○-) in isolated mitochondria. Production of hydrogen peroxide was measured using Amplex Red and horseradish peroxidase in medium containing glutamate (4 mM) and malate (1 mM) as respiratory substrates.

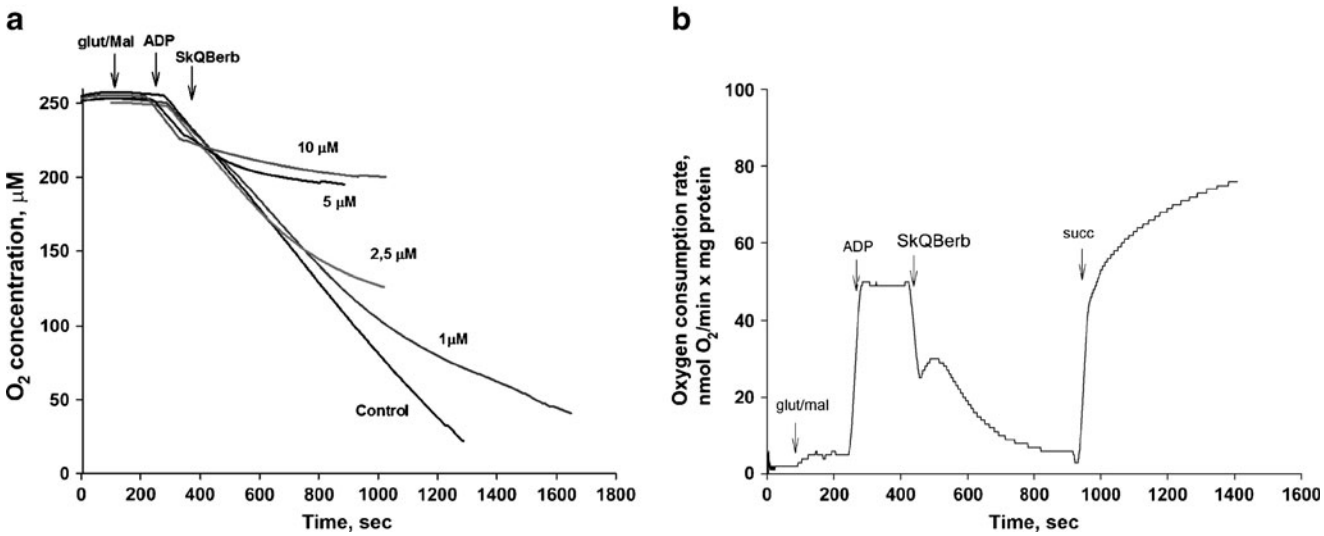

Fig. 7 Effects of SkQBerb on respiration of isolated mitochondria. (**a**) Time course of oxygen consumption in medium containing glutamate (4 mM) and malate (1 mM) as respiratory substrates and ADP (2.5 mM). SkQBerb was added when indicated. (**b**) Rate of respiration recorded by an Oxygraph-2 k (Oroboros) in the same medium as in (**a**). SkQBerb (5 μM) and succinate (5 mM) were added when indicated. For other additions, see (**a**).

in culture. Both compounds were accumulated in HeLa cells during 1–1.5 h as seen by increase in cell fluorescence (for SkQBerb, see Fig. 8a). The fluorescence did not change after accumulation, indicating that the living cell, just as isolated mitochondria, cannot reduce berberine and palmatine moieties of SkQBerb and SkQPalm (data not shown). Removal of SkQBerb and SkQPalm from the medium was followed by slow release of the compounds from the cells. The uncoupler FCCP prevented SkQBerb and SkQPalm accumulation and stimulated their efflux, confirming the membrane potential-driven nature of the accumulation of the cations in intracellular mitochondria. Verapamil, an inhibitor of P-glycoprotein, stimulated accumulation of SkQBerb and SkQPalm in HeLa cells, indicating that these compounds are substrates of the multidrug resistance (MDR) pump. Similar kinetics of accumulation and release of SkQBerb and SkQPalm were observed in human fibroblasts (data not shown). The only difference between HeLa cells and fibroblasts was that in the latter case verapamil was ineffective, which is in line with a low content of P-glycoprotein in fibroblasts in comparison with HeLa cells. Subcellular localization of SkQBerb and SkQPalm coincided with mitochondria stained with MitoTracker Red, indicating high selectivity of the novel mitochondria-targeted antioxidants (for SkQBerb, see Fig. 8b). All these observations closely resemble the behavior of SkQR1 (9,44,45). An important difference was found in parameters of release. The release of SkQR1 from both cell types was incomplete even in the presence of uncoupler, while SkQBerb and SkQPalm were released almost completely. These data might be related to a lower distribution coefficient of SkQBerb and SkQPalm in the octanol:water system as compared to SkQR1. Moreover, it is also possible that some irreversible modifications of cellular components inherent to SkQR1 is absent in the case of the novel antioxidants.

