Mitochondrial-Targeted Plastoquinone Derivatives. Effect on Senescence and Acute Age-Related Pathologies

M.V. Skulachev[1,2,3], Y.N. Antonenko[1], V.N. Anisimov[4], B.V. Chernyak[1], D.A. Cherepanov[5], V.A. Chistyakov[6], M.V. Egorov[3], N.G. Kolosova[7], G.A. Korshunova[1], K.G. Lyamzaev[1], E.Y. Plotnikov[1], V.A. Roginsky[8], A.Y. Savchenko[11], I.I. Severina[1,2], F.F. Severin[1], T.P. Shkurat[6], V.N. Tashlitsky[9], K.M. Shidlovsky[1], M.Y. Vyssokikh[1], A.A. Zamyatnin Jr[3], D.B. Zorov[1] and V.P. Skulachev[*,1,3,10]

[1]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Vorobyevy Gory 1, Moscow 119991, Russia*

[2]*Biological Faculty, Lomonosov Moscow State University, Vorobyevy Gory 1, Moscow 119991, Russia*

[3]*Institute of Mitoengineering, Lomonosov Moscow State University, Vorobyevy Gory 1, Moscow 119992, Russia*

[4]*Petrov Institute of Oncology, Pesochny pr. 2, St. Petersburg 197758, Russia*

[5]*Frumkin Institute of Physical Chemistry and Electrochemistry, 31 Leninsky Av., Moscow 119991, Russia*

[6]*Rostov State University, pr. Stachki 194/1, Rostov-on-Don 344104, Russia*

[7]*Institute of Cytology and Genetics, Novosibirsk 630090, Russia*

[8]*Institute of Chemical Physics, ul. Kosygina 4, Moscow 119977, Russia*

[9]*Chemical Faculty, Lomonosov Moscow State University, Vorobyevy Gory 1, Moscow 119991, Russia*

[10]*Faculty of Bioengineering and Bioinformatics, Lomonosov Moscow State University, Vorobyevy Gory 1, Moscow 119991, Russia*

[11]*FSA Therapeutic Products Regulatory Research Center, Petrovsky bulv. 8, Moscow, 103051, Russia*

Abstract: Plastoquinone, a very effective electron carrier and antioxidant of chloroplasts, was conjugated with decyltriphenylphosphonium to obtain a cation easily penetrating through membranes. This cation, called SkQ1, is specifically targeted to mitochondria by electrophoresis in the electric field formed by the mitochondrial respiratory chain. The respiratory chain also regenerates reduced $SkQ1H_2$ from its oxidized form that appears as a result of the antioxidant activity of $SkQ1H_2$. $SkQ1H_2$ prevents oxidation of cardiolipin, a mitochondrial phospholipid that is especially sensitive to attack by reactive oxygen species (ROS). In cell cultures, SkQ1 and its analog plastoquinonyl decylrhodamine 19 (SkQR1) arrest H_2O_2-induced apoptosis. When tested *in vivo*, SkQs (i) prolong the lifespan of fungi, crustaceans, insects, fish, and mice, (ii) suppress appearance of a large number of traits typical for age-related senescence (cataract, retinopathies, achromotrichia, osteoporosis, lordokyphosis, decline of the immune system, myeloid shift of blood cells, activation of apoptosis, induction of β-galactosidase, phosphorylation of H2AX histones, etc.) and (iii) lower tissue damage and save the lives of young animals after treatments resulting in kidney ischemia, rhabdomyolysis, heart attack, arrhythmia, and stroke. We suggest that the SkQs reduce mitochondrial ROS and, as a consequence, inhibit mitochondria-mediated apoptosis, an obligatory step of execution of programs responsible for both senescence and fast "biochemical suicide" of an organism after a severe metabolic crisis.

Keywords: Electric potential difference, mitochondria, drug targeting, antioxidant, plastoquinone, ischemia/reperfusion, kidney, rhabdomyolysis, cataract, retinopathies.

INTRODUCTION

About 40 years ago, our group found that mitochondria generate electric potential difference ($\Delta\psi$) across their inner membrane, the mitochondrial interior being negative [1-4]. This finding directly proved a crucial postulate of the Mitchell's chemiosmotic hypothesis which suggested that chemical energy released by respiratory chain is first transformed to electric energy of $\Delta\psi$ to be converted then to chemical energy of ATP [4a]. Development of these studies revealed the fact that the mitochondrial interior is a unique intracellular compartment that is negatively charged relative to cytosol (for review, see [5]). These findings were made using so-called penetrating ions, i.e. ionized molecules where the charge is delocalized over hydrophobic residues so

*Address correspondence to this author at the Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Vorobyevy Gory 1, 119991 Moscow, Russia; Tel: +74959395530; Fax: +74959390338; E-mail: skulach@belozersky.msu.ru

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that water dipoles cannot form a shell surrounding the ion and preventing it from entering into the hydrophobic membrane core [1-5]. Tetraphenylphosphonium (TPP) exemplifies such ions. In TPP, the positive charge is distributed over four phenyl residues. Mitochondria energized by respiration or ATP hydrolysis accumulate TPP, the TPP gradient being as a high as 1,000-fold at $\Delta\psi$ = 180 mV (10-fold gradient per 60 mV as described by the Nernst equation) [5]. We suggested that penetrating cations might be used as "electric locomotives" of molecular dimensions, transporting various compounds from cytosol to mitochondria. To this end, a penetrating cation should be conjugated to a transported compound [5, 6].

Recently, the electric locomotive principle was used successfully 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 [7]. Later, thiobutyl was replaced by α-tocopherol [8] 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) [9]. 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 $OH^{\bullet}$ *in vitro*, (iii) facilitates survival of a 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 [9-14]. Unfortunately, if too much MitoQ is added it can also operate as a prooxidant [10, 15-17], a feature which might limit its practical use *in vivo* in treating, e.g., retinopathies in mice [86] or Friedrich ataxia [19] or Parkinson disease [14] in humans.

STUDIES ON MODEL SYSTEMS AND MITOCHONDRIA *IN VITRO*

In 2002 we started a project of creation of mitochondria-targeted antioxidants more active than MitoQ. 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 [20-22]. 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. The acidic pH in the thylakoid interior also promotes oxidative stress due to protonation of $O_2^{\bullet-}$ to the much more aggressive $HO_2^{\bullet}$ [15, 27, 91].

Synthesis of New Compounds

Several plastoquinone derivatives combined with various penetrating ions were synthesized in our group. These compounds were called SkQs, where Sk is for penetrating cation ("Skulachev ion", a term introduced by David Green [23]), and Q is for quinone.

Plastoquinone differs from ubiquinone in that (i) the 2- and 3-methoxy residues are replaced by methyl groups and (ii) the 5-methyl residue is replaced by H. This is why we synthesized also 3-demethoxy ubiquinonyl decyltriphenylphosphonium (DMQ) and 5-methyl plastoquinonyl decyltriphenylphosphonium (SkQ3) derivatives which represent, in fact, compounds intermediate between ubiquinone- and plastoquinone-containing substances. As the penetrating cation, alkyltriphenylphosphonium residues were used in several synthesized compounds (SkQ1, SkQ3, SkQ5). However, in certain cases the penetrating cation was Rhodamine 19 (SkQR1) or Rhodamine B (SkQR4). In three cases, natural cations were conjugated with plastoquinone, namely methylcarnitine (SkQ2M), berberine (SkQB), and palmatine (SkQP). The latter two penetrating cations are used by plants as antibacterial agents [24]. Decane was usually used as a linker between the quinone and cation moieties, but in SkQ5 pentane was used. As a non-penetrating analog containing a cation with localized positive charge, plastoquinonyl decyltributylammonium (SkQ4) was synthesized. Moreover, compounds containing a penetrating cation and a linker (dodecyltriphenylphosphonium or dodecylrhodamine 19) but no quinone were synthesized (C_{12}TPP and C_{12}R1, respectively). Formulas of these substances are shown in Fig. (**1**) (for details of their synthesis, see [15]).

In vitro Studies on SkQs and Related Substances

First of all, the new compounds were tested for their ability to penetrate through hydrophobic membranes. Planar bilayer phospholipid membranes (BLM) were used for this purpose. Measurements of electric diffusion potential showed that SkQR1 or SkQR4 gradients within the concentration range 10^{-6}-10^{-5} M produced $\Delta\psi$ value close to the Nernstian prediction (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, MitoQ, SkQB, SkQP, C_{12}TPP, and C_{12}R1 produced $\Delta\psi$ of the same sign but lower than Nernstian value (Fig. **2**). In the case of SkQ2M, SkQ4, or SkQ5, $\Delta\psi$ was negligible or 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 a BLM to the other showed that this rate declines in the series SkQR1 > SkQ1 > SkQ3 > MitoQ [25]. On studying cations with low permeability, there is a danger that a diffusion potential will be shunted by other ion fluxes and as a result will drop below the theoretically expected value. Actually, concentrations of the studied cations could be increased, but in this case they all manifest detergent properties, thus damaging the BLM. This 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 $5{\cdot}10^{-5}$ to $5{\cdot}10^{-4}$ M concentration range, SkQ1 produced $\Delta\psi$ with a value corresponding to the Nernst equation [15].

Another mechanism that can shunt the diffusion potential of SkQ1, MitoQ, and other penetrating cations of similar structure is their mediation of transmembrane flux of fatty

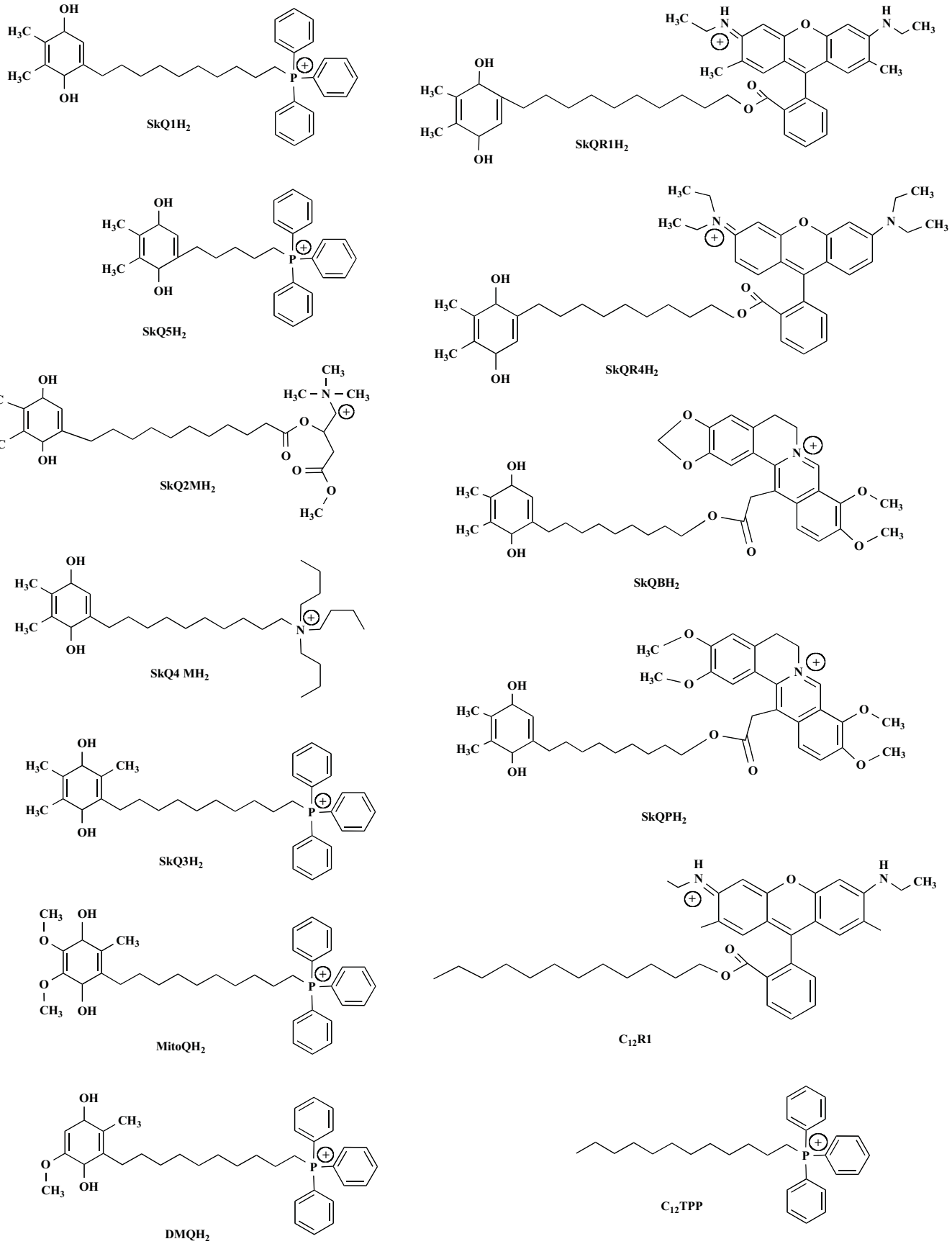


Fig. (1). Compounds synthesized and investigated in this study.

acid anions, which results in cycling of fatty acid and increase in the membrane H^+ conductance. Traces of free acids are always present in phospholipids used to form BLM [26]. Moreover, SkQR1 can operate as a protonophore even in the absence of fatty acids [27]. Both of these effects become impossible at pH 4, when H^+ cannot dissociate from a fatty acid or SkQR1. This is why for the experiments shown in Fig. (**2**) the pH value of the medium was chosen to be 4 [15].

