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Mitochondria-Targeted Plastoquinone Derivatives as Tools to Interrupt Execution of the Aging Program. 1. Cationic Plastoquinone Derivatives: Synthesis and *in vitro* Studies*

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Abstract—Synthesis of cationic plastoquinone derivatives (SkQs) containing positively charged phosphonium or rhodamine moieties connected to plastoquinone by decane or pentane linkers is described. It is shown that SkQs (i) easily penetrate through planar, mitochondrial, and outer cell membranes, (ii) at low (nanomolar) concentrations, posses strong antioxidant activity in aqueous solution, BLM, lipid micelles, liposomes, isolated mitochondria, and cells, (iii) at higher (micromolar) concentrations, show pronounced prooxidant activity, the "window" between anti- and prooxidant concentrations being very much larger than for MitoQ, a cationic ubiquinone derivative showing very much lower antioxidant activity and higher prooxidant activity, (iv) are reduced by the respiratory chain to $SkQH_2$, the rate of oxidation of $SkQH_2$ being lower than the rate of SkQ reduction, and (v) prevent oxidation of mitochondrial cardiolipin by $OH^{\bullet}$. In HeLa cells and human fibroblasts, SkQs operate as powerful inhibitors of the ROS-induced apoptosis and necrosis. For the two most active SkQs, namely SkQ1 and SkQR1, $C_{1/2}$ values for inhibition of the H_2O_2-induced apoptosis in fibroblasts appear to be as low as $1 \cdot 10^{-11}$ and $8 \cdot 10^{-13}$ M, respectively. SkQR1, a fluorescent representative of the SkQ family, specifically stains a single type of organelles in the living cell, i.e. energized mitochondria. Such specificity is explained by the fact that it is the mitochondrial matrix that is the only negatively-charged compartment inside the cell. Assuming that the $\Delta\psi$ values on the outer cell and inner mito-

Abbreviations: $\Delta\psi$, transmembrane electric potential difference; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; BLM, bilayer planar phospholipid membrane; BSA, bovine serum albumin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CM-DCF-DA, 5-(-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; C_{12}TPP, dodecyltriphenylphosphonium; DMQ, demethoxy-derivative of CoQ_0 lacking one of the methoxy groups; DPQ, decylplastoquinone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MDA, malondialdehyde; MitoQ, 10-(6′-ubiquinonyl) decyltriphenylphosphonium; NAC, N-acetyl cysteine; PQ, plastoquinone; ROS, reactive oxygen species; SF6846, 3,5-di(*tert*)butyl-4-hydroxybenzylidene malononitrile; SkQ, cationic derivative of plastoquinone or methyl plastoquinone; SkQ1, 10-(6′-plastoquinonyl) decyltriphenylphosphonium; SkQ2, 10-(6′-plastoquinonyl) decylcarnitine; SkQ2M, 10-(6′-plastoquinonyl) decylmethylcarnitine; SkQ3, 10-(6′-methylplastoquinonyl) decyltriphenylphosphonium; SkQ4, 10-(6′-plastoquinonyl) decyltributylammonium; SkQ5, 5-(6′-plastoquinonyl) amyltriphenylphosphonium; SkQR1, 10-(6′-plastoquinonyl) decylrhodamine 19; TMRE, tetramethylrhodamine ethyl ester; TPB, tetraphenylborate; TPP, tetraphenylphosphonium.

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chondrial membranes are about 60 and 180 mV, respectively, and taking into account distribution coefficient of SkQ1 between lipid and water (about 13,000 : 1), the SkQ1 concentration in the inner leaflet of the inner mitochondrial membrane should be $1.3 \cdot 10^8$ times higher than in the extracellular space. This explains the very high efficiency of such compounds in experiments on cell cultures. It is concluded that SkQs are rechargeable, mitochondria-targeted antioxidants of very high efficiency and specificity. Therefore, they might be used to effectively prevent ROS-induced oxidation of lipids and proteins in the inner mitochondrial membrane *in vivo*.

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Reactive oxygen species (ROS) are widely known to be dangerous for cells and organisms. Intramitochondrial ROS are especially damaging because they are produced in the hydrophobic region of the inner mitochondrial membrane, initiating free radical-mediated chain reactions of lipid peroxidation. This decreases the electrical resistance of the membrane (which normally is very high to avoid H^+ leakage and uncoupling of respiration from ATP synthesis). Moreover, oxidative damage to mitochondrial DNA, which is localized in close vicinity of the inner membrane, can occur [1]. Antioxidants conjugated with membrane-penetrating cations can be used to scavenge ROS inside mitochondria. This class of ions was described by our group 40 years ago [2]. The charge of the ionized atoms in such ions is distributed over a large hydrophobic molecule, allowing penetration through membranes in an electric transmembrane potential ($\Delta\psi$)-driven fashion [2-8]. Soon after the discovery of penetrating cations, we suggested that they might be used as "electric locomotives" to accumulate non-charged compounds in mitochondria [7, 9, 10]. Because the mitochondrial matrix is the only intracellular compartment that is negatively charged, a transported compound combined with a positively-charged penetrating cation should be specifically targeted to mitochondria, being accumulated inside the organelles, according to the Nernst equation, by a factor of 1000 when mitochondrial $\Delta\psi$ is 180 mV [6, 7]. Following this reasoning, combining an antioxidant and a penetrating cation might be a way to clean "the dirtiest place of the cell", i.e. the ROS-producing mitochondrial interior.

Recently, the electric locomotive principle was successfully employed by Murphy and Smith to accumulate certain antioxidants inside mitochondria. They synthesized a compound composed of a thiobutyl residue chemically linked to a phosphonium cation [11]. Later, thiobutyl was replaced by α-tocopherol [12] and, finally, by ubiquinol to obtain a rechargeable antioxidant (oxidized forms of ubiquinol, appearing as a result of its antioxidant activity, can be converted back to the original reduced form by the mitochondrial respiratory chain) [13]. It was found that the latter compound, called MitoQ, (i) accumulates inside energized mitochondria in a $\Delta\psi$-dependent fashion, (ii) prevents mitochondrial lipids from being oxidized by $OH^{\cdot}$ *in vitro*, (iii) facilitates survival of an ROS-hypersensitive cell line at concentrations much lower than those of CoQ or α-tocopherol, and (iv) prolongs lifespan of cultivated fibroblasts at high O_2 tension [13-18].

Unfortunately, depending on the amount added, MitoQ can also operate as a prooxidant [14, 19, 20], a feature limiting its practical use *in vivo* in treating, e.g. retinopathies in mice [21] or Friedrich ataxia in humans [22].

The goal of the present work was to find therapeutically useful, rechargeable antioxidants operating without risk of prooxidant side effects. To this end, we decided to use plastoquinone instead of ubiquinone in the cationic antioxidant construct. Plastoquinone operates in the chloroplast electron transfer chain, whereas the mitochondrial electron transfer chain is served by ubiquinone. Why are two different quinones used in the same plant cell? An explanation might be that plastoquinone is a better antioxidant than ubiquinone [23-25]. This feature could be of crucial importance for the oxygen-producing chloroplasts which are exposed to much stronger oxidative stress than oxygen-consuming mitochondria. Moreover, light is likely to produce more singlet oxygen in chloroplasts than in mitochondria because of the much higher light absorbance of these chlorophyll-containing organelles. Acidic pH in the thylakoid interior is also favorable for oxidative stress due to protonation of $O_2^{\cdot-}$ to much more aggressive $HO_2^{\cdot}$.

We synthesized several plastoquinone derivatives combined with various penetrating ions and tested them in model membranes, mitochondria, cells, and organisms. In this paper, we report that cationic plastoquinone derivatives penetrate planar phospholipid membranes, are electrophoretically accumulated in mitochondria, being practically absent in other cellular compartments, and can be reduced by the respiratory chain. These compounds were coined SkQs where Sk is for penetrating cation ("Skulachev ion", a term introduced by David Green [8]) and Q is for quinone. In isolated mitochondria, SkQ1 (a member of the SkQ family) was found to operate as a very potent antioxidant protecting (in the

nanomolar concentration range) cardiolipin against oxidation by $OH^{\cdot}$. Micromolar SkQ1, like MitoQ, showed strong prooxidant effect. However, for mitochondria *in vivo* the "window" between anti- and prooxidant concentrations for SkQ1 fortunately proved to be as wide as about 1000, whereas for its ubiquinone-containing analog MitoQ it was less than 2 under the same conditions. In cell cultures, SkQ1 inhibited H_2O_2-induced apoptosis at very low (picomolar) concentrations. The same concentrations prevented fission of extended mitochondrial filaments to small roundish mitochondria ("the thread → grain transition"), induced by H_2O_2, and stimulated fusion of the majority of the filaments to electrically-united mitochondrial reticulum. (For preliminary communication, see [26].)

MATERIALS AND METHODS

Synthesis of penetrating cations is shown in Fig. 1 and described in Supplementary Information (section 1) presented in the electronic version of this article (http://www.protein.bio.msu.ru/biokhimiya).

All reagents for cell cultures were from Gibco (USA). Mitotracker Red, Mitotracker Green, CM-DCF-DA (5-(-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate), and TMRE (tetramethylrhodamine ethyl ester) were from Molecular Probes (USA). The other reagents (if not indicated otherwise) were from Sigma-Aldrich (USA).