Table I Inhibition of Mitochondrial Respiration by Berberine- and Palmatine-Containing Compounds. Mitochondria were Preincubated for 20 min with the Tested Compounds, and Half-Maximal Inhibitory Concentrations ($C_{1/2}$) were Measured in the Presence of Glutamate, Malate and ADP as in Fig. 7a

Compound	$C_{1/2}$, M
SkQBerb	2.5×10^{-7}
SkQPalm	1.5×10^{-6}
C10Berb	3.5×10^{-7}
C10Palm	6.0×10^{-6}

Our earlier studies using mitochondria-targeted antioxidants demonstrated (9,44,46) that oxidative stress induced by exogenous H_2O_2 results in an excessive production of endogenous ROS in mitochondria. That is why we have exploited this model in the following experiments. One of the earliest consequences of oxidative stress is fragmentation of extended tubular mitochondria into small spherical ones (the "thread–grain transition" (47)). It was shown earlier that incubation with SkQ1 at nanomolar concentrations for 2 h prevents the H_2O_2-induced fragmentation of mitochondria even while the level of intracellular ROS (measured with (5-(−6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, CM-DCF-DA) remains unaffected (44). This indicates that mitochondrial fragmentation is a result of oxidation of some components of mitochondrial membrane initiated by ROS produced inside mitochon-

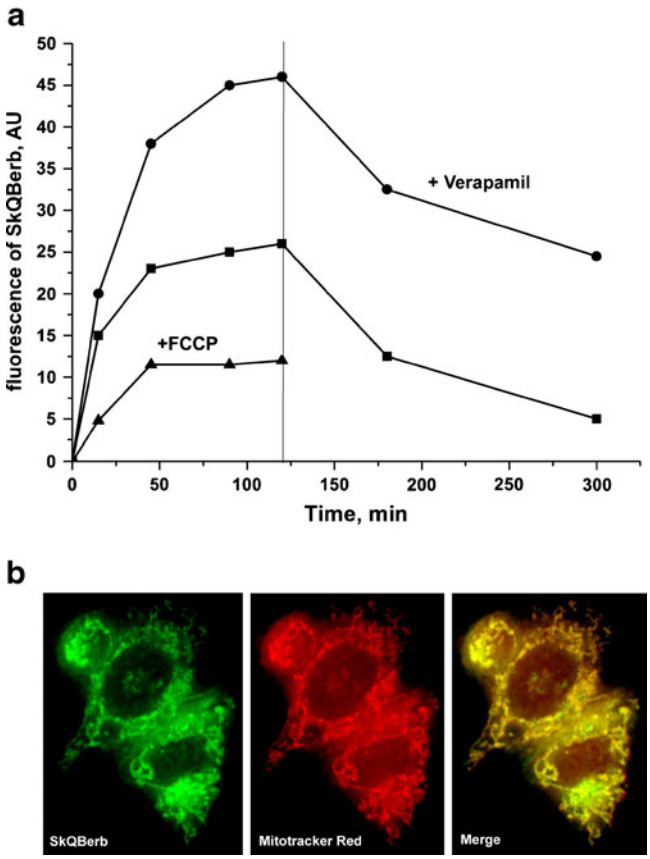

Fig. 8 Accumulation of SkQBerb in cells. (**a**) HeLa cells were incubated with 5 μM SkQBerb . 50 μM verapamil and 10 μM FCCP were added to the cells 15 min before SkQBerb. After 2 h, the medium was changed to the similar one but without SkQBerb (indicated with *vertical line*). Fluorescence (excitation/emission maxima, ~350/530 nm) was measured after detachment, washing, and lysis of the cells with detergent mixture. (**b**) HeLa cells were incubated with 0.5 μM SkQBerb for 60 min and 200 nM MitoTracker Red was added, incubated for 15 min, and analyzed with an Axiovert microscope (Carl Zeiss) equipped with a Neofluar 100 × NA 1.3 objective.

dria. These observations were confirmed with SkQBerb and SkQPalm (Fig. 9). The control compounds C10Berb and C10Palm were ineffective in protection of mitochondrial structure, indicating that the nanomolar concentrations oxidized forms of berberine and palmatine moieties of SkQBerb and SkQPalm do not have detectable antioxidant properties in living cells as well as in isolated mitochondria and model membranes.