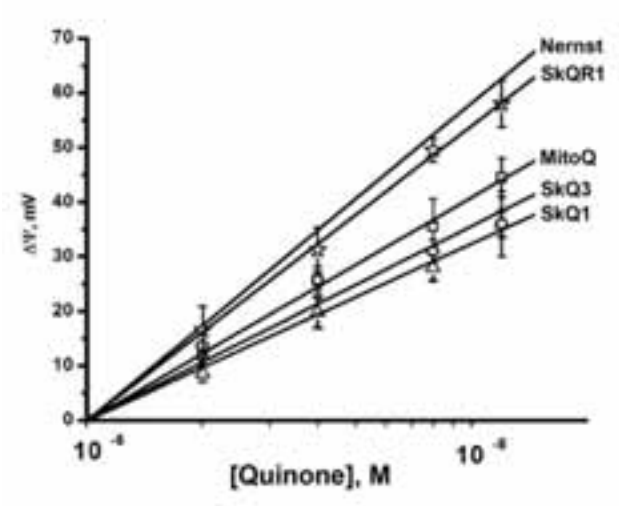


Fig. (2). Generation of diffusion electric potential on planar bilayer phospholipid membrane by SkQ1 and related compounds. In the right compartment, the concentration of the studied compound was 10^{-6} M. In the left compartment, this concentration is indicated by the abscissa. The incubation mixture contained 10 mM Tris-HCl, 10 mM MES, 10 mM KCl, pH 4.0. (*From Antonenko et al. [15]*).

Anti- and prooxidant activities of the SkQs were tested in model systems, i.e. aqueous solutions, lipid/detergent micelles, liposomes, and planar membranes. To measure the ability of SkQ1 to quench $OH^{\bullet}$ and other ROS 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) [28, 29]. It was found that 0.5 μM $SkQ1H_2$ (reduced form of SkQ1) strongly decreased chemiluminescence. The reduced MitoQ ($MitoQH_2$) showed much lower efficiency than $SkQH_2$ [15].

Prooxidant activities of $SkQ1H_2$ and $MitoQH_2$ in an aqueous solution are compared in Figs. (**3A** and **3B**). As a measure of such activities, rate of reduction O_2 to $O_2^{\bullet-}$ by these quinols was chosen. Formations of quinones from corresponding quinols or $O_2^{\bullet-}$ from O_2 were followed with a spectrophotometer (Fig. **3A**) or an ESR spectrometer (Fig. **3B**), respectively. Both methods clearly showed much higher prooxidant activity of $MitoQH_2$ compared to $SkQ1H_2$.

Antioxidant properties of the compounds were also analyzed using lipid/detergent micelles. In this case, per-oxidation of the methyl ester of linoleate in micelles of Triton X-100 was followed by measuring O_2 consumption. The antioxidant 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 quinol moiety of penetrating cations (QH_2):

$$LO_2^{\bullet} + QH_2 \rightarrow LOOH + QH^{\bullet} \quad (1)$$

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

$$LO_2^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad (2)$$

where $L^{\bullet}$ is a carbon-centered radical of methyl linoleate.

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 [30]).

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 $LO_2^{\bullet}$ is almost four times higher than that of $MitoQH_2$ (Table **1**). This is consistent 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 [21, 22]. The oxi-dized forms of the cations (SkQ1 and MitoQ) were comp-letely inactive in this system.

To model damage to membrane proteins caused by ROS, a new method was developed, i.e. measuring ROS sensitivity of BLM conductance created by gramicidin D [31]. Tryptophan residues of this channel-forming polypeptide are attacked by ROS, resulting in the inactivation of the channels. Ascorbate, $FeSO_4$, and *tert*-butyl hydroperoxide

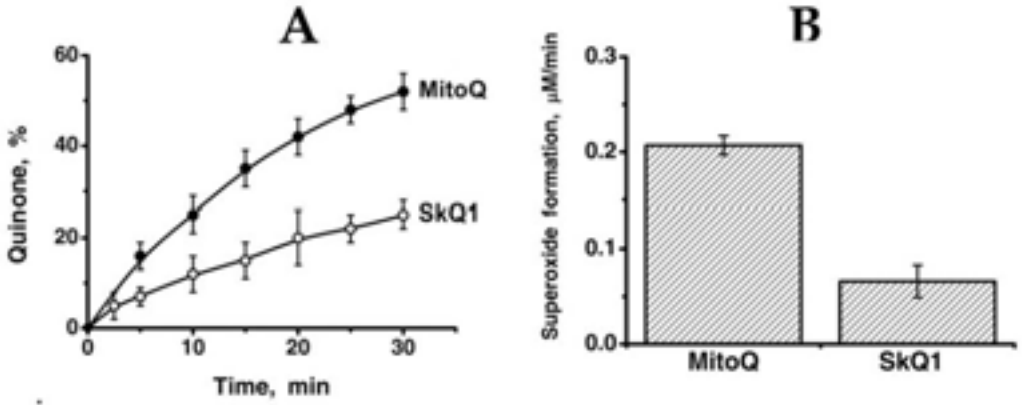


Fig. (3). Prooxidant effects of $SkQ1H_2$ and $MitoQH_2$ in aqueous solution. **A**, Autooxidation of $SkQ1H_2$ and $MitoQH_2$ by O_2: rates of SkQ1 and MitoQ formation from $SkQ1H_2$ and $MitoQH_2$, respectively. **B**, rates of $O_2^{\bullet-}$ formation due to the reduction of O_2 by $SkQ1H_2$ or $MitoQH_2$. 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. Concentration of quinols, 5 μM. (*From Antonenko et al. [15]*).

were used as a ROS-generating system. It was shown that the ROS-induced inactivation of the gramicidin-mediated current could be prevented by $SkQ1H_2$ or $MitoQH_2$, with $SkQ1H_2$ being more effective. Another version of the gramicidin experiment was the use of light to generate ROS with Methylene Blue as a photosensitizer. The antioxidant effect of $SkQ1H_2$ was revealed at lower concentrations than that of $MitoQH_2$ ($C_{1/2}$ values were 1.8 and 4.5 μM, respectively) [15].

Table 1. Kinetic Parameters Characterizing the Antioxidant Activity of the Reduced Forms of Mitochondria-Targeted Quinones in Methyl Linoleate (ML)/Triton X-100 Micelles. Incubation Mixture, 20 mM ML, 50 mM Triton X-100, 50 mM Phosphate Buffer, pH 7.4, 37°C. Oxidation of ML was Initiated by 3 mM AAPH. Figures in Brackets are the Number of Independent Experiments. (*From Roginsky et al. [30]*)

Quinol	k_1/k_2	$k_1 \times 10^5$, $M^{-1}s^{-1}$
$SkQR1H_2$	3800 ± 500 (5)	2.3 ± 0.3
$SkQ1H_2$	3670 ± 280 (7)	2.2 ± 0.2
$SkQ3H_2$	2720 ± 210 (4)	1.6 ± 0.1
$SkQ5H_2$	2670 ± 180 (5)	1.6 ± 0.1
$DMQH_2$	1260 ± 85 (4)	0.76 ± 0.5
$MitoQH_2$	970 ± 55 (6)	0.58 ± 0.03

Interaction of SkQs with Isolated Mitochondria

M.S. Muntyan showed in our group that uptake of SkQ1 by mitochondria can be monitored with a hydrophobic cation-sensitive electrode. Addition of uncoupler discharging Δψ initiated partial release of SkQs to the medium, the effect being larger with SkQ5 than with the more hydrophobic SkQ1. This was consistent with the octanol/water distribution coefficients which were found to be 13,000 : 1 and 500 : 1 for SkQ1 and SkQ5, respectively (cf. 3,000 : 1 for MitoQ [10]) [15].

Then it was tested whether SkQ1 and other plastoquinone derivatives could be reduced by the respiratory chain of animal mitochondria. It was found that these compounds can be reduced by both NAD-linked substrates and by succinate. The reduction of SkQ1 was completely arrested by antimycin A but not myxothiazol [15]. These relationships can be explained assuming that SkQ1, like plastoquinone [33-36], 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 nonenzymatic (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. This means that in respiring mitochondria SkQ1 should be mainly in its reduced state that is competent in antioxidant activity [15].

What are the relationships between the anti- and prooxidant effects of SkQ1 and related substances on mitochondria? To estimate antioxidant activity, we measured inhibition by these compounds of malondialdehyde (MDA) formation initiated by Fe^{2+} and ascorbate in rat heart mitochondria. The antioxidant activity of SkQ1 was measurable at much lower concentration than that of MitoQ. The efficiency of various compounds in inhibiting MDA formation was compared in Table **2**. In this respect, SkQ1 and SkQR1 were the most active, whereas MitoQ is the least active among the quinone-containing compounds bearing a cationic residue. Decylplastoquinone (DPQ) lacking a cation was even less active than MitoQ, whereas $C_{12}TPP$ lacking a quinone residue was quite inactive at the concentrations studied. As further analysis revealed, it is cardiolipin that is first oxidized in heart mitochondria under conditions of $OH^{\bullet}$ generation by Fe^{2+} + ascorbate. The amount of cardiolipin was strongly decreased after the Fe^{2+} + ascorbate treatment, the effect being significantly inhibited by 100 nM SkQ1 (Fig. **4**). In other experiments, it was shown that SkQ1 and other hydrophobic cations displace N-nonyl acridine orange from a complex with phospholipids (presumably with cardiolipin) in rat heart mitochondria, MitoQ being less efficient than SkQ1 and much less efficient than SkQR1 [15, 32] (for method, see [37]).

Table 2. Concentrations of Compounds Decreasing Twofold the MDA Formation in Rat Heart Muscle Mitochondria Treated with Fe^{2+} and Ascorbate (*From Antonenko et al. [15]*)

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

To quantify the prooxidant action of the cationic quinones, we measured stimulation by the same compounds of 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 the cationic quinones. The prooxidant effect was much smaller with $C_{12}TPP$. Prooxidant activity of SkQ1 becomes observable at slightly higher [SkQ1] than [MitoQ]. As a result, the "window" for pure antioxidant effect 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) [15].

At concentrations of SkQ1, SkQ3, and MitoQ above 10^{-6} M, uncoupling effect was manifested. In particular, stimulation of respiration rate in State 4 and steady decrease in Δψ were observed. Stimulation of the State 4 respiration disappeared at concentrations above $5 \cdot 10^{-6}$ M [15, 32].

The mechanism of uncoupling involves the formation of a membrane-permeable ion pair of the studied cations and fatty acid anions. In fact, SkQ1 or $C_{12}TPP$ operates as carrier

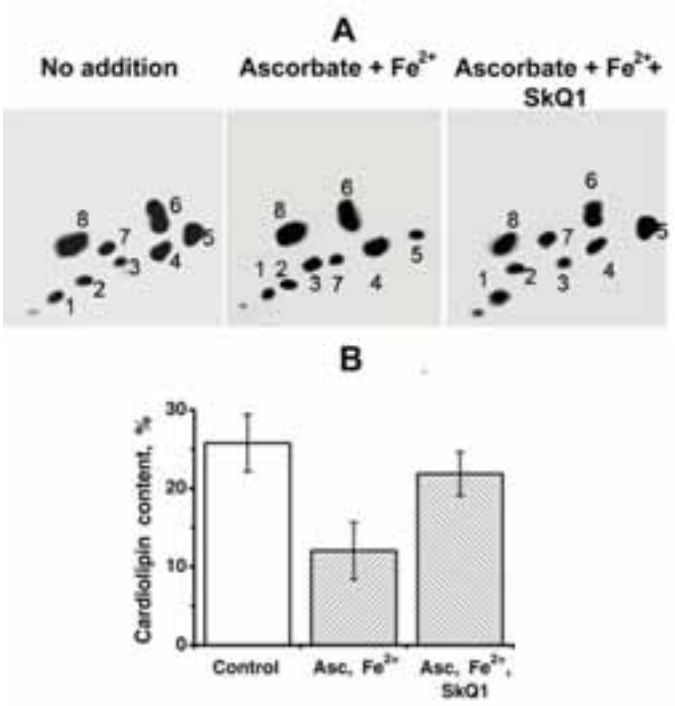


Fig. (4). SkQ1 specifically protects mitochondrial cardiolipin from oxidation by $OH^{\bullet}$. Mitochondria were incubated with ascorbate and $FeSO_4$. Where indicated, 100 nM SkQ1 was added. Mitochondrial lipids were chromatographed on Silica gel-60 plates with chloroform–methanol–water, 65 : 25 : 5 (horizontal) and chloroform–acetone–methanol–acetic acid–water, 3 : 4 : 1 : 1 : 0,5 (vertical). Lipid spot designations: (1) dilysocardiolipin; (2) phosphatidylinositol; (3) monolysocardiolipin; (4) phosphatidylglycerol; (5) cardiolipin; (6) phosphatidylethanolamine; (7) phosphatidic acid; (8) phosphatidylcholine (*From M. Yu. Vyssokikh unpublished, and Antonenko et al. [15]*).