Permeability of bilayer phospholipid membrane (BLM). A bilayer phospholipid membrane was formed on a 0.6-mm aperture in a Teflon septum separating the experimental chamber into two compartments. To form BLM, *Escherichia coli* phospholipids were used (57% phosphatidylethanolamine, 15% phosphatidylglycerol, 10% cardiolipin, 18% others). The phospholipids were dissolved in decane (final concentration, 2%). The membrane separated two compartments containing equal concentrations of the studied cation. Then an additional amount of the cation was put into one compartment. If the cation can easily penetrate through BLM, it diffuses from the compartment of higher concentration to that of lower concentration, generating thereby a transmembrane electric potential difference ($\Delta\psi$), the lower concentration compartment being positively charged. For a monovalent cation, a 10-fold gradient should, according to the Nernst equation, form $\Delta\psi$ of about 60 mV. Electric parameters were measured with AgCl electrodes and a VA-J-5 electrometer (for details, see [27]).

Isolation of mitochondria from rat heart and liver. Mitochondria were isolated as in [28, 29]. Isolated mitochondria were suspended in medium containing 250 mM sucrose, 10 mM Mops-KOH, pH 7.4, 1 mM EGTA, and 0.1% BSA (for heart mitochondria) or 0.3% BSA (for liver mitochondria). Protein concentration of mitochondria (here and elsewhere) was measured with bicinchoninic acid according to the producer's instruction (Pierce, USA) using BSA as a standard protein solution.

Malondialdehyde measurement. Lipid peroxidation levels of the mitochondrial membranes were evaluated by measuring thiobarbituric acid (TBA)-reactive substances (TBARS) by the method of Yagi [30] as modified by Biesalski [31]. Amounts of TBARS (adducts of MDA and TBA) were expressed as nanomoles of malondialdehyde per mg protein. Light absorption was read at 535 and 572 nm on an Aminco DW2000 dual wavelength spectrophotometer.

Measurement of hydrogen peroxide. The Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; Invitrogen, USA) was used to detect the release of H_2O_2 from mitochondria. Resorufin, the fluorescent product of hydrogen peroxide-induced Amplex Red oxidation, was measured by monitoring its fluorescence (excitation/emission maxima, ~550/595 nm). Mitochondrial suspension was added to a 1 ml plastic cuvette to be diluted by isolation medium without BSA (see above) down to protein concentration of 0.25 mg/ml. Then horseradish peroxidase (final content 1 U/ml) and 4 μl 10 mM Amplex Red reagent solution in dimethylsulfoxide were added. The fluorescence was recorded with a Hitachi MPF4 spectrofluorimeter (Japan).

Mitochondrial membrane potential measurement. Safranin O was used as a membrane potential probe [32]. The 555- to 523-nm light absorption was measured with an Aminco DW-2000 spectrophotometer (dual-wavelength regime). The medium for measurement contained 250 mM sucrose, 10 mM Mops-KOH, pH 7.4, 0.1 mM EGTA, 5 mM succinate, 2 μM rotenone, and 15 μM safranin O. Mitochondrial protein concentration was 0.7 mg/ml.

Cell culture and transfections. HeLa cells and human skin fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, streptomycin (100 U/ml), and penicillin (100 U/ml). HeLa cells were transfected with Mito-YFP construct (Clontech, USA) containing yellow fluorescent protein (YFP) fused with the mitochondrial localization signal of subunit VIII of cytochrome *c* oxidase. Transfections were performed using Lipofectamine 2000 reagents according to the manufacturer's protocol (Invitrogen). The transfected cells were transferred to DMEM containing geneticin (500 μg/ml) (Invitrogen).

Visualization of intracellular mitochondria and immunostaining. Mitochondria were visualized with Mitotracker Red or Mitotracker Green (200 nM, 15 min, 37°C). For immunostaining, cells grown on glass cover slips were fixed with 3.7% formaldehyde in phosphate-buffered saline solution (PBS) for 15 min at room temperature and stained with monoclonal antibodies against cytochrome *c* (6H2.B4; BD Pharmingen, USA) or with

anti-BAX polyclonal antibodies (13666E; BD Pharmingen). Images were analyzed with an Axiovert microscope (Carl Zeiss, Germany) and with an LSM 510 confocal microscope (Carl Zeiss). In experiments on fragmentation of mitochondria, 300 cells were counted in each sample.

Photodynamic treatment. HeLa cells were loaded with 200 nM Mitotracker Red for 15 min at 37°C. Then the cells were transferred onto plastic dishes and illuminated by the tungsten lamp of an Axiovert 200 M microscope (Carl Zeiss) using a green filter (bandpass, 545/25 nm) and Neofluor 20× objective, for 1.0-1.5 min to obtain illumination equal to 34.8 J/cm^2. Necrosis was measured 5 h after illumination.

Detection of ROS production. For induction of oxidative stress, cells were treated with H_2O_2 (200 μM, 45 min) and then loaded with 5 μM CM-DCF-DA for 15 min at 37°C. Fluorescence was analyzed using a Partec PAS-III flow cytometer (Partec GmbH, Germany), equipped with a 488-nm argon laser.

Analysis of cell death. Apoptotic cells were stained with Hoechst 33342 (1 μg/ml, 40 min). Five hundred nuclei were counted in each sample [33]. The cells with condensed and fragmented chromatin usually showed staining with Annexin V-Cy3 fluorescent conjugates indicating phosphatidyl serine externalization. On the other hand, they were not stained with propidium iodide, indicating intactness of the plasma membrane. At high concentrations of H_2O_2, a significant fraction of the cells was double stained with Annexin V and propidium iodide due to a secondary necrosis. Necrotic cells in photodynamic treatment experiments were counted after staining with propidium iodide (1 μg/ml, 5 min).

Electron microscopy. HeLa cells were fixed with 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 2 h and then treated with buffered 1% osmium tetroxide for 1.5 h. The sample was then dehydrated with ethanol solutions (50% ethanol, 30 min; 60% ethanol, 30 min, twice; 70% ethanol with 1.5% uranyl acetate, 11 h; 100% ethanol, 30 min; acetone, 30 min). Then the sample was embedded into Epon-812 epoxide resin and 700-800 A slices were prepared using an Ultracat (Leica, Germany) ultramicrotome. Slices were stained with lead. The resulting preparations were investigated and photographed using HU-12 or H-700 electron microscopes (Hitachi).

Measurement of intracellular reduced glutathione. Non-protein thiols (mainly reduced glutathione) were measured after disruption of HeLa cells in PBS with 5% $HClO_4$, 2.5% Triton X-100, and 2.5 mM EDTA. Denatured proteins were separated by centrifugation. Thiols were measured by reduction of 6 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 250 mM sodium phosphate (pH 7.5) and 6 mM EDTA. Protein was measured with the Lowry reagent. Light absorption at 412 nm was estimated with a Hitachi 557 spectrophotometer.

RESULTS

Synthesis of SkQs. The following new compounds composed of plastoquinone or its methylated derivative conjugated with phosphonium, ammonium, rhodamine 19, or methyl carnitine cations via decane or pentane linkers have been synthesized and characterized: 10-(6′-plastoquinonyl) decyltriphenylphosphonium (SkQ1), methylated 10-(6′-plastoquinonyl) decylcarnitine (SkQ2M), 10-(6′-methylplastoquinonyl) decyltriphenylphosphonium (SkQ3), 10-(6′-plastoquinonyl) decyltributylammonium (SkQ4), 5-(6′-plastoquinonyl) amyltriphenylphosphonium (SkQ5), and 10-(plastoquinonyl) decylrhodamine 19 (SkQR1). Moreover, two ubiquinone-containing penetrating cations were synthesized, namely 10-(6′-ubiquinonyl) decyltriphenylphosphonium (MitoQ) and its demethoxy-derivative lacking one of the two methoxy groups (DMQ). As a control compound possessing the cationic and linker constituents but no quinone moiety, dodecyltriphenylphosphonium (C_{12}TPP) was synthesized. For formulas, see Supplementary Information (Figs. S1 and S2)[1] or [26]. The methods of synthesis are shown in Fig. 1 (this article) and Figs. S3-S6. The micelle formation constant of SkQ1 (Fig. S7) and absorption and ESR spectra (Fig. S8) were determined.

Penetration of cationic plastoquinone derivatives through lipid bilayers. First of all, the compounds were tested for their ability to penetrate through hydrophobic membranes. Measurements of electric diffusion potential showed that SkQR1 gradient within the concentration range 10^{-6}-10^{-5} M produced $\Delta\psi$ value close to the Nernstian (10-fold gradient formed $\Delta\psi$ of about 60 mV with "+" sign in the compartment with lower cation concentration). At the same concentrations, SkQ1, SkQ3, and MitoQ produced $\Delta\psi$ of the same sign but lower value than the Nernstian one (Fig. 2). In the case of SkQ2M, SkQ4, and SkQ5, $\Delta\psi$ was significantly lower. These results could be explained by different permeability of the BLM for the studied cations. Direct measurements of the transfer rate of these cations from one lipid monolayer of BLM to the other showed that this rate declines in the series SkQR1 > SkQ1 > SkQ3 > MitoQ [50]. On studying cations with low permeability, there is a danger that a diffusion potential will be shunted by the other ion flows and as a result will drop below theoretically calculated value. Actually, concentrations of cations under study could be elevated, but in this case they all manifest detergent properties thus damaging the BLM. The problem can be solved by replacing the BLM with a thick planar phospholipid membrane. In this case, SkQ1 concentration can be increased to 10^{-4} M without damaging the membrane. Within the $5\cdot10^{-5}$ to $5\cdot10^{-4}$ M concentration

[1] Here and further, for figures designated with "S", see Supplementary Information presented in the electronic version of the article (http://www.protein.bio.msu.ru/biokhimiya).