In contrast to protection of mitochondrial structure, prevention of fibroblast cell death induced by exogenous H_2O_2 required prolonged (24 h) preincubation with SkQ1, and the protective effect was correlated with the decrease in intracellular ROS accumulation (9,44). Prevention of the cell death by SkQBerb and SkQPalm also required 24 h preincubation, and these antioxidants (as well as SkQ1) were effective at nanomolar concentrations (Fig. 10). Again, C10Berb and C10Palm were ineffective in protection of the cells. The possible explanation of delayed antioxidant and

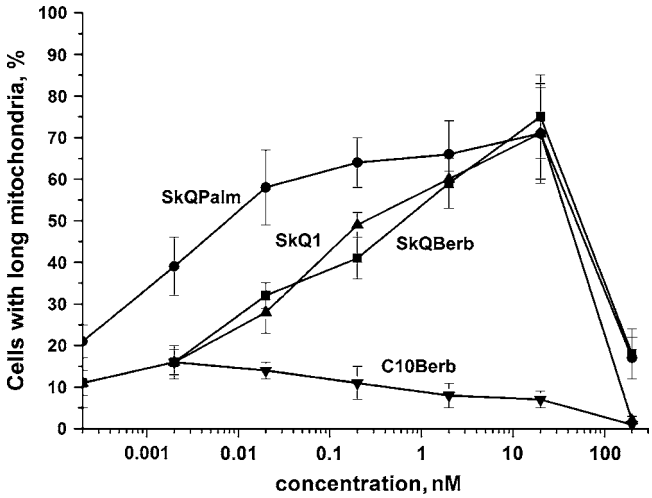

Fig. 9 SkQBerb and SkQPalm prevented fragmentation of mitochondria induced by H_2O_2. Fibroblasts were incubated with antioxidants for 2 h and treated with 400 μM H_2O_2 for 3 h. Mitochondria were visualized with MitoTracker Green, and cells with elongated mitochondria were counted.

protective effects of mitochondria-targeted antioxidants suggests slow redistribution of this compound in the population of mitochondria in the cell. It was shown in various cell types, including fibroblasts, that the cells contain a fraction of mitochondria with low membrane potential that would accumulate cationic antioxidants only slowly (46). These same "impaired" mitochondria probably produced the major part of ROS under oxidative stress (as observed in other cell models (48,49). Apparently, just these ROS determine the fate of the cell. An alternate explanation is that slow development of the effects of mitochondria-targeted antioxidants could be related to induction of endogenous antioxidant systems in the cell.

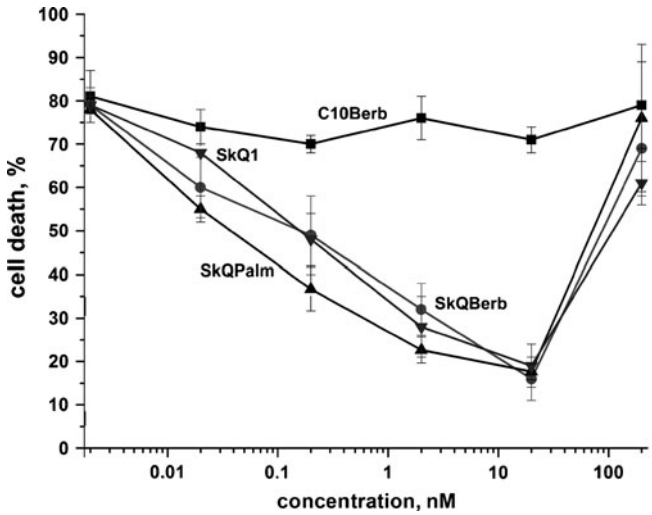

Fig. 10 SkQBerb and SkQPalm protected cells against H_2O_2-induced damage. Fibroblasts were incubated with the antioxidants for 24 h and treated with 400 μM H_2O_2 for the next 24 h. Cell viability was determined with CellTiter-Blue reagent (Promega).

However, at nanomolar concentrations of SkQBerb and SkQPalm, we did not detect any sign of prooxidant action of these compounds, which might be a stimulus for induction. No induction of expression of the major antioxidant enzymes at the level of mRNA was detected (data not shown). Prooxidant activity of SkQBerb and SkQPalm was pronounced at micromolar concentrations, resulting in reversal of the protective action on mitochondrial structure and cell viability under oxidative stress (Figs. 9, and 10).

In conclusion, novel mitochondria-targeted antioxidants where plastoquinone is conjugated with berberine or palmatine moieties (SkQBerb and SkQPalm) were synthesized and tested in various models. Their antioxidant activity in living cells depends on the plastoquinone moieties and is not significantly different from that of conjugates of plastoquinone with synthetic cations triphenylphosphonium (SkQ1) or rhodamine 19 (SkQR1). The isoquinoline plant alkaloids derived from the *Berberidaceae* family have been used in traditional Chinese medicine for a long time and reveal numerous therapeutic effects. The new mitochondria-targeted antioxidants constructed exclusively from natural components could be promising candidates for therapeutic applications.

ACKNOWLEDGMENTS & DISCLOSURES

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