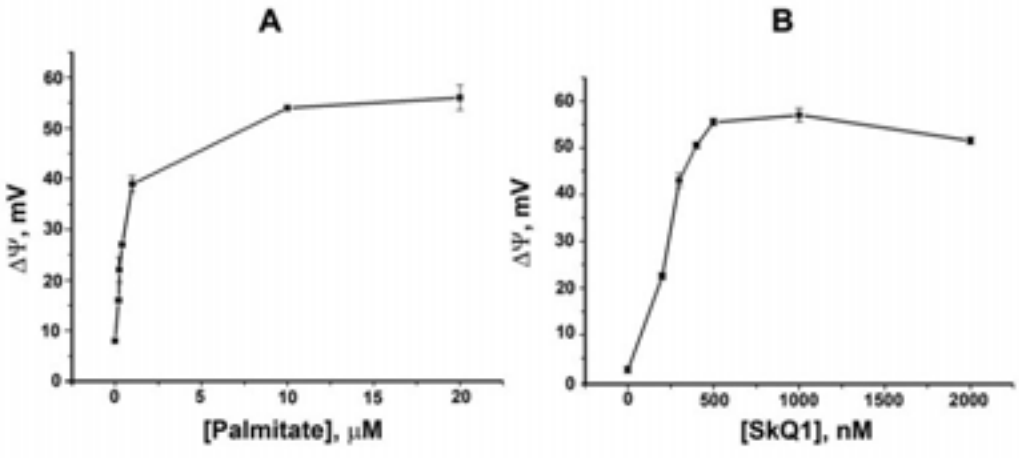


Fig. (5). Penetrating cations mediate protonophorous effect of fatty acids in BLM. Incubation mixture, 10 mM Tris-MES, 10 mM KCl. The pH was 7 in one compartment and 6 in the other compartment. When palmitate and SkQ1 were added, the more acidic compartment was negatively charged. (**A**) 1 μM SkQ1 is present in all cases; for [Palmitate], see abscissa. (**B**) 10 μM palmitate in all cases; for [SkQ1], see abscissa. (*From Severin et al. [26]*).

for anions of free fatty acids [26, 27]. This effect in cooperation with back-flow of protonated fatty acids and of free SkQ1 (C_{12}TPP) cations mediates H^+ conductance directed from the outside to the inside of the energized mitochondrion.

The above-described scheme was directly proved in experiments of our group on BLM separating two solutions differing in pH value. In this system, addition of a protonophore (FCCP) resulted in a transmembrane H^+ flux generating an electric potential difference across the BLM, the more acidic compartment being negatively charged. Fig. (**5**) shows that palmitate and a penetrating cation (SkQ1) added together can effectively substitute for FCCP. The $\Delta\psi$ increases when pH values of the BLM-separated solutions are higher than the pK value of the fatty acid. At pH 4 in one compartment and pH 5 in the other compartment, $\Delta\psi$ was as small as 13 mV, whereas at pH 6 *versus* 7 (or 7 *versus* 8) it reached the theoretical Nernstian value, approaching 60 mV [26]. C_{12}TPP, SkQR1, SkQR4, and C_{12}R1 were found to substitute for SkQ1.

Formation of a complex of $C_{12}R1$ and palmitate in an aqueous solution was directly proved in a study performed by our group. It was found that fluorescence of the rhodamine moiety is strongly decreased by palmitate. Simultaneously, the light absorption spectrum of $C_{12}R1$ was dramatically changed [27]. The above-mentioned relationship can be described by eqs. 3-9.

(A) On the membrane surface facing the more acidic compartments:

palmitate anion + H^+ → palmitic acid (3)

palmitate anion•penetrating cation complex → palmitate anion + penetrating cation (4)

(B) Inside membrane:

palmitic acid near acidic membrane surface → palmitic acid near opposite (alkaline) surface (5)

penetrating cation near acidic surface → penetrating cation near alkaline surface + $\Delta\psi$ (6)

(C) On the membrane surface facing the more alkaline compartment:

palmitic acid → palmitate anion + H^+ (7)

palmitate anion + penetrating cation → palmitate anion•penetrating cation complex (8)

(D) Inside the membrane:

palmitate anion•penetrating cation complex near alkaline surface → palmitate anion•penetrating cation complex near acidic surface (9)

In rat heart mitochondria studied *in vitro*, we revealed that not only SkQ1 but also $C_{12}TPP$ decreases H_2O_2 production under State 4 conditions when reversed electron transport from succinate to NAD^+ is operative. However, the $C_{1/2}$ value of effect of SkQ1 was 10-15-fold lower than for $C_{12}TPP$ (Fig. **6A**). Fat-free bovine serum albumin abolished the action of $C_{12}TPP$ but was failed to arrest the inhibitory effect of low SkQ1 concentrations [26]. The effect of albumin suggests that endogenous fatty acids are somehow

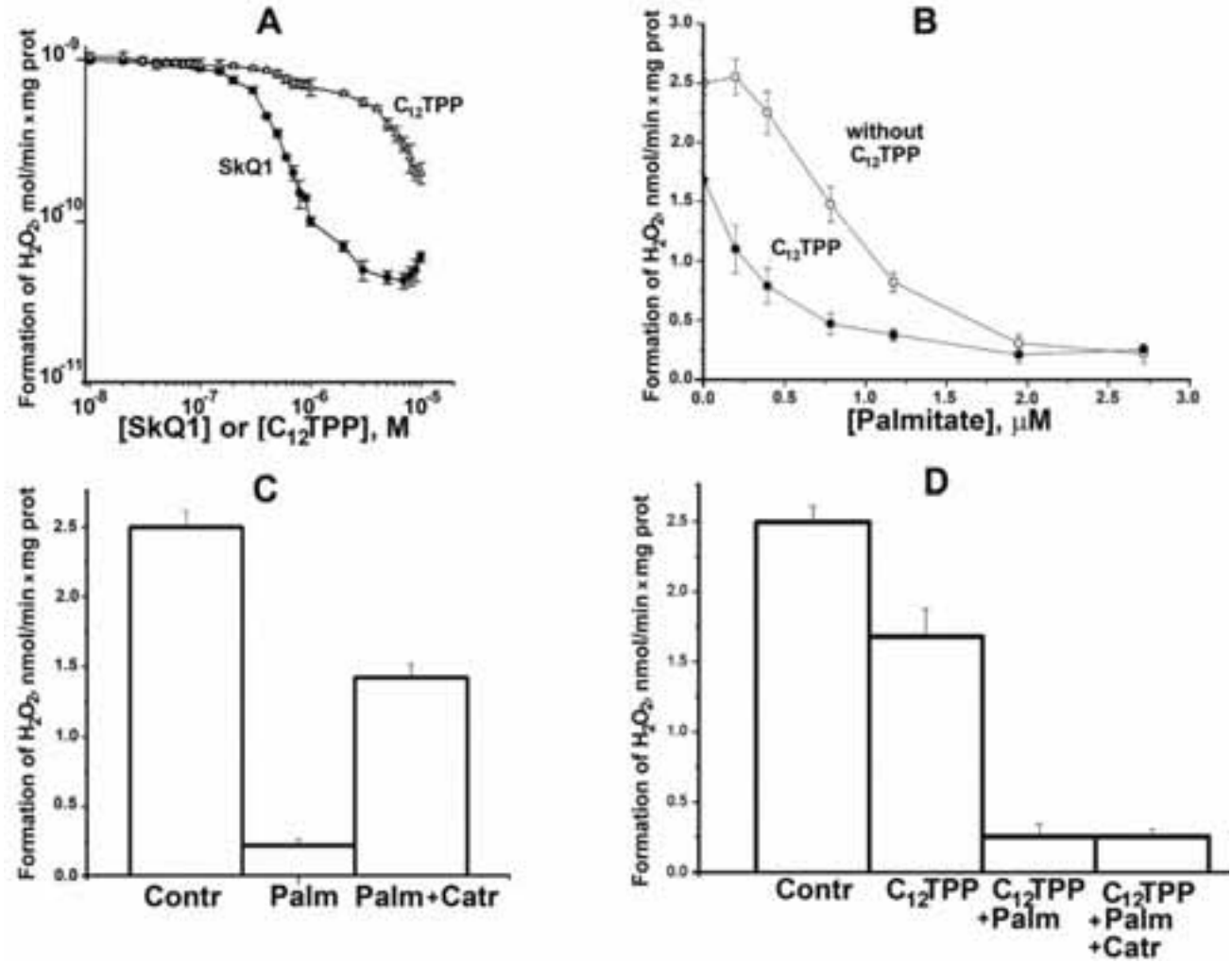


Fig. (6). Inhibition of H_2O_2 formation in isolated rat heart mitochondria by penetrating cations and palmitate. State 4 conditions with succinate as the respiratory substrate. **A**, Comparison of the efficiencies of SkQ and $C_{12}TPP$ in inhibiting H_2O_2 formation. Incubation mixture, 250 mM sucrose, 1 mM EGTA, 10 mM MOPS-KOH (pH 7.4), 5 mM succinate, 2 μM Amplex Red, horseradish peroxidase (9 units), and rat heart mitochondria (0.15 mg protein/ml). **B**, 1 μM $C_{12}TPP$ makes palmitate more efficient in inhibiting H_2O_2 formation. Condition as in A. **C**, **D**, Palmitate stimulates inhibitory effect of $C_{12}TPP$ on H_2O_2 formation in a Catr-resistant manner. Incubation mixture as in A. Additions, 2 μM palmitate, 1 μM Catr and 1 μM $C_{12}TPP$. (*From Severin et al. [26]*).

involved in the action of C_{12}TPP. To test this possibility, we added palmitate in the same experimental system (Figs. **6B-D**). Palmitate completely inhibited H_2O_2 formation by mitochondria under conditions of reverse electron transfer ($C_{1/2}$ for palmitate was $1x10^{-6}$ M). Addition of $1x10^{-6}$M C_{12}TPP lowered the palmitate $C_{1/2}$ value by factor of five (Fig. **6B**). The effect of palmitate without C_{12}TPP was strongly decreased by carboxyatractyloside (Catr), an inhibitor of the ATP/ADP antiporter, as previously shown by our group [39]. However, Catr was completely ineffective if 1 μM C_{12}TPP was added (Fig. **6C, D**). Under these conditions, we are reminded of Shönfeld's observation [40] that tetraphenylphosphonium (TPP) stimulates uncoupling activity of fatty acids. This stimulation, according to data of our group, is Catr-sensitive [41]. Such relationships were explained assuming that TPP facilitates translocation of fatty acid anions to nucleotide anion-binding positively charged amino acid residues of the antiporter, which are involved in the fatty acid cycling in the mitochondrial membrane [41-43]. The more hydrophobic C_{12}TPP is apparently competent in the transmembrane movement of fatty acid anions with no ATP/ADP antiporter or other proteins involved.