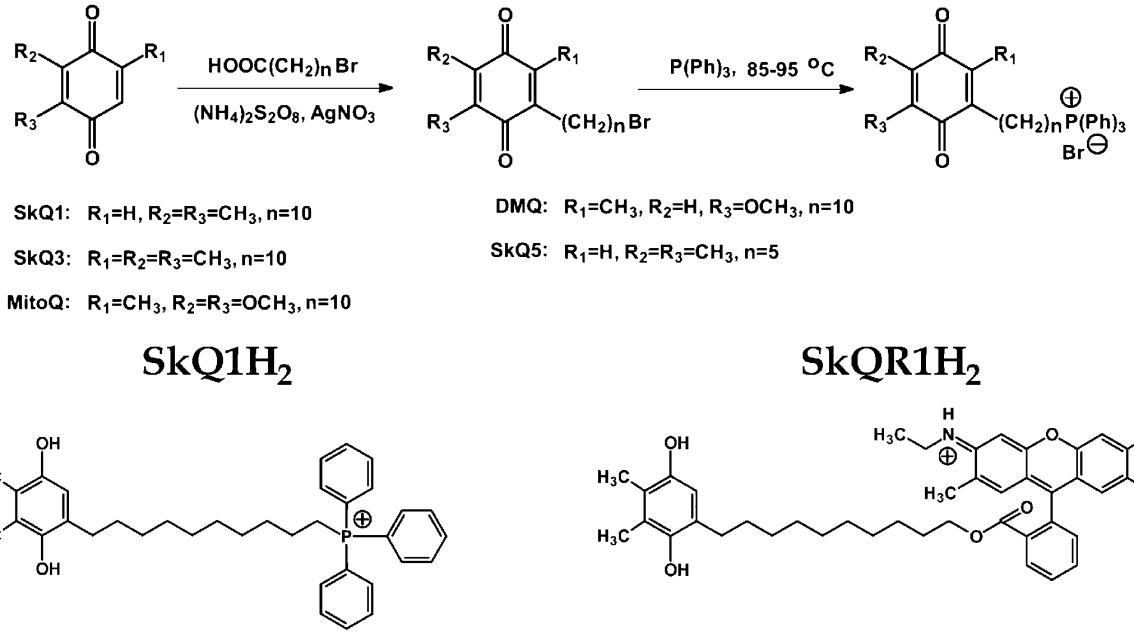

Fig. 1. Principle of synthesis of derivatives of quinone with alkyltriphosphonium as a cationic group.

range, SkQ1 produced $\Delta\psi$ with a value corresponding to the Nernstian (see Fig. S9a in Supplementary Information). Thus, SkQ1, SkQ3, and SkQR1 have the highest membrane permeability among these novel compounds synthesized by us. They were chosen for further experiments.

Antioxidant properties of SkQs in various model systems. Anti- and prooxidant activities of the SkQs were tested in model systems, i.e. aqueous solutions, micelles, liposomes, and planar membranes. To measure the ability of SkQ1 to quench $OH^{\bullet}$ in an aqueous solution, we used its effect on a luminal-mediated chemiluminescence induced by an azo-initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) [34, 35]. It is seen (Fig. 3, a and b) that 0.5 μM $SkQ1H_2$ (reduced form of SkQ1) strongly decreases chemiluminescence, oxidized SkQ1 being much less effective. As for $MitoQH_2$, it showed lower efficiency than $SkQH_2$. MitoQ proved to be completely ineffective.

Prooxidant activities of SkQ1 and MitoQ in an aqueous solution are compared in Figs. 3c and 3d. As a measure of such activities, rate of reduction O_2 to $O_2^{\bullet-}$ was chosen. To this end, formations of quinones from corresponding quinols or $O_2^{\bullet-}$ from O_2 were followed with a spectrophotometer (Fig. 3c) or an ESR spectrometer (Fig. 3d), respectively. Both methods clearly showed much higher prooxidant activity of MitoQ compared to SkQ1.

Antioxidant activity of the studied compounds was also analyzed using lipid micelles. In this case, peroxidation of methyl ester of linoleate in micelles of Triton X-100 was followed by measuring O_2 consumption. This activity of $SkQ1H_2$ and $MitoQH_2$ was characterized by the rate constant k_1 for the reaction between the peroxyl radical of oxygenated linoleate residue ($LO_2^{\bullet}$) and a quinol (QH_2):

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

which competes with the reaction of the chain propagation (rate constant k_2):

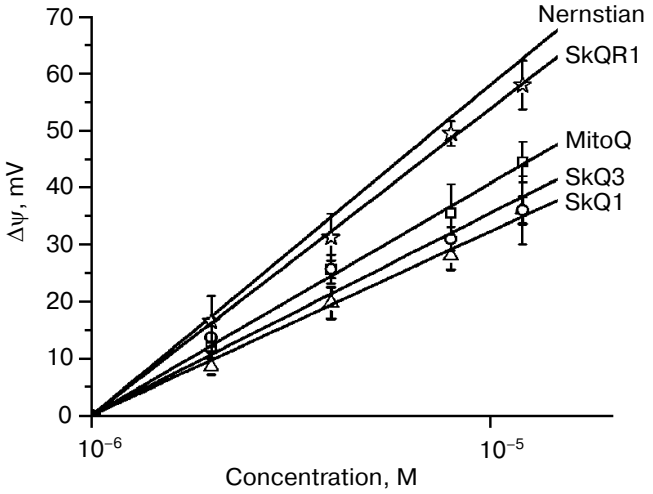

Fig. 2. Generation of diffusion electric potential on planar bilayer phospholipid membranes by SkQ1 and related compounds. In the right compartment, the concentration of the studied compound was 10^{-6} M. In the left compartment, this concentration is indicated by the abscissa. The incubation mixture contained 10 mM Tris-HCl, 10 mM Mes, 10 mM KCl, pH 4.0.