Studies on Cellular Level

The next question was whether protonophorous effect of fatty acid/penetrating cation pair is realized in mitochondria of intact cells. If an intact cell contains a certain level of non-esterified fatty acids, C_{12}TPP should specifically increase H^+ conductance of the mitochondrial membrane, other membranes being unaffected since C_{12}TPP is targeted to mitochondria. This prediction was verified by experiments of our group on intact *S. cerevisiae* cells at low pH of the growth medium. As shown in Fig. (**7A**), lowering of pH in the medium to 3.0 allowed FCCP to stimulate cell respiration only at low uncoupler concentrations. If the concentration was increased, the stimulation disappeared. However, both low and high concentrations of C_{12}TPP caused a large increase in the respiration rate (Fig. **7B**). These relationships can be accounted for by assuming that high [FCCP] increased H^+ conductance not only of the mitochondrial but also of the plasma membrane. This should result in acidification of the cytosol if the pH outside the cell is low. Lowering of intracellular pH should, in turn, inhibit mitochondrial respiration. As was shown in our group, decrease of pH in the incubation mixture from 7.5 to 6.0 resulted in 7-fold lowering the respiration rate of isolated *S. cerevisiae* mitochondria [26]. As to C_{12}TPP, it electrophoretically accumulates in mitochondria and, hence, in cooperation with endogenous fatty acids, increases H^+ conductance of the mitochondrial (but not of the plasma) membrane. If this is the case, inhibition of respiration at pH_{out} = 3.0 should be achieved by adding a penetrating weak acid instead of the protonophore FCCP. This was confirmed when acetic acid was added to the yeast cells [26].

In human HeLa cells or fibroblasts, SkQR1, a fluorescent SkQ derivative, was specifically accumulated by mitochondria, showing the same intracellular localization as mitochondria-targeted jellyfish fluorescent protein YFP [15] or Mitotracker Green (Fig. **8A**). As shown in Fig. (**8B**), the staining of mitochondria inside the living cell by SkQR1 took about 1 h, and subsequent incubation of the cells in medium without SkQR1 resulted in slow SkQR1 release ($t_{1/2}$ = 2.5 h). The uncoupler FCCP prevented the SkQR1 staining and stimulated its efflux if added to non-stained and stained cells, respectively [15].

In another series of experiment, we investigated whether SkQ derivatives possess antiapoptotic and antinecrotic effects if cell death is induced by ROS. The experiments showed that SkQs prevented ROS-linked cell death. Especially low concentrations of SkQ1 and SkQR1 were effective when human fibroblasts were pretreated with SkQs for a week before initiation of apoptosis by H_2O_2 (following the protocol described by Saretzki *et al*. [12] 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. **8C**). Dissipation of $\Delta\psi$ by uncoupler FCCP prevented

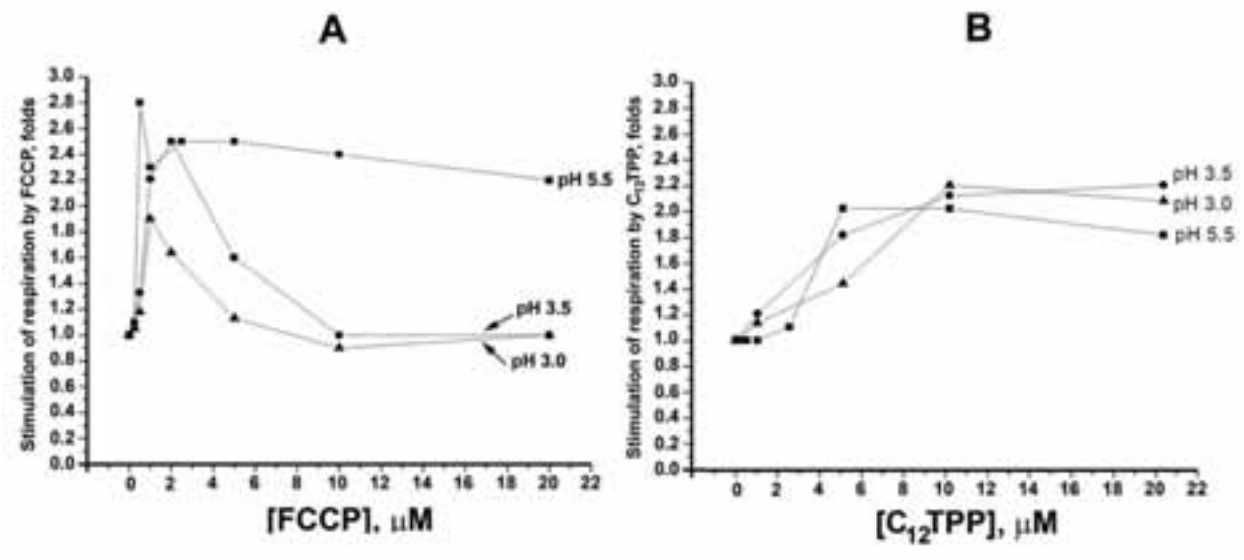


Fig. (7). Effects of FCCP (**A**) and C_{12}TPP (**B**) on respiration of *S. cerevisiae* cells at different extracellular pH values. (*From Severin et al. [26]*).

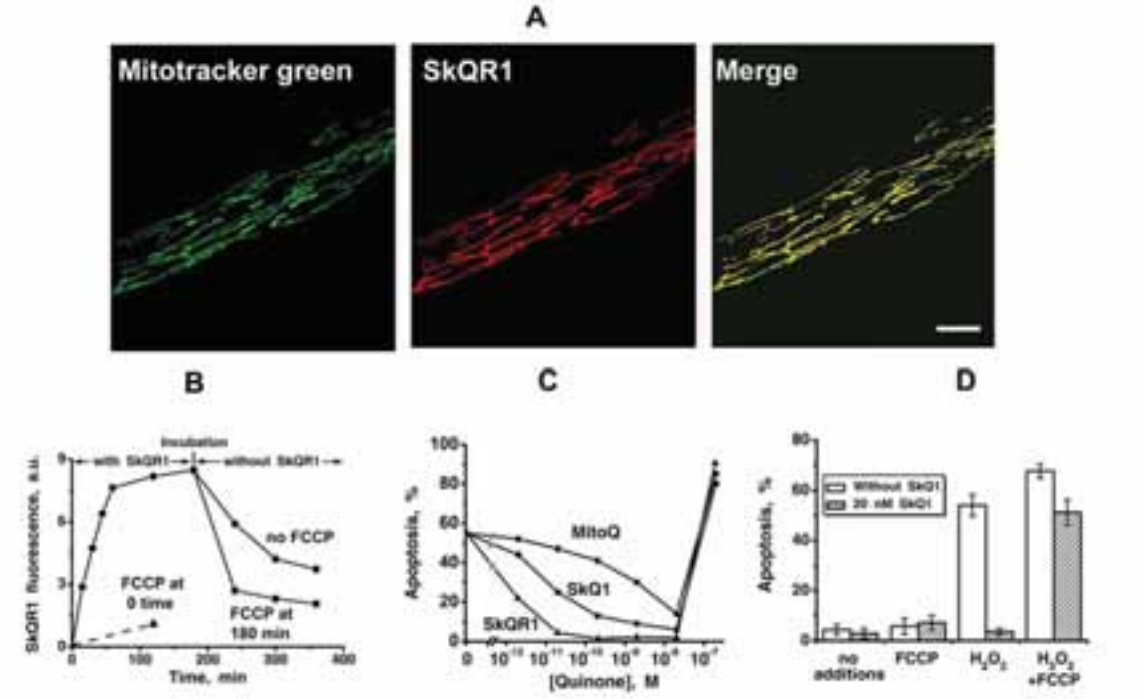


Fig. (8). Effects of SkQ1, SkQR1, and MitoQ on human cell cultures. **A,** Colocalization of SkQR1 (a fluorescent SkQ derivative) and mitochondria-specific fluorescent dye Mitotracker green in human fibroblasts. Confocal microscopy. Bar, 15μm (*From Izyumov et al. [102]*). **B,** Kinetics of SkQR1 uptake to and release from HeLa the cells. At zero time, 50 nM SkQR1 was added to the cells. Where indicated, 10 μM FCCP was added. The SkQR1 uptake and release were measured fluorometrically with a Beckman-Coulter FC500 flow cytometer. **C,** SkQ1 and SkQR1 are more efficient than MitoQ in arresting the H_2O_2-induced apoptosis in human fibroblasts (For conditions see D). **D,** 7-day pretreatment 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. (*From Antonenko et al. [15]*).

the antiapoptotic effect of such a low SkQ1 concentration (Fig. **8D**). The protective effect of small amounts of SkQ1 could be overcome by increasing [H_2O_2] to 500 μM [15]. 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 the cytosol. Neither DPQ nor C_{12}TPP at nanomolar concentrations could substitute for SkQ1 [15]. Toxic effect of SkQ1 was observed at 0.1 μM concentrations and strengthened in the presence of H_2O_2. This effect was probably due to prooxidant activity of high [SkQ1].

Addition of a small amount of H_2O_2 to HeLa cells induces a burst in endogenous ROS formation. Earlier we showed that ROS formation under these conditions is respiratory chain-linked and stimulated by respiratory inhibitors rotenone (Complex I) and myxothiazol (Complex III) [44]. This phenomenon, described as a ROS-induced ROS release [45], was completely abolished by pretreatment of the cells with 20 nM SkQ1 [15]. Staining of mitochondria by Mitotracker Green was found to sensitize the cells to light-induced necrosis, the effect being abolished by 1 h pretreatment with 1 μM SkQ1 [15]. Pretreatment with very low [SkQ1] or [SkQR1] prevented the ROS-initiated decomposition of mitochondrial filaments into small mitochondria ("the thread–grain transition" [46]). In this case, preincubation of fibroblasts with the cationic quinones for 2 h was sufficient to observe the protective effect, which increased in the series: MitoQ < SkQ1 < SkQR1. Measurable activity was observed at $2 \cdot 10^{-13}$ M SkQR1. C_{12}TPP was ineffective [15].

To explain such extremely high efficiency of SkQs, 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 the $\Delta\psi$ value 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 mitochondrial matrix), resulting in 10^4-fold SkQ gradient between extracellular medium and mitochondrial matrix. Then, membrane/water distribution coefficient, which is of about $1 \cdot 10^4$ for SkQ1 [18], should be taken into account. Thus, the concentration gradient of SkQ between the inner leaflet of mitochondrial membrane and extracellular medium can reach a value as high as 10^8-fold as shown schematically in Fig. (**9**).

Moreover, it is important that (i) antioxidant activity of quinones is inherent only in the reduced form (in our case, $SkQH_2$ [15, 30]) and (ii) reduction of SkQ to $SkQH_2$ occurs in mitochondria [15]. These facts are favorable factors for mitochondria-tropic effects of SkQs as compounds specifically defending the cell from *mitochondrial* ROS. It is significant that SkQs can be reduced only at one site of the mitochondrial respiratory chain. This is site *i* of Complex III (heme b_h), localized on the *matrix* side of the mitochondrial membrane, i.e. in the inner half-membrane leaflet of this membrane where SkQ is electrophoretically accumulated. In contrast, MitoQ is also reduced at several other sites of the respiratory chain. $MitoQH_2$ and $SkQH_2$ are oxidized by Complex III at site *o* as well as directly by O_2. These

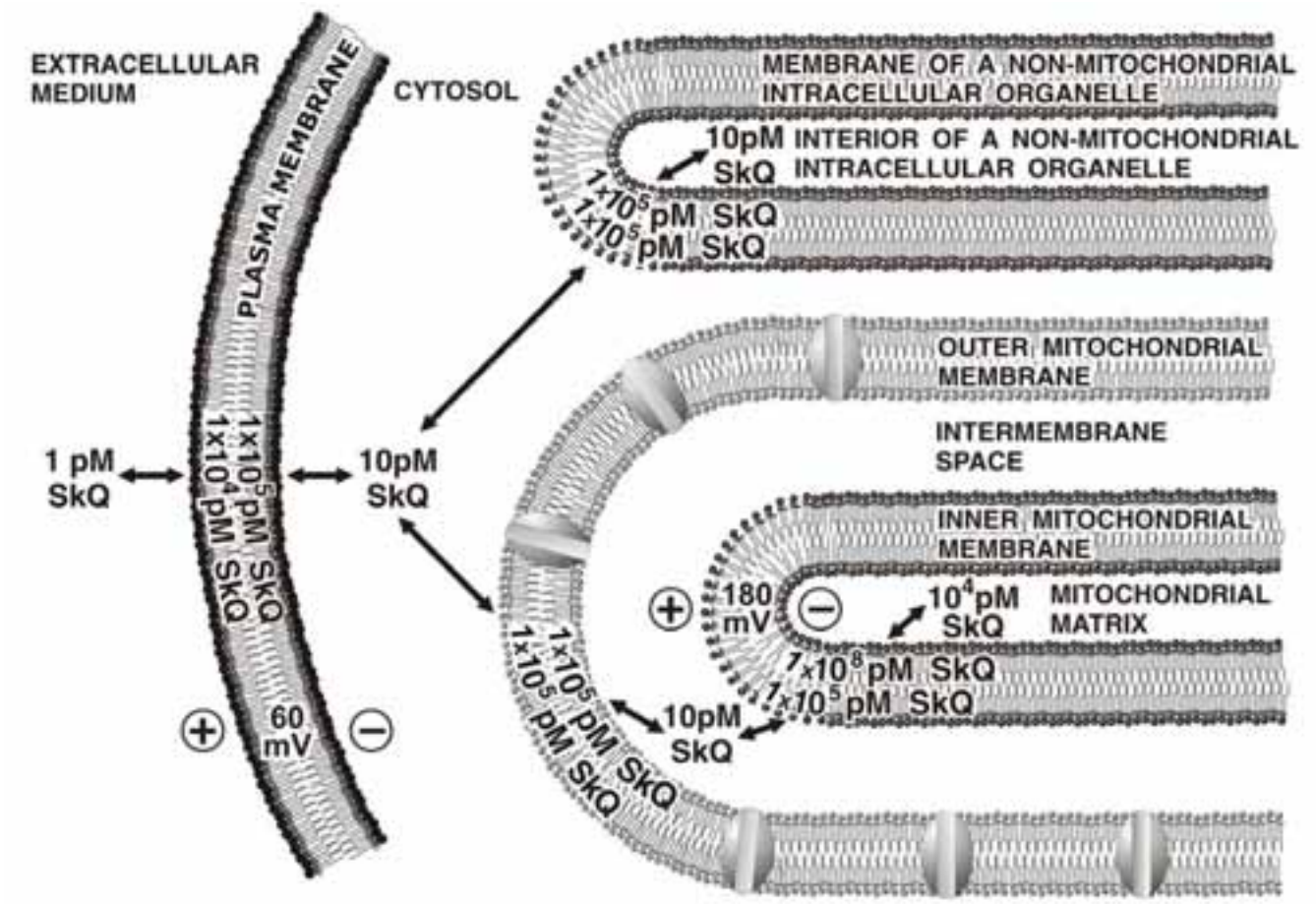


Fig. (9). Scheme illustrating accumulation of SkQ in inner leaflet of the inner mitochondrial membrane. The Δψ values on the plasma membrane and the inner mitochondrial membrane are assumed to be 60 and 180 mV, respectively. Membrane/water distribution coefficient for SkQ is assumed to be 10,000 : 1.

activities are lower for $SkQ1H_2$ than for $MitoQH_2$, which means that in steady state the SkQ1 pool should be more reduced than that of MitoQ.

Molecular dynamics calculations showed that there are two cardiolipin molecules localized in Complex III in the vicinity of heme b_h. Eight fatty acyl residues of these cardiolipins form a well with negative phospholipid charges on the bottom. This structure fits rather well to localize $SkQ1H_2$ near its oxidants (peroxyl radicals at C_9 and C_{13} of linoleate residues of cardiolipins [27]) and not far from the site of its reduction (heme b_h) (Fig. **10**). An interesting possibility is that oxidation of $SkQ1H_2$ to $SkQ1H^{\bullet}$ should result in its deprotonation:

$$SkQ1H_2 \rightarrow SkQ1H^{\bullet} \rightarrow SkQ1^{\bullet -} + H^+ \quad (10)$$

So that the cationic phosphonium group of SkQ1 is neutralized by anionic groups of semiquinone. As a result, a zwitterion "scorpion-shape" conformation of SkQ1 could appear, which already could not be bound by phosphate anions of cardiolipins. Thus, $SkQ1^{\bullet -}$ could leave hydrophobic cardiolipin well and come to heme b_h to be reduced back to $SkQ1H_2$. It is remarkable that the distance between cardiolipins and heme b_h is as short as 2 nm.

Special attention should be given to comparison of SkQs and MitoQ. The simplest explanation for the advantage of SkQs is the much larger concentration window between anti- and prooxidant activities for SkQs than for MitoQ [15]. However, Murphy and coauthors insist that prooxidant activity of MitoQ is never revealed under *in vivo* conditions [47]. They failed to find any indications of oxidative stress when administrating 0.5 mmol MitoQ/kg per day to mice for up to 28 weeks. Nevertheless, our experiments on simple model systems, isolated mitochondria, and living cells revealed some advantages of SkQs over MitoQ as antioxidants. This was more apparent with mitochondria and especially with cells than in the model systems. Such relationships appear to be due to the fact that in mitochondria and cells several favorable properties of SkQ potentiate each other, resulting in the final effect when SkQ is many times more active as antioxidant compared to MitoQ. These properties are the following.

(1) In aqueous solution $SkQ1H_2$ quenches $OH^{\bullet}$ several times better than $MitoQH_2$ [15].

(2) In aqueous solution, $SkQ1H_2$ is oxidized by O_2 to form $O_2^{\bullet -}$ three times slower than $MitoQH_2$ (Fig. **3**).

(3) $SkQ1H_2$ inhibits peroxidation of methyl linoleate in micelles four times more effectively than $MitoQH_2$ (Table **1**).

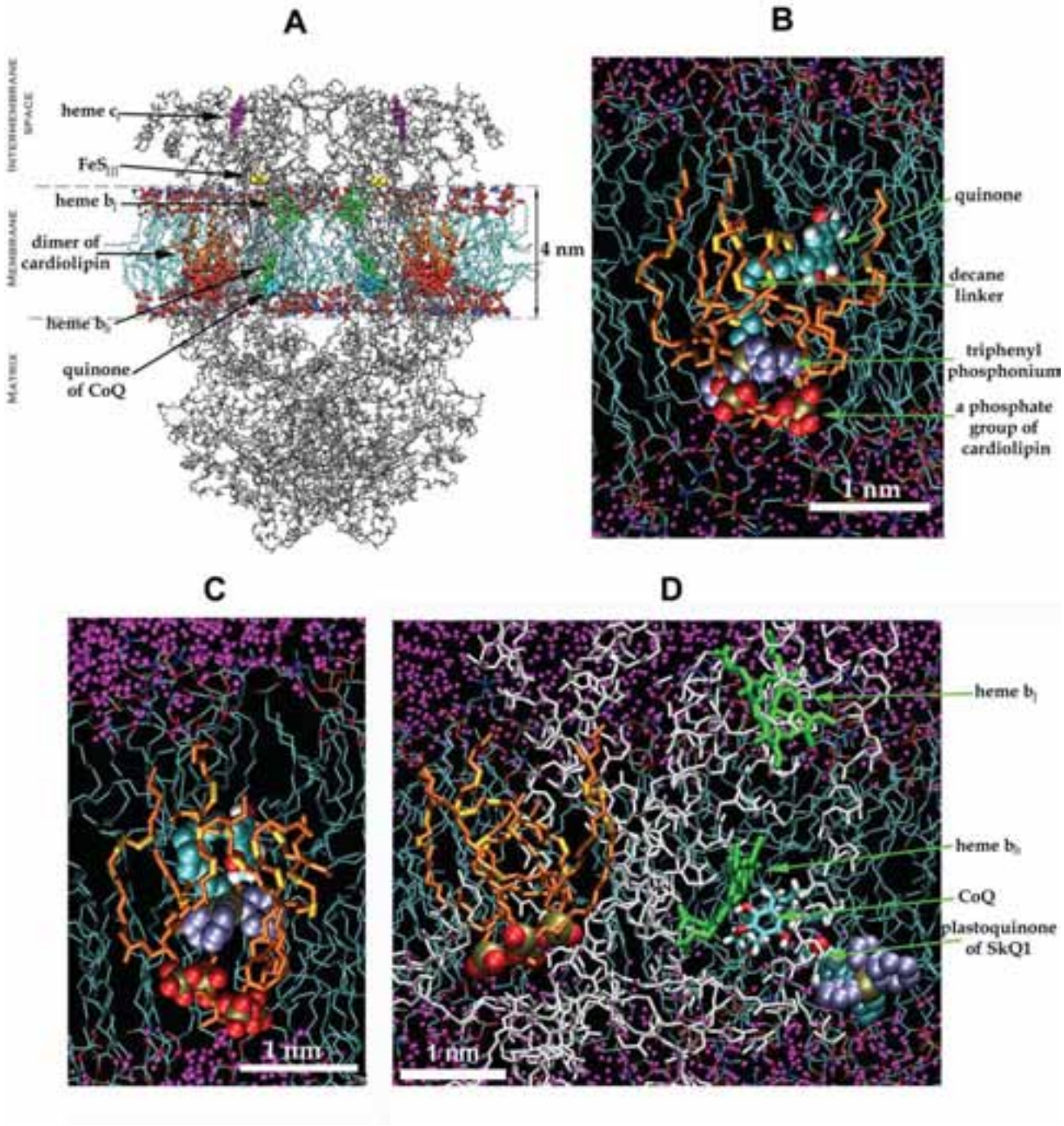


Fig. (10). Molecular modeling of SkQ1 binding with Complex III and cardiolipin. **A,** Crystal structure of the bovine mitochondrial bc_1 complex embedded into a lipid bilayer. The protein backbone, gray thin lines; 1-palmitoyl 2-oleoyl phosphatidyl choline, (green thin lines) (*From Huang et al. [103]*). **B,** extended conformation of $SkQ1H_2$ in the cavity formed by cardiolipin; **C,** folded conformation of the SkQ1 semiquinone in the cardiolipin cavity; **D,** extended conformation of the SkQ1 semiquinone at the *i* site of complex III. SkQ1 is shown by large spheres (violet, phenyl rings; brown, phosphorus; cyan, carbon; red, oxygen; white, hydrogen). Cardiolipin is show by orange (aliphatic carbon atoms) and yellow (unsaturated bonds); its anionic groups are shown by large spheres (brown, phosphorus; red, oxygen). 1-palmitoyl 2-oleoyl phosphatidyl choline is blue. Oxygen atoms of water are shown by small violet spheres. Protein backbone, white thin lines. The molecular dynamics were simulated with the CHARMM 27 force field [104] using the program NAMD [105]. All calculations were performed at the SKIF Chebyshev supercomputer, Moscow State University. The system was energy minimized and equilibrated in a 10 ns-long molecular dynamics run, which allowed the bilayer area to relax. (*From Cherepanov, in preparation*).

(4) In BLMs, $SkQ1H_2$ protects gramicidin from being attacked by ROS at concentrations three times lower than that of $MitoQH_2$ [15].

(5) The affinity of mitochondrial cardiolipin for SkQ1 is 2.5 times higher than for MitoQ.

(6) SkQ1 is four times more hydrophobic than MitoQ.

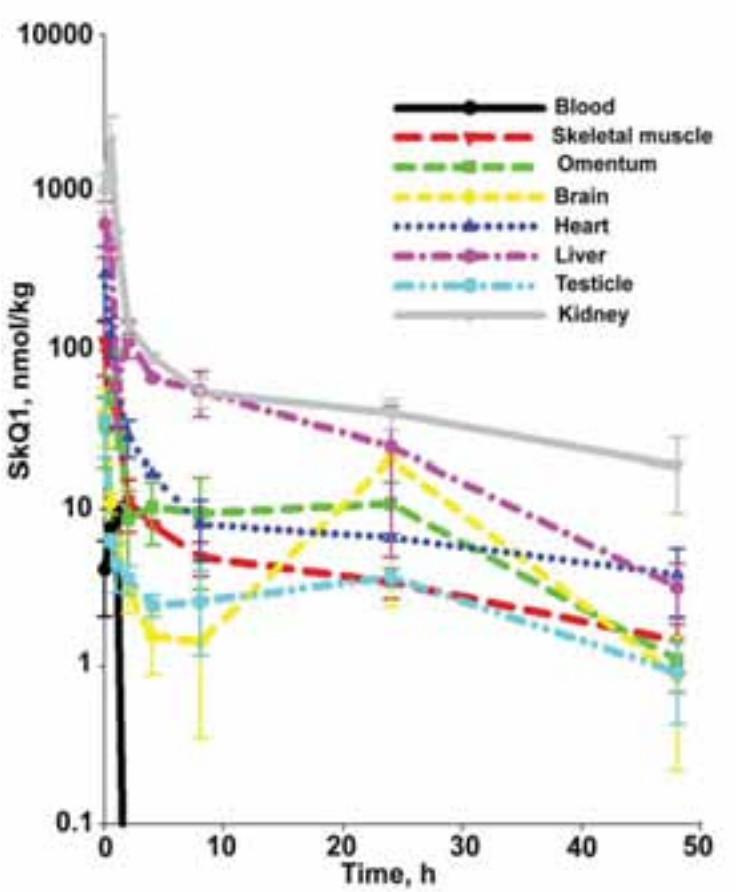


Fig. (11). Pharmacokinetics of SkQ1 after its intravenous injection into a rat. (*From Tashlitsky et al., in preparation*).