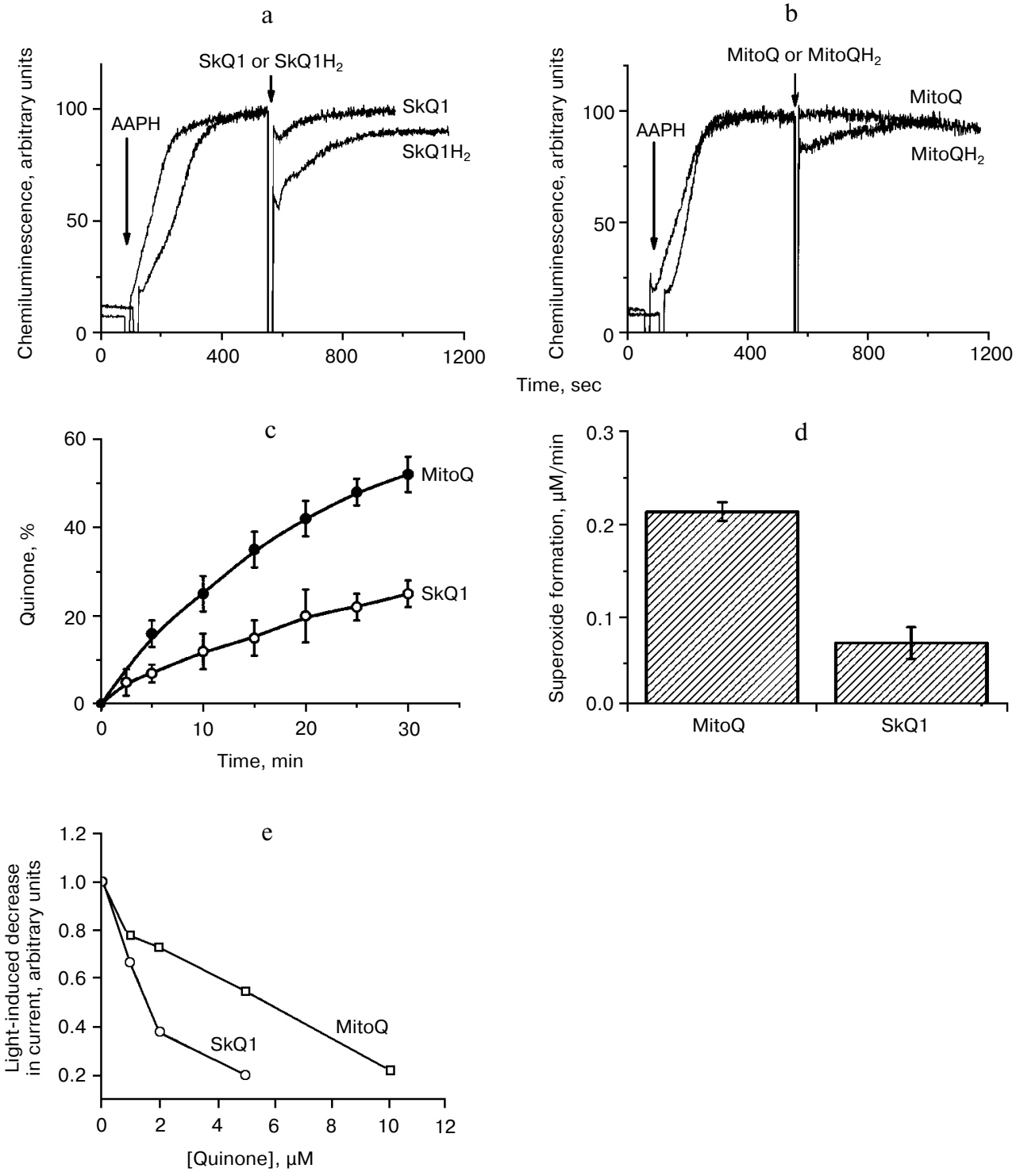

Fig. 3. Antioxidant (a, b, e) and prooxidant (c, d) effects of oxidized and reduced forms of SkQ1 and MitoQ in aqueous solutions (a-d) and BLM (e). a, b) Quenching of the AAPH-induced chemiluminescence of luminal. c, d) Autooxidation of $SkQ1H_2$ and $MitoQH_2$ by O_2: rates of SkQ1 and MitoQ formation from $SkQ1H_2$ and $MitoQH_2$, respectively (c); rates of $O_2^{\cdot-}$ formation (d). SkQ1 and MitoQ were dissolved in ethanol and reduced by $NaBH_4$. Formation of superoxide radicals was measured in 50% ethanol–PBS reaction medium (pH 8.0) using an ESR spectrometer. TIRON (sodium 4,5-dihydrobenzene-1,3-disulfonate) was used as a spin trap. Final concentration of quinones in mixtures was 5 μM. e) SkQ1 and MitoQ defend gramicidin channels in BLM against photoinactivation in the presence of Methylene Blue. The BLM was formed from diphytanoyl phosphatidylserine dissolved in decane. Incubation medium: 100 mM KCl, 10 mM Tris, 10 mM Mes (pH 7.0), 0.5 μM Methylene Blue.

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

The k_1/k_2 value was determined from the kinetic curve for O_2 consumption. The absolute k_1 values were calculated from k_1/k_2 values assuming $k_2 = 60$ $M^{-1}\cdot sec^{-1}$. For more details, see [24, 25].

The values of k_1 for $SkQ1H_2$ and $MitoQH_2$ were found to be $2.2\cdot10^5$ and $0.58\cdot10^5$ $M^{-1}\cdot sec^{-1}$, respectively. Thus, the reactivity of $SkQ1H_2$ towards $LO_2^{\bullet}$ was almost 4 times higher than that of $MitoQH_2$. This is in line with data obtained for simpler analogs of $SkQ1H_2$ and $MitoQH_2$, namely trimethyl-1,4-hydroquinone and 2,3-dimethoxy-5-methyl-1,4-hydroquinone [24, 25]. It should be noted that oxidized forms SkQ1 and MitoQ were inactive in this system (not shown in the figures).

In a further series of experiments, we examined the antioxidant action of SkQ1 and MitoQ in liposomes. First, we used one of the most conventional methods to assess lipid peroxidation, i.e. TBARS assay. Lipid peroxidation in azolectin liposomes was induced by the addition of 100 μM $FeSO_4$, 1 mM ascorbate, and 30 mM *tert*-butylhydroperoxide (tBOOH). Under these conditions, TBARS formation was mainly due to production of malondialdehyde. This process was found to be substantially slower in the presence of 10 μM $SkQ1H_2$ as compared to the control, SkQ1 being much less effective (not shown in the figures).

To directly follow the kinetics of oxidative lipid destruction, we used C11-BODIPY(581/591), a fluorescent lipid peroxidation probe [36, 37]. It was revealed that the addition of 5 μM Fe^{2+} led to a very strong inhibition of the C11-BODIPY fluorescence in azolectin liposomes. The Fe^{2+}-induced fluorescence changes were almost completely prevented in the presence of 0.5 μM $SkQ1H_2$. Oxidized SkQ1 was without any effect (not shown in the figures).

To model membrane protein damage caused by ROS, a new method was developed, i.e. measuring ROS sensitivity of the BLM conductance created by gramicidin D [38]. Tryptophan residues of this channel-forming polypeptide were shown to be attacked by ROS, resulting in the inactivation of the channels. Ascorbate, $FeSO_4$, and *tert*-butyl hydroperoxide were employed as a ROS-generating system. It was shown that the ROS-induced inactivation of the gramicidin-mediated current could be prevented by SkQ1 or MitoQ, with SkQ1 being more effective (Fig. S10). Another version of the gramicidin experiment is shown in Fig. 3e. Here light was used to generate ROS, Methylene Blue being a photosensitizer. It is seen that antioxidant effect of SkQ1 was revealed at lower concentrations than that of MitoQ ($C_{1/2}$ values were 1.8 and 4.5 μM, respectively).

Interaction of SkQs with isolated mitochondria. Uptake of SkQ1 by mitochondria was monitored with a hydrophobic cation-sensitive electrode. Addition of uncoupler discharging $\Delta\psi$ initiated partial release of SkQs to the medium, the effect being larger with SkQ5 than with the more hydrophobic SkQ1 (Fig. S11). This was in line with octanol/water distribution coefficients which were found to be 13,000 : 1 and 500 : 1 for SkQ1 and SkQ5, respectively (cf. 3000 : 1 for MitoQ [14]).

Next, we tested whether SkQ1 and other plastoquinone derivatives could be reduced by the animal mitochondrial respiratory chain. Either NAD-linked substrates or succinate were found to be used as electron donors for SkQ1 reduction (Figs. 4a and S12), which was completely arrested by antimycin A but not myxothiazol (not shown). The relationships can be explained assuming that SkQ1, like plastoquinone [39-42], is reduced by endogenous $CoQH_2$ bound in center *i* of Complex III. Oxidation of $SkQ1H_2$ was found to be enzymatic (by myxothiazol-sensitive complex III of the respiratory chain) or non-enzymatic (by either lipid radicals or O_2). The overall rate of $SkQ1H_2$ oxidation was lower than that of SkQ1 reduction by the respiratory chain (Fig. S12). This means that in respiring mitochondria SkQ1 should be mainly in its reduced state, which is competent in antioxidant activity.

What are the relationships of the anti- and prooxidant effects of SkQ1 and related substances on mitochondria? To estimate antioxidant activity, we used prevention by these compounds of malondialdehyde (MDA) formation initiated by Fe^{2+} and ascorbate in rat heart mitochondria. Figure 4b shows that antioxidant activity of SkQ1 is measurable at much lower concentration than that of MitoQ. The efficiency of the various compounds in inhibiting MDA formation is compared in Table 1. It is seen that in this respect SkQ1 and SkQR1 are the most active, whereas MitoQ is the least active among the quinone-containing compounds bearing a cationic residue. Decylplastoquinone (DPQ) lacking a cation is even less active than MitoQ, whereas $C_{12}TPP$ lacking a quinone residue is quite inactive at the concentrations studied.

Table 1. Concentrations of compounds decreasing twofold the MDA formation in rat heart muscle mitochondria treated with Fe^{2+} and ascorbate

Compound	$C_{1/2}$, nM
SkQ1	25
SkQR1	25
SkQ3	400
DMQ	110
MitoQ	1000
DPQ	2000
$C_{12}TPP$	no effect at 2000