(7) SkQR1 and SkQ1 cross the phospholipid bilayer ten and two times faster than MitoQ, respectively [15, 32, 49].

It was not surprising, therefore, that *in vivo* SkQs proved to be much more efficient than MitoQ.

IN VIVO EFFECTS OF SkQ

A Single Injection of SkQ Protects Rats from the Kidney Ischemia-Induced Death

We chose kidney ischemia as an *in vivo* model for SkQ effects. This was done since direct measurements of distribution of SkQ1 between various organs after a single intravenous SkQ1 injection showed that the SkQ1 level in kidney is higher than in seven other organs and tissues tested (Fig. **11**). In the beginning, effects of SkQ1 were studied in a kidney ischemia/reperfusion model. Kidney ischemia/reperfusion is known to result in severe oxidative stress [50]. We investigated whether a single *in vivo* SkQ treatment could prevent this stress.

In experiments on kidney epithelial cell cultures, antioxidant properties of SkQs were tested. Fig. (**12**) shows that preincubation with SkQ1 increases survival of these cells and decreases apoptosis after 24 h anoxia followed by 24 h reoxygenation (not shown). Optimal concentrations of SkQ1 were between 30 and 250 nM [50]. Fig. (**13**) shows that SkQ1 prevents fission of elongated mitochondrial filaments to small roundish mitochondria induced by the anoxia/reoxygenation procedure in the same cells [50].

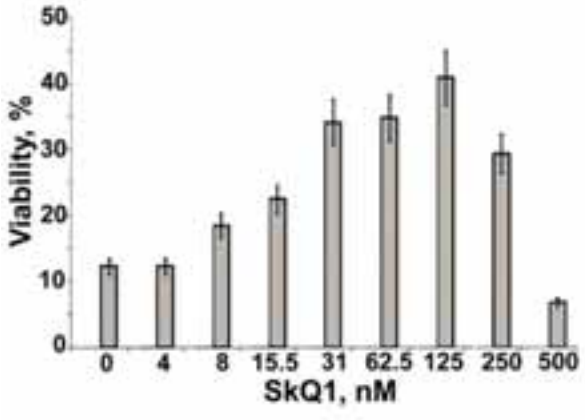


Fig. (12). A five day preincubation with SkQ prevents the kidney epithelial cells from death caused by 24 h anoxia followed by 24 h reoxygenation. A/R, anoxia/reoxygenation. A, cell viability as a function of [SkQ1]. (*From Bakeeva et al. [50]*).

Then we studied the distribution of SkQR1, a fluorescent SkQ1 derivative, in kidney slices after either intravenous or intraperitoneal injection of SkQR1 into rats. The fluorescence was clearly seen in glomerular zones of the kidney 60 min after injection of SkQR1 into the *inferior vena cava* (cf. Figs. **14A-C**). After 2 h, fluorescence decreased in these regions with parallel appearance of fluorescence in the kidney tubular epithelial cells (Figs. **14D, E**). In 3 days,

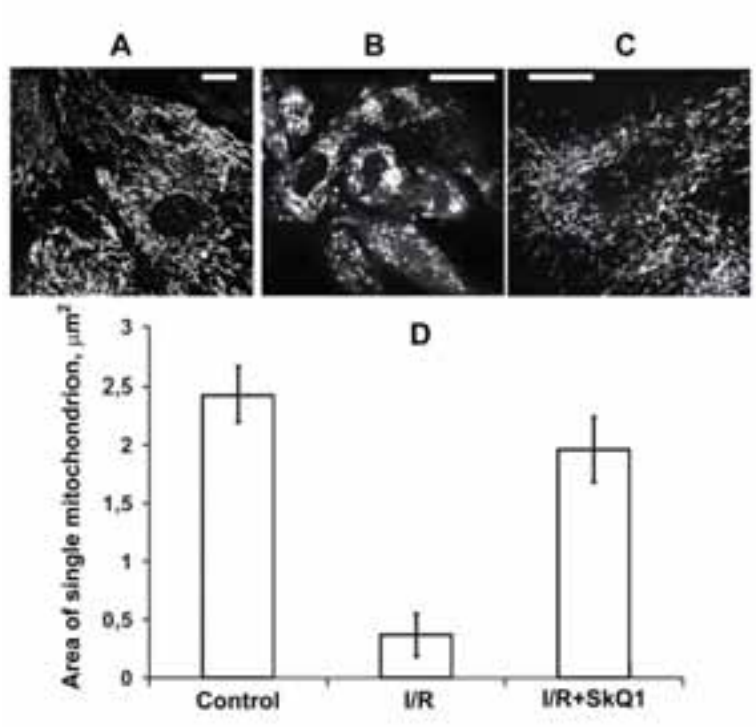


Fig. (13). Effect of SkQ1 on mitochondrial structure of kidney epithelial cells. Cells were stained with 200 nM TMRE. **A**, Control cells; **B**, cells exposed to 24 h anoxia followed by 24 h reoxygenation; **C**, cells were pretreated with 60 nM SkQ1 for 5 days before 24 h anoxia/24 h reoxygenation. Bar, 20 μm. **D**, Mean area occupied by a single mitochondrion. A/R, anoxia/reoxygenation. (*From Bakeeva et al. [50]*).

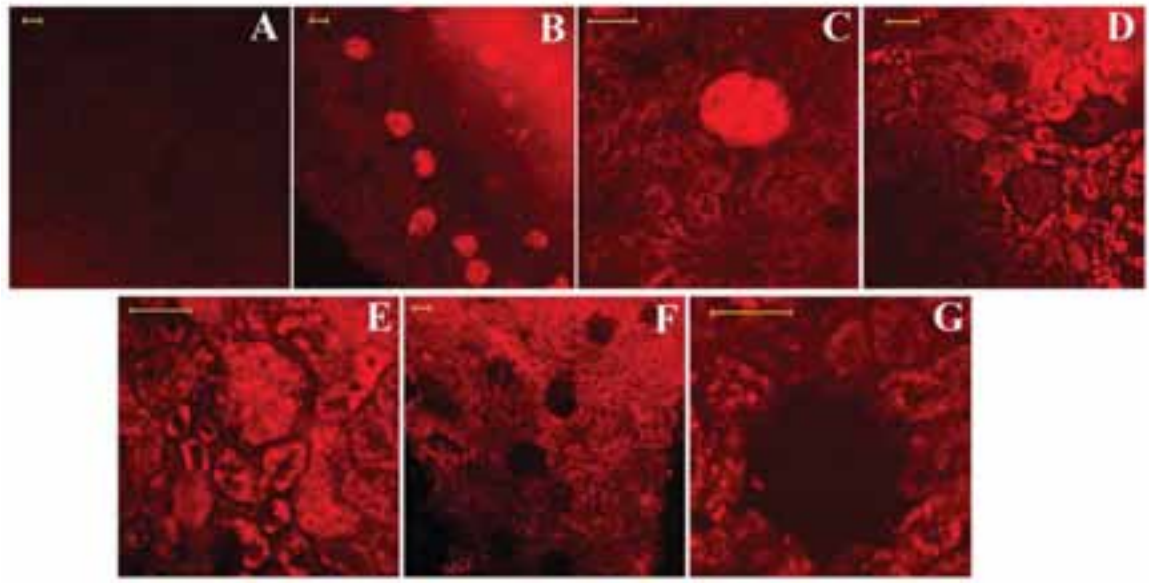


Fig. (14). Pharmacokinetics of SkQR1 distribution over kidney compartments after intravenous SkQR1 injection. **A,** 10 min after injection of 300 μl of 1 mM SkQR1 to *vena cava inferior*; **B, C,** 60 min after injection of 200 μl of 1 mM SkQR1; **D, E,** 120 min after injection of 200 μl of 0.1 mM SkQR1; **F, G,** 3 days after injection of 200 μl of 0.1 mM SkQR1. Bar, 50 μm. (*From Bakeeva et al. [50]*).

SkQR1 further accumulated in the epithelium, and fluorescence almost completely disappeared in the glomerular zones (Figs. **14F, G**). Incubation of a kidney slice taken from a rat 120 min after intraperitoneal injection of SkQR1 in the presence of mitochondrial fluorescent dye (Mitotracker Green) revealed colocalization of the two dyes in the kidney cells (not shown in figures).

After a 40-min *ex vivo* kidney ischemia followed by reperfusion, ROS production (detected by DCF fluorescence in kidney slices) was found to be significantly elevated (Fig. **15**). Earlier we demonstrated that this elevated DCF fluorescence originated from mitochondria [51]. A single intraperitoneal injection of SkQR1 (1 μmol/kg) a day before ischemia caused partial normalization of the ROS level (Fig. **15A**).

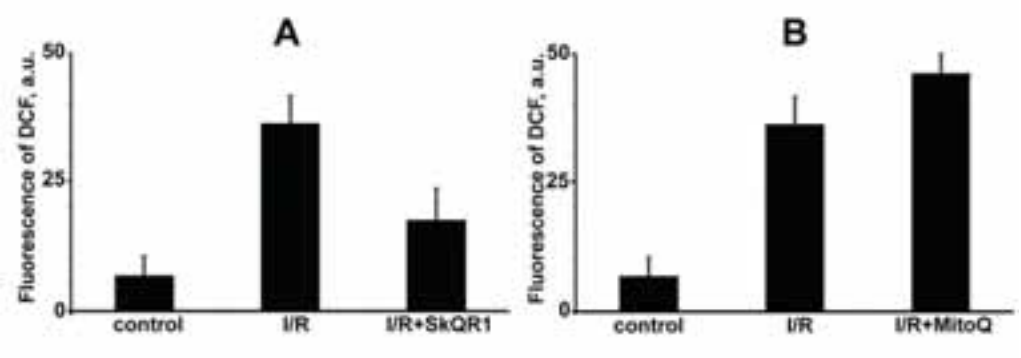


Fig. (15). Effects of ischemia/reperfusion, SkQR1, and MitoQ on kidney in single-kidney rats. ROS generation in kidney slices detected by DCF fluorescence. Control, isolated kidney; I/R isolated kidney after 40 min ischemia followed by 10 min reperfusion. I/R + SkQR1 (MitoQ), as I/R but single intraperitoneal injection of 1 μmol SkQR1 (or MitoQ)/kg one day before I/R. (*From Bakeeva et al. [50]*).

Injection of the same amount of MitoQ (Fig. **15B**) had no effect.

Some markers of kidney dysfunction after ischemia/ reperfusion are presented in Fig. (**16**). These experiments were done on animals having a single kidney (the other was removed before the ischemia). This model, recently applied, e.g., by Serviddio *et al*. [52], was chosen to put higher load on kidney function since unilateral ischemia in animals carrying two kidneys was shown to be without crucial effect on the organism (all animals survived after such a procedure). In the case of single-kidney animal experiments, there was an obvious beneficial action of SkQR1 (partial normalization of diuresis, blood creatinine concentration, and Ca^{2+} reabsorption, facts showing that this compound protects kidney tissue from a damage induced by ischemia/ reperfusion, Fig. (**16**)). However, the chosen amount of SkQ1 did not have any influence such a key marker as blood creatinine level [50]. An effect was found when the interval between of the SkQ1 treatment and ischemia was increased from 1 to 3 days. A similar situation was observed when blood urea level was measured. Favorable effect of SkQ1 on Ca^{2+} reabsorption was observed when the dose of SkQ1 was decreased from 1 μmol to 5 nmol/kg [50].

Kidney ischemia (90 min) followed by reperfusion was extremely dangerous to rats having only one kidney. The majority of the rats died on days 3-6 after such treatment. However, injection of SkQR1 or SkQ1 almost totally prevented the death of the experimental animals (Fig. **17**). The rats that survived on the critical day 6 were found to completely restore their vital functions and were alive for at least half a year after the ischemia.

In certain experiments, another kind of the SkQR1 treatment of single-kidney rats was tried, i.e. SkQR1 injections 3 h before ischemia/reperfusion and then at hours 1, 18, 30, and 42 after ischemia/reperfusion. As shown in Fig. (**18**), such treatments saved the lives of the majority of the animals and significantly prevented increase in creatinine concentration in the blood. If the SkQR1 pretreatment was excluded and SkQR1 was injected only after the ischemia/ reperfusion

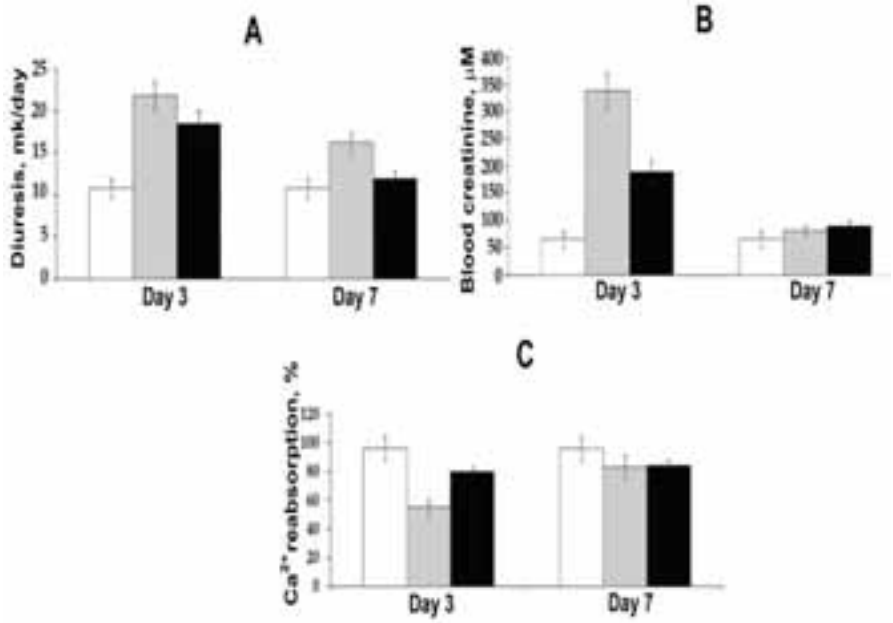


Fig. (16). Effects of SkQR1 (1 μmol/kg, intraperitoneally injected 1 day before 90 min ischemia) on kidney functioning in single-kidney rats. **A,** diuresis; **B,** blood creatinine concentration; **C,** Ca^{2+} reabsorption. White, control; gray, I/R; black, I/R + SkQR1 (*From Bakeeva et al. [50]*).

procedure, the effect of SkQR1 on survival of rats was decreased only slightly, whereas normalization of creatinine concentration completely disappeared (E. Plotnikov *et al*., in preparation).

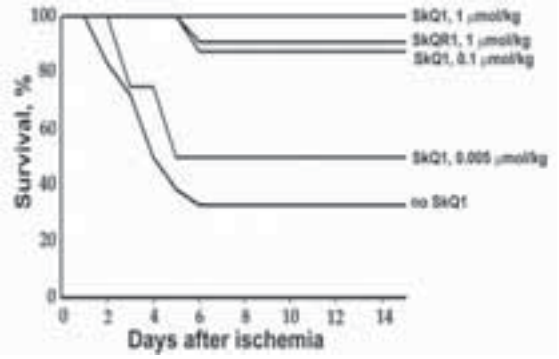


Fig. (17). Survival of single-kidney rats exposed to 90-min kidney ischemia followed by reoxygenation. Before application of the vascular clamp, another kidney was excised. SkQ1 or SkQR1 were intraperitoneally injected 1 day before the ischemia. (*From Bakeeva et al. [50]*).

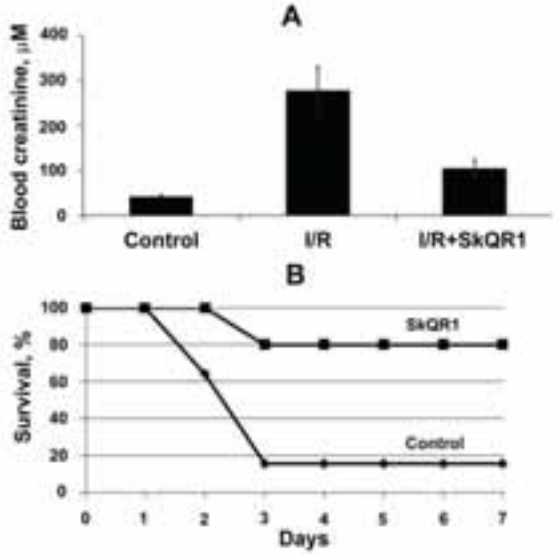


Fig. (18). Effect of 5 intraperitoneal injections of 100 nmol SkQR1/kg on single-kidney rats exposed to 40 min kidney ischemia followed by reperfusion. The injections were made 3 h before and 1, 18, 30, and 42 h after ischemia/reperfusion (*From Plotnikov et al., in preparation*). I/R, ischemia/reperfusion.

Rhabdomyolysis-Linked Kidney Failure is Successfully Treated by SkQ

In Stedman's Medical Dictionary, rhabdomyolysis is described as "an acute, fulminating, potentially fatal disease of skeletal muscle that entails destruction of skeletal muscle as evidenced by myoglobinemia and myoglobinuria" [53].

Myoglobinemia causes kidney failure that is accompanied (or even induced) by oxidative stress occurring due to cessation of normal blood circulation in this organ [54, 55]. Our study showed that mitochondria are involved in this oxidative stress. It was found that the malondialdehyde (MDA) level in kidney mitochondria isolated from rats strongly increases 4-48 h after injection of glycerol into a leg (a treatment inducing rhabdomyolysis [54]). This effect was decreased by injections of 100 nmol SkQR1/kg at 12, 24, and 36 h after initiation of rhabdomyolysis (Fig. **19**). Moreover, SkQR1 strongly lowered the level of cytochrome *c* appearing in the blood during rhabdomyolysis (Fig. **20A**). Catastrophic elevations of blood urea and creatinine were significantly prevented by the SkQR1 treatment (Fig. **20B, C**). SkQ1 and C_{12}R1 failed to substitute for SkQR1 in prevention of increase in urea and creatinine (not shown) [54]. These relationships can be explained assuming that (i) SkQR1 is much better penetrant for biomembranes than SkQ1 [49] and (ii) the plastoquinone moiety is required for the protective effect [55, 86, 108].

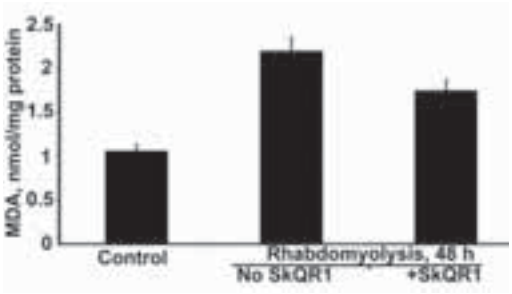


Fig. (19). MDA level in kidney mitochondria after rhabdomyolysis. Effect of injections of 100 nmol SkQR1/kg (4 injections 12, 24, 36 and 38 h after initiation of rhabdomyolysis) (*From Plotnikov et al. [55, 108]*).

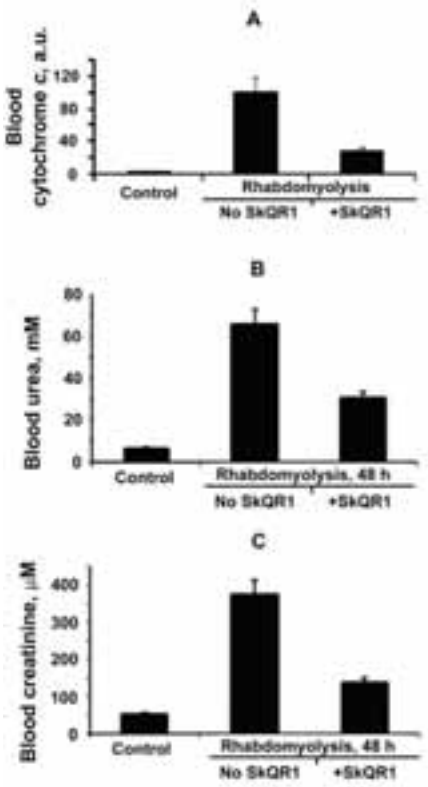


Fig. (20). (**A**) Release of cytochrome *c* to the blood 48 h after rhabdomyolysis and its inhibition by SkQR1 injections made as in Fig. (19) (*From Plotnikov et al. [54, 108]*). Increase in [urea] (**B**) and [creatinine] (**C**) in the blood 48 h after rhabdomyolysis and its inhibition by SkQR1 injections made as in Fig. (19). (*From Plotnikov et al. [54, 86]*).

Some *in vivo* Effects of Two Week SkQ Treatment

In the next experiments, we studied effects of 25 or 250 nmol SkQ1/kg (0.1 ml of SkQ1 solutions in 0.2% ethanol) administered into the cheek pouches of Wistar rats daily during two weeks. Three parameters were measured, i.e. (i) total peroxides in blood serum, (ii) 8-hydroxy-2'-deoxyguanosine in blood serum and (iii) chromosome aberrations in corneal epitheliocytes. It was shown [56] that 250 nmol SkQ1/kg per day causes more than two-fold decrease in the level of serum peroxides (Fig. **21A**) and smaller but statistically significant lowering of concentration of oxidized deoxyguanosine (Fig. **21B**). Chromosomal aberrations in the cornea were lowered almost twofold (Fig. **21C**). The majority of the chromosomal aberrations were found to be chromatid fragments in anaphase. Mitotic index was affected by SkQ1 only slightly (1.9 ± 0.13; 1.7 ± 0.1, and 1.5 ± 0.15% in rats without SkQ1 treatment and with 25 and 250 nmol SkQ1/kg per day, respectively).

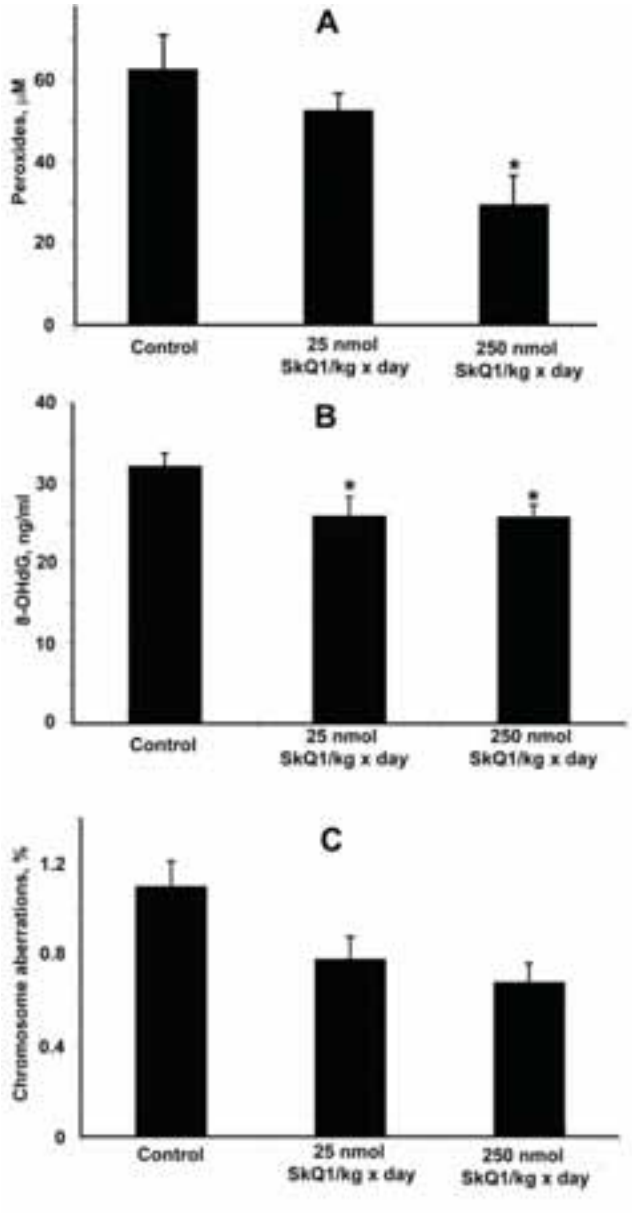


Fig. (21). Effect of SkQ1 on concentrations of peroxides (**A**) and 8-OHdG (**B**) in rat blood serum and on chromosome aberrations in cornea epitheliocytes (**C**). SkQ1 treatment, 14 days (details in text). *, <0.05. (*From Chistyakov et al. [56]*).

Life-Long SkQ Treatments Increase Median Lifespan

We investigated effect of SkQ1 on lifespan of fungi, invertebrates, fish, and mammals. We found that SkQ1 at extremely low concentrations is a geroprotector, increasing lifespan and preventing development of a number of typical traits of aging.