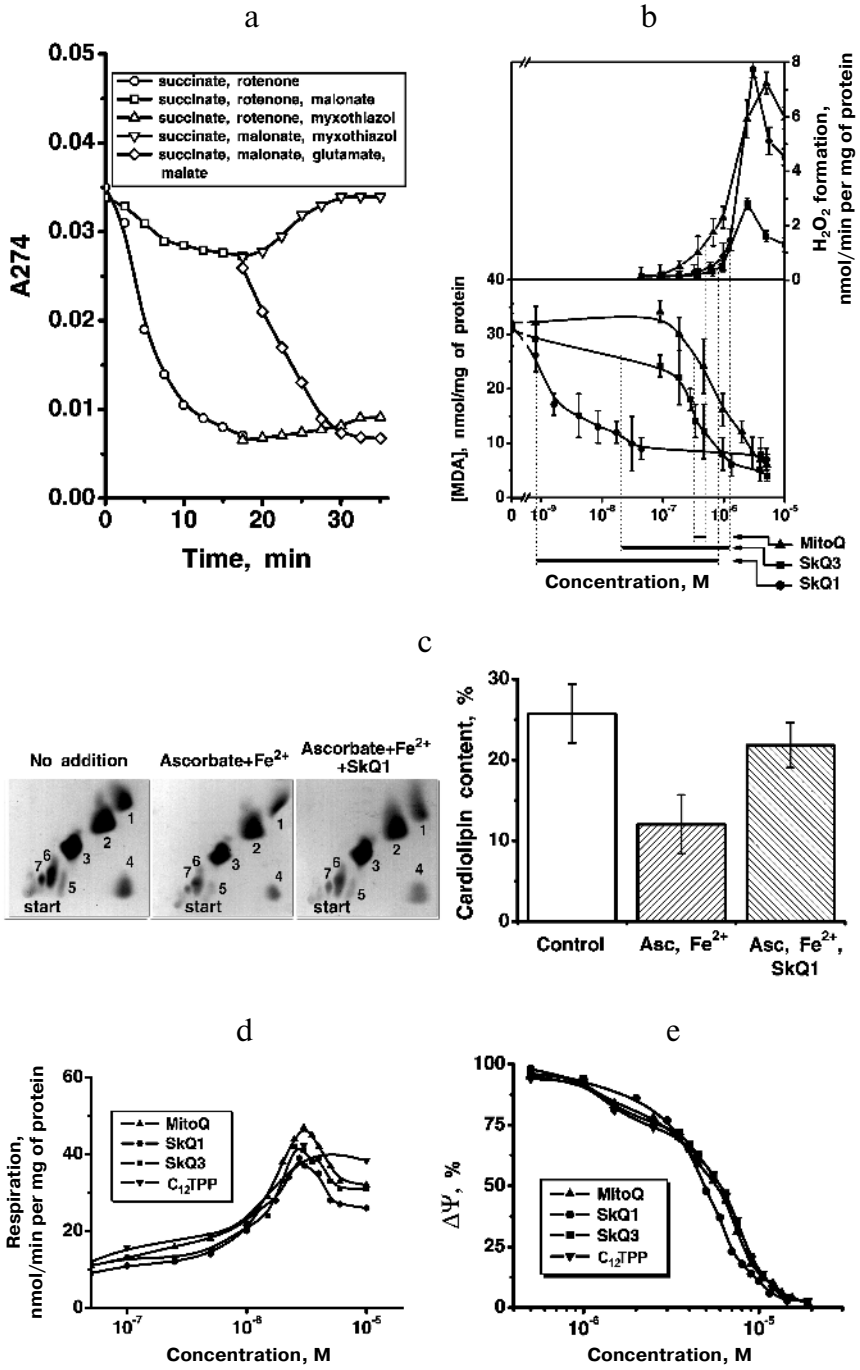

Fig. 4. SkQ effects on isolated rat heart mitochondria. a) Reduction of SkQ1 and oxidation of formed $SkQ1H_2$ by isolated rat heart mitochondria. The reduction and oxidation were measured by decrease and increase in optical density at 274 nm, respectively. Where indicated, 2.5 mM succinate and either 2 μM rotenone or 2.5 mM malonate were added at zero time. After 18-min incubation, 1 μM myxothiazol or 4 mM glutamate and 1 mM malate were added. The medium contained 250 mM sucrose, 10 mM Mops-KOH, 0.1 mM EGTA, pH 7.4, and mitochondria (0.05 mg protein/ml). b) Antioxidant activity of SkQ1, SkQ3, and MitoQ was measured as inhibition of MDA formation initiated by 10 mM ascorbate and 100 μM $FeSO_4$ in mitochondria oxidizing 4 mM glutamate and 1 mM malate (other components as in (a)). Prooxidant activity was estimated as stimulation of H_2O_2 formation under similar conditions (glutamate + malate) but without ascorbate and $FeSO_4$. Line segments below the abscissa show "windows" between concentrations of given quinone derivative causing 20% anti- and 20% prooxidant effects. c) SkQ1 specifically protects mitochondrial cardiolipin from damage by $OH^{\cdot}$. Mitochondria were incubated with ascorbate and $FeSO_4$ as described above in (b). Where indicated, 100 nM SkQ1 was added. Chromatography of mitochondrial lipids on Silica gel-60 plates was performed with chloroform–methanol–water, 65 : 25 : 4 (horizontal) and chloroform–acetone–methanol–acetic acid–water, 6 : 8 : 2 : 2 : 1 (vertical). Lipid spot designations: 1) cardiolipin; 2) phosphatidylethanolamine; 3) phosphatidylcholine; 4) phosphatidic acid; 5) phosphatidylserine; 6) phosphatidylinositol; 7) lysophosphatidylinositol. d, e) Effects of SkQ1, SkQ3, MitoQ, and $C_{12}TPP$ on State 4 respiration (d) and membrane potential (e) of mitochondria. The medium contained succinate and rotenone as indicated in (a).

As further analysis revealed, it is cardiolipin that is first oxidized under conditions of $OH^{\bullet}$ generation by the Fe^{2+} + ascorbate in heart mitochondria. The amount of cardiolipin was strongly decreased after the Fe^{2+} + ascorbate treatment, the effect being almost completely prevented by 100 nM SkQ1 (Fig. 4c). In other experiments, it was shown that SkQ1 and other hydrophobic cations remove N-nonyl acridine orange from complex with phospholipids (presumably with cardiolipin) in rat heart mitochondria, MitoQ being less efficient than SkQ1 and much less efficient than SkQR1 (Table 2; for method see [43]).

As prooxidant action of quinones, we measured stimulation by the same compounds of the H_2O_2 production in mitochondria oxidizing glutamate and malate in the absence of ADP (State 4). Uncouplers as well as respiratory chain inhibitors (rotenone, myxothiazol, and cyanide) strongly lowered the rate of H_2O_2 formation in the presence of micromolar concentrations of cationic quinones. The prooxidant effect was much smaller with C_{12}TPP (Fig. S13, b and c). Prooxidant activity of SkQ1 becomes observable at slightly higher [SkQ1] than [MitoQ]. As a result, the "window" for the pure antioxidant effect not accompanied by a prooxidant action appears to be much larger for SkQ1 (from 0.9 to 850 nM), than for MitoQ (from 350 to 550 nM), SkQ3 occupying an intermediate position (from 25 to 1600 nM).

At concentrations of SkQ1, SkQ3, and MitoQ above 10^{-6} M, the uncoupling effect of these compounds was manifested. In particular, stimulation of respiration rate in State 4 and irreversible decrease in $\Delta\psi$ were observed (Figs. 4d and 4e, respectively). At concentrations above $5 \cdot 10^{-6}$ M, all the quinone derivatives inhibited State 4 respiration (Fig. 4d), which explains some decrease in the rate of H_2O_2 formation (Fig. 4b). Uncoupled respiration was also inhibited by MitoQ, SkQ1, SkQ3, or C_{12}TPP (which may be due to a damaging effect of these hydrophobic cations on respiratory chain enzymes), K_i values being around 2-15 μM (not shown in figures). The concentration range for the SkQ uncoupling effect was at least 1000 times higher than that for their antioxidant activity. For MitoQ, this "window" was again much smaller.

Table 2. Hydrophobic cations compete with N-nonyl acridine orange (NAO) for cardiolipin in rat heart mitochondria. K_a, association constant of NAO with mitochondria in the absence and in the presence of hydrophobic cations

Cation	K_a, M^{-1}
–	$1.2 \cdot 10^6$
SkQ3	$5.5 \cdot 10^6$
MitoQ	$1.0 \cdot 10^5$
SkQ1	$2.5 \cdot 10^5$
C_{12}TPP	$1.0 \cdot 10^4$
SkQR1	$8.0 \cdot 10^4$

Thus, data on lipid micelles, liposomes, BLM, and isolated mitochondria indicate that SkQs are very effective antioxidants. As to their prooxidant, uncoupling, and respiratory enzyme-inhibiting activities, they are revealed at much higher concentrations.

SkQs protect human cells in the culture from ROS-induced apoptosis and necrosis. In HeLa cells, SkQR1, a fluorescent SkQ derivative, was found to be specifically accumulated by mitochondria, showing the same intracellular localization as mitochondria-targeted jellyfish fluorescent protein YFP (Fig. 5; see color insert) or Mitotracker Green (not shown). As shown in Fig. 6a, the SkQR1 staining of mitochondria inside the living cell took about 1 h, and subsequent incubation of the cells in a medium without SkQR1 resulted in slow SkQR1 release ($t_{1/2}$ = 2.5 h). Uncoupler FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) prevented the SkQR1 staining and stimulated its efflux if added to non-stained and stained cells, respectively.

In the further study, we asked whether SkQ derivatives possess antiapoptotic and antinecrotic effects if cell death is induced by ROS. As the experiments showed, SkQs prevent ROS-linked cell death. Especially low concentrations of SkQ1 and SkQR1 were effective when human fibroblasts were pre-treated with SkQs for a week before initiation of apoptosis by H_2O_2 (following the protocol described by Saretzki et al. [16] for MitoQ). In this case, 0.2 nM SkQ1 completely abolished apoptosis of human fibroblasts induced by 400 μM H_2O_2. SkQR1 was more effective and MitoQ much less effective compared to SkQ1 (Fig. 6c). Dissipation of $\Delta\psi$ by uncoupler FCCP prevented the antiapoptotic effect of such a low SkQ1 concentration (Fig. 6b). The protective effect of small amounts of SkQ1 could be overcome by increasing [H_2O_2] to 500 μM (Fig. S14a). Low [SkQ1] prevented development of the main stages of H_2O_2-caused apoptosis, i.e. migration of proapoptotic protein BAX to mitochondria and cytochrome *c* release to cytosol (Fig. S14b). Neither DPQ nor C_{12}TPP at nanomolar concentrations could substitute for SkQ1 (Fig. S14c). Toxic effect of SkQ1 was observed at micromolar concentrations and strengthened in the presence of low H_2O_2 concentrations (which were non-toxic without SkQ1; not shown). This effect is probably due to prooxidant effect of SkQ1 at micromolar concentrations.

Addition of small amount of H_2O_2 to HeLa cells was found to induce a burst in endogenous ROS formation. Earlier we showed that ROS formation under these conditions is determined by the activity of the respiratory chain and stimulated by respiration inhibitors rotenone (Complex I) and myxothiazol (Complex III) [44]. This phenomenon described in our group as a ROS-induced ROS release [45] was completely abolished by pre-treatment of the cells with 20 nM SkQ1 (Fig. 6d).

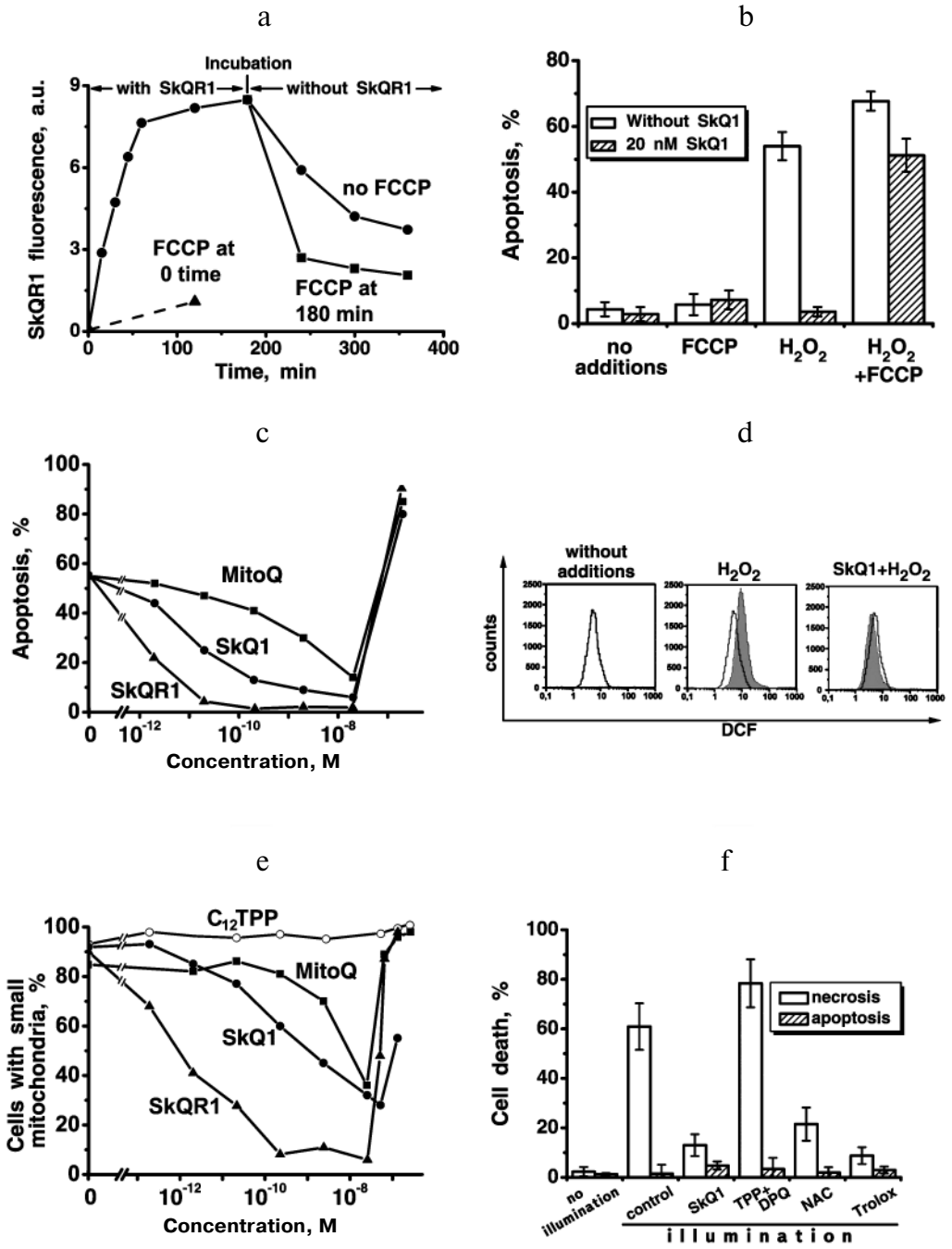

Fig. 6. Effects of SkQ1, SkQR1, and MitoQ on human cell cultures. a) Kinetics of SkQR1 uptake to and release from HeLa cells. At zero time, 50 nM SkQR1 was added to cells. Where indicated, 10 μM FCCP was added. The SkQR1 uptake and release were measured fluorimetrically with a Beckman-Coulter FC500 flow cytometer (USA). b) A 7-day pre-treatment of human fibroblasts with 20 nM SkQ1 arrests apoptosis induced by subsequent treatment with H_2O_2. Apoptotic cells were counted 24 h after adding H_2O_2. Additions: 1 μM FCCP, 400 μM H_2O_2. c) SkQ1 and SkQR1 are more efficient than MitoQ in arresting the H_2O_2-induced apoptosis in human fibroblasts. Conditions as in (b). d) A 7-day pre-treatment of HeLa cells with 20 nM SkQ1 prevents an increase in the intracellular H_2O_2 level induced by 45 min treatment with 200 μM H_2O_2. The cells were stained with 4 μM CM-DCF-DA for 15 min and then analyzed with a flow cytometer. e) Preventive effects of SkQR1, SkQ1, and MitoQ on H_2O_2-induced fission of elongated mitochondria. Human fibroblasts were preincubated with SkQR1, SkQ1, or MitoQ for 2 h and then treated with 400 μM H_2O_2 for 3 h. f) SkQ1 prevents necrosis induced by a photodynamic treatment of HeLa cells. The cells stained for 15 min with 200 nM Mitotracker Red were illuminated for 15 min with green light (545 nm, 34.8 J/cm^2). Necrosis was measured 5 h after illumination. The cells were pre-treated for 1 h with 1 μM SkQ1, mixture of 1 μM TPP (tetraphenylphosphonium) and 1 μM DPQ (decylplastoquinone), 20 mM NAC (N-acetylcysteine), or 1 mM Trolox.

In other experiments, the level of reduced glutathione was measured in HeLa cells (Fig. 7), and it was found to be lowered by adding H_2O_2, which pointed to development of oxidative stress [46]. Glutathione oxidation was prevented by SkQ1, the $C_{1/2}$ being around $2 \cdot 10^{-12}$ M. FCCP prevented the SkQ1 effect (Fig. 7). These data confirm that mitochondria are the origin of endogenous ROS and suggest that it is the ROS production that causes the oxidative stress and apoptosis.

Pre-treatment with very low [SkQ1] or [SkQR1] prevented decomposition of mitochondrial filaments into small mitochondria (the thread–grain transition, an early consequence of the action of apoptogens [47]) (Fig. 8). In this case, preincubation of fibroblasts with the cationic quinones for 2 h was sufficient to observe the protective effect, which increased as follows: MitoQ < SkQ1 < SkQR1. Measurable activity was observed at $2 \cdot 10^{-13}$ M SkQR1. C_{12}TPP was ineffective (Fig. 6e). Such variations in the quinone efficiencies could be explained by different permeability of membranes to various cationic quinone derivatives, by different size of the "antioxidant window", and/or by various affinities of the cations to cardiolipin. An increase in the quinone concentrations abolished the protective action (Fig. 6e), which was most probably due to their pro-oxidant action. This finding was confirmed in experiments on HeLa cells (Fig. S15), which showed that (i) antioxidant Trolox inhibits decomposition of mitochondrial filaments by H_2O_2 in the presence of high concentrations of SkQ1, and (ii) micromolar SkQ1 added without H_2O_2 produces partial decomposition of mitochondrial filaments.

Low concentration SkQ1 without H_2O_2 stimulated formation of the mitochondrial network (Fig. 9; see color insert). Local damage to this network by a very narrow laser beam resulted in $\Delta\psi$ collapse over the entire mitochondrial reticulum. Under the same conditions but without SkQ1, $\Delta\psi$ was found to be discharged in a small part of the cell only. This suggests that the SkQ1-induced decrease in the endogenous ROS level stimulates fusion of the majority of mitochondria into an electrically-united mitochondrial reticulum [48].

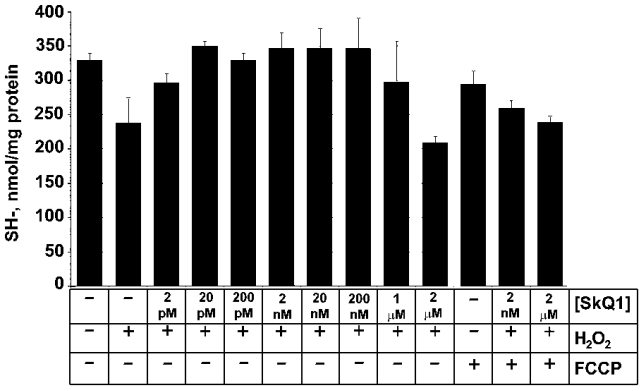

Fig. 7. Effect of SkQ1 on the content of SH-compounds (mainly reduced glutathione) in HeLa cells. The cells were preincubated with or without SkQ1 for 4 h and then treated with 1 mM H_2O_2 for 2 h. Where indicated, 5 μM FCCP was added to the preincubation medium.

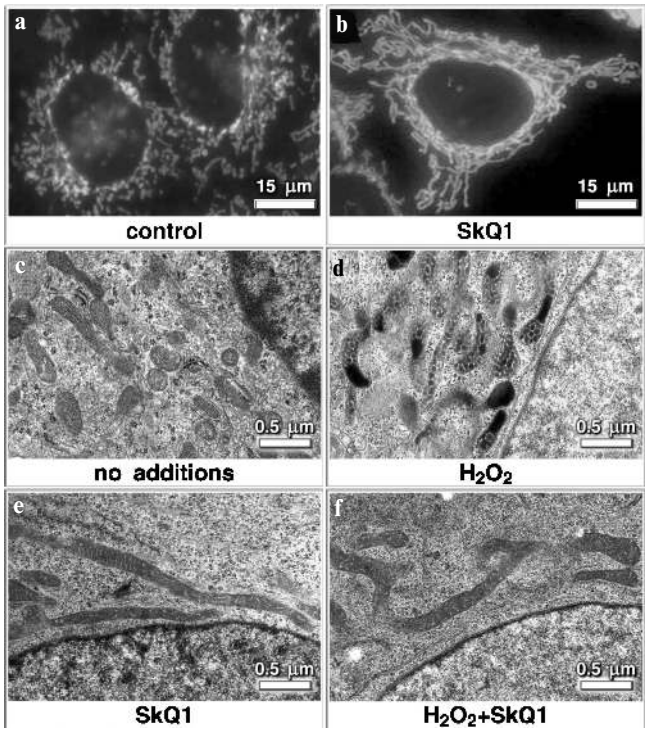

Fig. 8. Effect of SkQ1 on mitochondrial morphology in HeLa cells. a, b, c-f) Confocal and electron microscopy, respectively; a, c, d) without SkQ1; b, e, f) after 7-day treatment with 20 nM SkQ1. After 7-day preincubation, the cells were kept without (a-c, e) or with (d, f) 100 μM H_2O_2. In (a, b) mitochondria were visualized with Mitotracker Green.

In other experiments, pre-treatment of HeLa cell with 1 μM SkQ1 for 1 h arrested necrosis caused by illumination-induced ROS (Fig. 6f) (mitochondria-targeted fluorescent dye Mitotracker Red was used as a photosensitizer). Complete protection against necrosis was observed at 0.5-1.0 μM SkQ1 and 1-h preincubation, and half-maximal effect was achieved at about 200 nM (Fig. S16). Under the same conditions, a mixture of 1 μM TPP and 1 μM DPQ was inefficient, whereas NAC (N-acetylcysteine) and Trolox could substitute for SkQ1 but at much higher concentrations (20 and 1 mM, respectively) (Fig. 6f).

The data of this section suggest that SkQs effectively operate as antioxidants at the cell level, preventing ROS-induced apoptosis and necrosis.

DISCUSSION

Our results confirmed the original suggestion that cationic plastoquinone derivatives (SkQs) are of high

mitochondria-targeted antioxidant activity. This suggestion initially based on some theoretical conjectures is supported by data on model membranes, isolated mitochondria, and human cell cultures. Screening of the synthesized compounds in planar membranes identified those that are readily permeable through lipid barriers. This property was found to be inherent in SkQ1, SkQ3, and SkQR1 (as well as in MitoQ introduced by Murphy and co-workers [13]) (Fig. 2). On the other hand, BLM was of lower permeability for SkQ4, SkQ5, and SkQ2M. This is why SkQ1, SkQ3, and SkQR1 were chosen for further studies. It was found that in BLM inlaid with gramicidin D, SkQ1 protects gramicidin channels from being inactivated by ROS. In water solution and all the model systems studied (methyllinoleate micelles, liposomes, and BLM), antioxidant activity of SkQ1 was significantly higher than that of MitoQ (Figs. 3 and S10).

As experiments on mitochondria showed, SkQ1, like MitoQ, can be reduced by the mitochondrial respiratory chain (Fig. 4a). As to the $SkQ1H_2$ oxidation, it proved to be slower than SkQ1 reduction (Fig. S12), indicating that in mitochondria SkQ1 should be mainly in its reduced form. Thus, SkQ1 can be regarded as a rechargeable antioxidant. Measurable antioxidant effect was observed at [SkQ1] as low as 1 nM. Increase in [SkQ1] to about 1 μM resulted in appearance of its prooxidant activity (Fig. 4b). MitoQ started to operate as antioxidant at much higher concentrations than SkQ1 (0.3 μM), which was only slightly lower than the level when its prooxidant activity becomes measurable (0.5 μM).

It should be stressed that the prooxidant effect of micromolar concentrations of cationic quinone derivatives is very strong. In fact, the rate of H_2O_2 production by mitochondria in the presence of micromolar MitoQ or SkQ1 was much higher than that in State 4 with succinate or in the presence of an uncoupler and antimycin A, i.e. under conditions which are regarded as optimal for mitochondrial ROS production (Figs. 4b and S13, b and c). It is the prooxidant activity of cationic quinones that determines their toxic effect on cells at micromolar concentrations under oxidative stress conditions. Thus, our study revealed a new requirement for a cationic quinone derivative to be used as a mitochondria-targeted antioxidant. It is that the "window" between quinone concentrations causing anti- and pro-oxidant effects must be large enough to minimize risk of enhancing mitochondrial ROS level instead of lowering it. Such a risk is much lower with SkQ1 (antioxidant effect becomes measurable at about 1000 times lower quinone concentration than prooxidant) compared with MitoQ, for which this value is less than 2 times. These relationships should be taken into account in attempts to apply cationic quinones as antioxidants in medicine or biotechnology. (For role of prooxidant activity of quinones and their toxicology, see [49].)

An antioxidant effect of SkQ1 consists in preventing peroxidation of cardiolipin, a phospholipid specific for the inner mitochondrial membrane (Fig. 4c). In fact, it is cardiolipin, which is peroxidized in mitochondria under oxidative stress, whereas other phospholipids remain more or less intact. Such specificity is due to much higher content of polyunsaturated fatty acid residues in cardiolipin than in any other type of mitochondrial phospholipids. Polyunsaturated fatty acids are extremely sensitive to peroxidation; they easily form peroxyl radicals ($LO_2^{\bullet}$) as a result of oxygenation of a methylene group localized between two double bond-containing moieties of the fatty acid hydrocarbon chain (–CH=CH–$\mathbf{CH_2}$–CH=CH–). $SkQ1H_2$ most probably interrupts the chain reaction of lipid peroxidation reacting with $LO_2^{\bullet}$ [50], thereby effectively protecting cardiolipin from oxidation. Complex formation between hydrophobic cationic quinones and cardiolipin (which appeared to be tighter for SkQ1 and particularly for SkQR1 compared to MitoQ) may take part in this process.

There are several reasons why cardiolipin peroxidation appears to be a key event in mitochondrial oxidative stress. First, it is the most sensitive to the ROS-induced peroxidation and its peroxidation initiates a chain reaction "setting on fire" other membrane constituents. Second, oxidized cardiolipin fails to bind cytochrome *c* to the mitochondrial membrane [51]. As a result, cytochrome *c* releases to the mitochondrial intermembrane space and gains cardiolipin-peroxidase activity, initiating a vicious cycle of further cardiolipin peroxidation [52]. Third, the loss of cardiolipin inactivates all the respiratory chain complexes, H^+-ATP-synthase, ATP/ADP-antiporter, etc. and induces an increase in permeability of the inner mitochondrial membrane and, as a consequence, $\Delta\psi$ collapse, swelling of matrix, disruption of the outer mitochondrial membrane, and release of cytochrome *c* and other intermembrane proapoptotic proteins into the cytosol. Thus, cardiolipin oxidation initiates apoptosis [53], and SkQ1 blocking this oxidation can prevent cell death.

In human cell cultures, very low (nanomolar and even picomolar) [SkQ] were found to prevent ROS-induced apoptosis. Even more effectively, plastoquinone derivatives block such an early apoptotic event as fission of elongated mitochondria. In this case, the measurable protective effect is observed at $8\cdot10^{-13}$ M SkQR1 or $1\cdot10^{-11}$ M SkQ1 on intact cells (Figs. 6e and S15). To explain such extremely high efficiency, one should take into account that (i) SkQs are rechargeable antioxidants and (ii) they are specifically accumulated in mitochondria. The accumulation coefficient can be estimated taking into account $\Delta\psi$ on the outer cell membrane (about 60 mV, with negative charging of the cytoplasm) and on mitochondrial membrane (about 180 mV, with negative charging of the matrix), resulting in 10^4-fold SkQ gradient between extracellular medium and mitochondrial matrix. Then, membrane/water distribution coefficient, which is of about $1\cdot10^4$ for SkQ, should be taken into

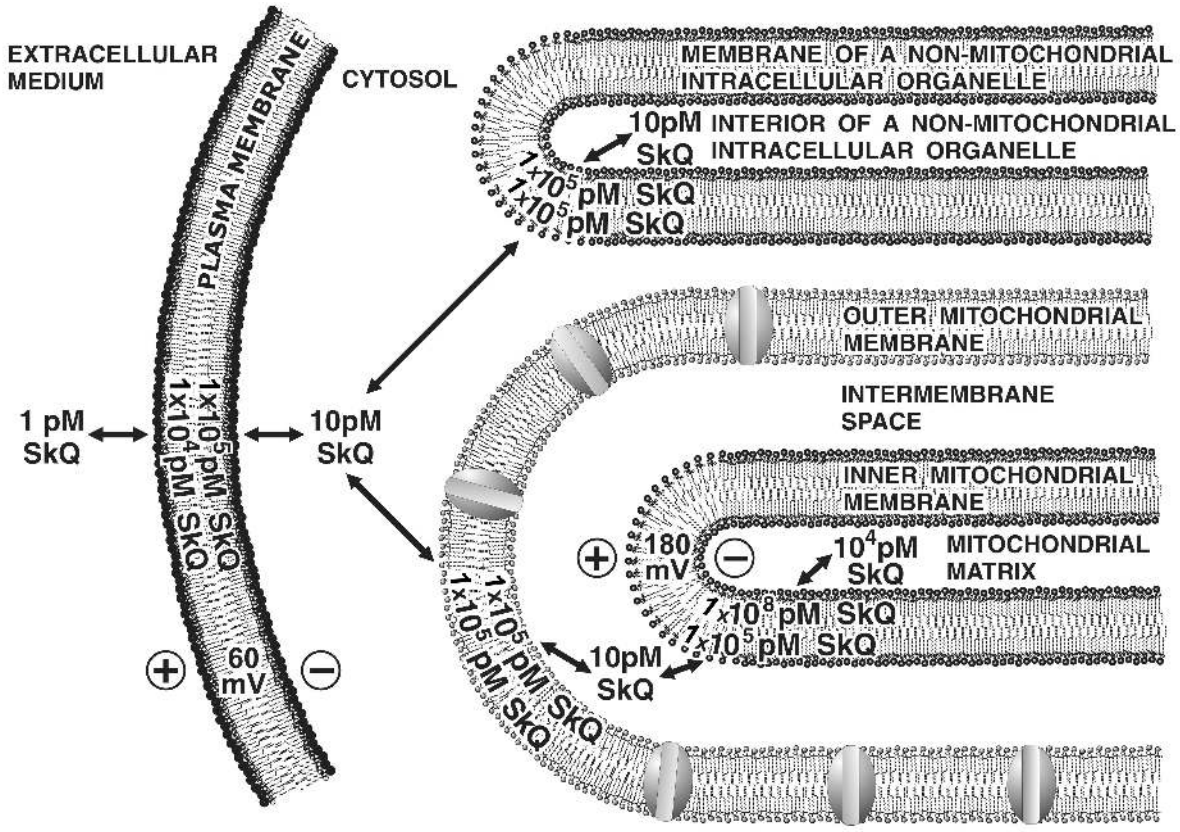

Fig. 10. Scheme illustrating accumulation of SkQ in inner leaflet of the inner mitochondrial membrane. The $\Delta\psi$ values on the plasma membrane and the inner mitochondrial membrane are assumed to be 60 and 180 mV, respectively. Membrane/water distribution coefficient for SkQ is assumed to be 10,000 : 1.

account. Thus, the concentration gradient of SkQ between the inner leaflet of mitochondrial membrane and extracellular medium may reach a value as high as 10^8-fold (Fig. 10).

It is not clear why substitution of phosphonium group by rhodamine as the cationic moiety of SkQ results in an increase in the SkQ efficiency in experiments on cell cultures (see Fig. 6, c and e), in spite of the fact that in isolated mitochondria for SkQ1 and SkQR1 the antioxidant $C_{1/2}$ values are similar (Table 1). As our experiments showed, SkQR1 is a substrate of ABC ATPase, a nonspecific pump of the outer cell membrane ejecting hydrophobic cations and some non-charged compounds of high hydrophobicity (this pump is responsible for the phenomenon known as multidrug resistance). Most probably, SkQ1 can also be expelled from the cell by ABC ATPase. The higher efficiency of SkQR1 than SkQ1 might be accounted for by assuming that SkQ1 is better substrate of ABC ATPase. Such an assumption predicts that inhibition of ABC ATPase should make SkQ1 as efficient as SkQR1. This possibility in now under investigation within the framework of our project.

Effective accumulation of cationic quinones in mitochondria results in that already at micromolar concentrations they demonstrate prooxidant activity and stimulate apoptosis under oxidative stress conditions. In case of MitoQ, the protective effect is replaced by the opposite effect if the quinone concentration is increased slightly. The experiments showed that SkQs are significantly more efficient antioxidants compared to others presently known. Apoptosis caused by oxidative stress seems to play a crucial role in both cellular [54] and organism [55, 56] senescence. The results obtained give hope that SkQs could become an effective remedy for curing senescence pathologies and for extending the healthy period of life.

Results on *in vivo* and *ex vivo* effects of SkQs will be described in accompanying papers [57-60].

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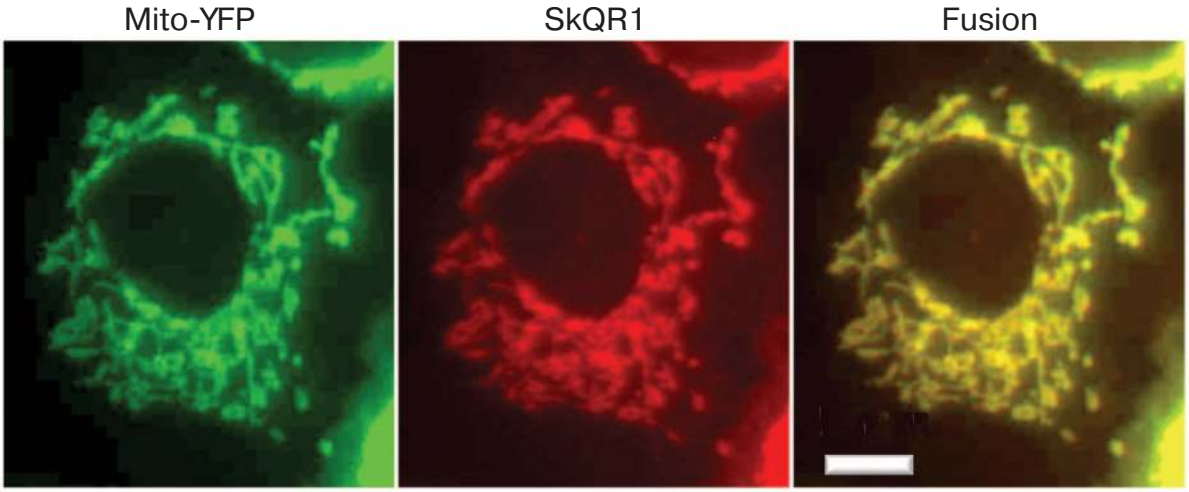

Fig. 5. (Y. N. Antonenko et al.) Co-localization of SkQR1 (a fluorescent SkQ derivative) and mitochondria-targeted jellyfish yellow fluorescent protein YFP fused with the leader sequence of cytochrome oxidase subunit VIII. HeLa cells were transfected with Mito-YFP (Clontech) and incubated for 15 min with 100 nM SkQR1. Bar, 15 μm.

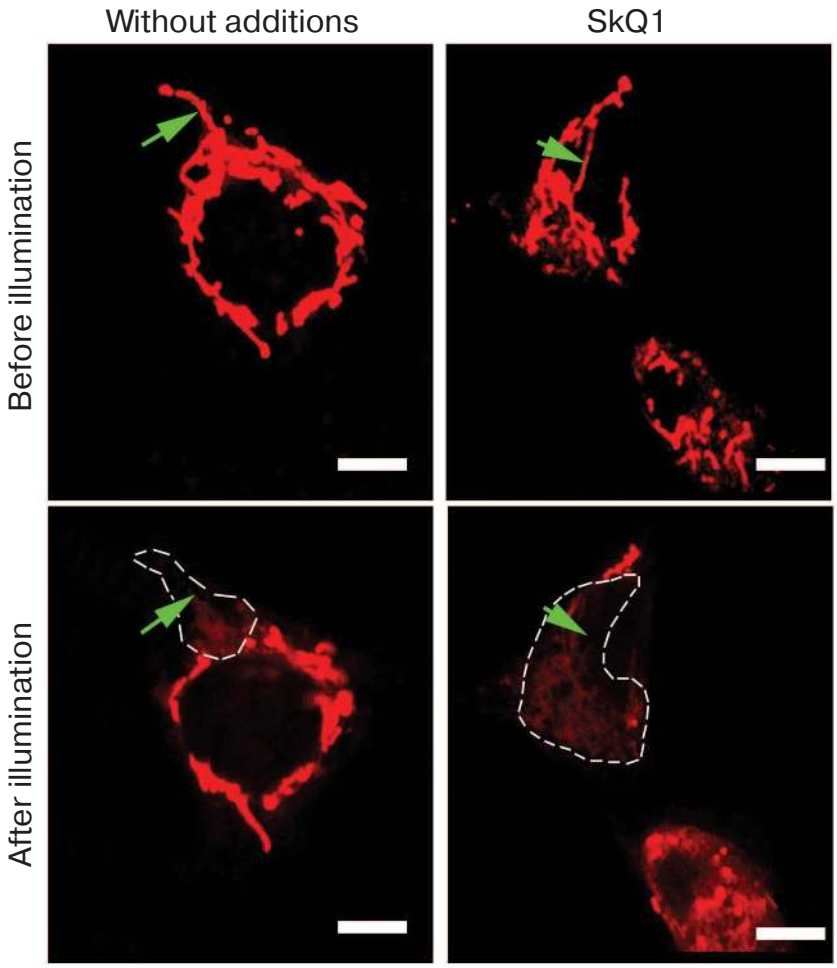

Fig. 9. (Y. N. Antonenko et al.) SkQ1 initiates formation of a united mitochondrial electric network in HeLa cells. Arrow shows the place of illumination of a mitochondrion with a narrow argon laser beam of a Zeiss LSN 510 confocal microscope (488 nm; 50 sec; light spot, 6 × 60 pixels). Cells were stained with 100 nM TMRE for 15 min and analyzed 15 min after the laser illumination. Where indicated, cells were pre-treated with 20 nM SkQ1 for 7 days. Bar, 15 μm.