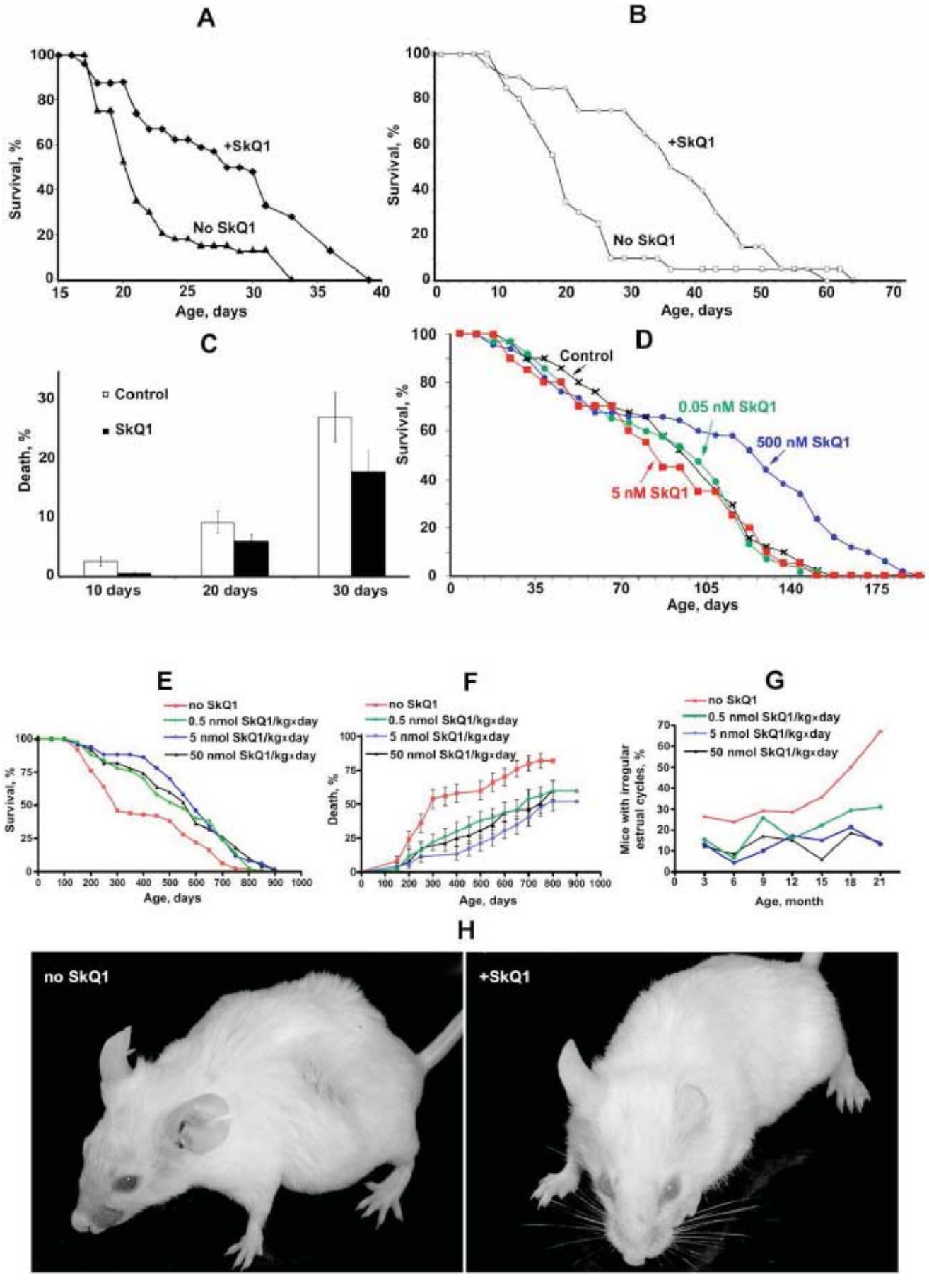
During the last decade, the mycelium of the fungus *Podospora anserina* has become a subject of interesting experiments on regulation of lifespan. In particular, it was found that duration of life of this short-lived organism can be significantly changed experimentally by affecting mitochondrial respiration, morphology, and reactive oxygen species (ROS) formation [57, 58]. We used this organism for gerontological study of the effect of SkQs.

Experiments performed by our group (Fig. **22A**) revealed that 100 nM SkQ1 added to the agar medium increases the lifespan of *P. anserina*. SkQ1 was more effective at early stages of aging. Thus, on day 21, 70% of the fungi had died without SkQ1 and only 25% had died with SkQ1. The median lifespan was increased by SkQ1 by 50%. The difference decreased with age, so that lifespan of the last 10% of the fungi was increased by only 20% (Fig. **22A**). Higher (1 μM) and lower (20 nM) SkQ1 concentrations were less effective than 100-400 nM. The effect of SkQ1 was accompanied by retardation of development of such a trait of mycelium aging as disappearance of its protrusion into the air phase [59].

The crustacean *Ceriodaphnia affinis* is a convenient subject for lifespan research since its imago life cycle is usually as short as 15-20 days and cultivation under laboratory conditions is rather easy. Another advantage is that the concentration of SkQ1 affecting the living subject can be known exactly since the *C. affinis* can live in an aqueous solution of SkQ1 of given concentration. A disadvantage of this species is the genetic heterogeneity of its population, which can affect the results. Fig. (**22B**) shows that 0.55 nM SkQ1 increased the lifespan of *C. affinis* twofold. Increasing the concentration to 55 nM SkQ1 had the opposite effect (not shown) [59].

Drosophila melanogaster is a useful model system for gerontological studies due to the availability of genetically well characterized lines and due to short lifespan. Experiments performed by our group showed that SkQ1 prolonged the lifespan of flies even though the effect was not as strong as for *C. affinis*. Extremely low SkQ1 doses were effective. Once per week, 0.1 ml of 20 pM SkQ1 solution was applied to the surface of wheat–sugar–raisin mixture and dried overnight at 25°C. This amount of SkQ1 was sufficient to increase the median lifespan by 15% (Fig. **22C**). The same effect was produced by 20 nM SkQ1, whereas 20 fM and 20 μM were in [61, 97] effective (not shown) [59].

It should be noted that the idea concerning mitochondrially-targeted quinones as geroprotectors was introduced by one of us (VPS) (for details, see [60] and by Murphy


A
Survival, %
+SkQ1
No SkQ1
Age, days
B
Survival, %
+SkQ1
No SkQ1
Age, days
C
Control
SkQ1
Death, %
10 days
20 days
30 days
D
Control
0.05 nM SkQ1
500 nM SkQ1
5 nM SkQ1
Survival, %
Age, days
E
no SkQ1
0.5 nmol SkQ1/kg×day
5 nmol SkQ1/kg×day
50 nmol SkQ1/kg×day
Survival, %
Age, days
F
no SkQ1
0.5 nmol SkQ1/kg×day
5 nmol SkQ1/kg×day
50 nmol SkQ1/kg×day
Death, %
Age, days
G
no SkQ1
0.5 nmol SkQ1/kg×day
5 nmol SkQ1/kg×day
50 nmol SkQ1/kg×day
Mice with irregular estrual cycles, %
Age, month
H
no SkQ1
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(Fig. 22) Contd…..

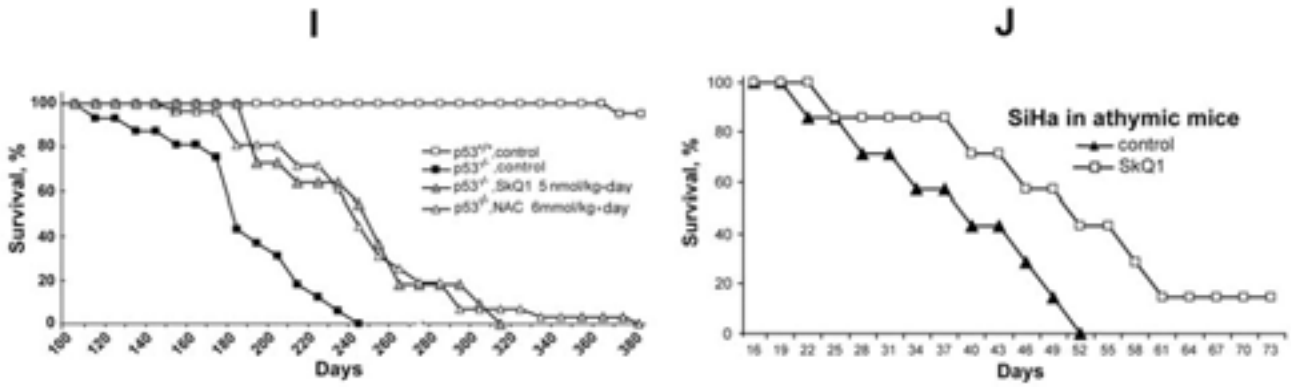

Fig. (22). Effect of SkQ1 on the lifespan of various living creatures. **A,** Effect of 100 nM SkQ1 on the lifespan of the fungus *Podospora anserina*. SkQ1 was added to the growth medium of the mycelium (*From Anisimov et al. [59]*). **B,** Effect of 0.55 nmol SkQ1 on the lifespan of crustacean *Ceriodaphnia affinis*. SkQ1 was added to the growth medium. (*From Anisimov et al. [59]*). **C,** Effect of 20 pmol SkQ1 on the mortality of *Drosophila melanogaster* w^{1118} line. One hundred microliters of 20 pM ethanol solution of SkQ1 was applied once per week on the surface of the food and dried overnight at 25 °C. (*From Anisimov et al. [59]*). **D**, Effect of different concentrations of SkQ1 on the lifespan of fish *Nothobranchius furzeri*. SkQ1 was added to the aquarium water. (From K. Shidlovsky, in preparation). **E,** SkQ increases lifespan and prevents appearance of traits of senescence in mice. The figure summarizes results of two experiments on the lifespan measurement of 200 females. In each experiment, four groups of females (25 mice in each group) were studied. **F**, Age-dependent mortality of mice studied in E, for reasons other than tumors. **G**, SkQ1 prevents disappearance with age of regular estrous cycles. **H,** 630-day-old mice receiving (right photo) and not receiving (left photo) 0.5 nmol SkQ1/kg per day. (*From Anisimov et al. [59]*). **I** and **J,** Effect of SkQ1 on the lifespan of $p53^{-/-}$ mice and athymic mice with SiHa xenografts, respectively. A log–rank test comparing the group of non-treated $p53^{-/-}$ mice and the groups treated with 5 nmol SkQ1/kg per day or 6 mmol NAC/kg per day showed two-sided distribution, $p < 0.005$. (*From Agapova et al. [69]*).

and coauthors [62]. In Murphy's group [63], an attempt was made to prolong the lifespan of *D. melanogaster* with MitoQ. No geroprotective effect was observed on wild type flies. MitoQ was effective only on females of a short-lived mutant that was deficient in mitochondrial superoxide dismutase and which showed several traits of progeria. We tried 20 pM MitoQ and observed some geroprotective effect even in wild type flies. The effect could be revealed only at early ages. The median lifespan changed insignificantly. It seems possible that for such a post-mitotic organism as a fly, mean lifespan is not a sensitive trait to reveal potential geroprotectors. It is remarkable that the geroprotective effect of SkQ1 was much more pronounced when an age-dependent *in vivo* damage to the fly muscle mitochondria was measured in our group [59].

The African fish *Nothobranchius furzeri* is an interesting subject for studying senescence. This organism has the shortest known lifespan among fish, only four months. Such a time corresponds to duration of a rainy season. The fish lives in puddles that disappear when the rainy season is over. During these four months, *N. furzeri* spawns several times and shows many typical traits of senescence, including appearance with age of lipofuscin granules in liver and β-galactosidase in dermis, neurofibrillary degeneration in brain, decline of locimitory activity as well of learning ability [64]. We measured the lifespan of *N. furzeri* in aquaria containing various concentrations of SkQ1. At 500 nM concentration, SkQ1 significantly prolonged the lifespan of the fish, whereas lower SkQ1 concentrations were ineffective (Fig. **22D**). Remarkably, the effect of SkQ1 on the shape of the survival curve proved to be very similar to that of resveratrol in experiments of another group [64].

Two long-term experiments were performed to study the effect of SkQ on the lifespan of outbred SHR mice. One of them was started in December 2004, and another in June 2005. In each experiment there were four groups of females (25 animals in each group): one group for control mice (not-treated with SkQ1), and three others for mice receiving with drinking water 0.5, 5, or 50 nmol SkQ1/kg per day. The results of the two experiments are summarized in Fig. (**22E**). All the tested SkQ1 doses significantly prolonged the life-span, the middle (5 nmol) dose being optimal. Moreover, as in the above-described experiments on fungi and invertebrates, the effect was especially strong on initial and middle stages of life rather than on maximal lifespan, which increased only slightly (so-called rectangularization of the survival curve). With the optimal SkQ1 dose, the median lifespan doubled. For mammals, such a strong positive shift of the survival curve is very rare. On day 300, more than 50% of the control mice had died, whereas in the group of 5 nmol SkQ1 dose almost all of the animals were still alive [59].

Analysis of the reasons for death revealed that the SkQ1-induced increase in lifespan is mainly due to strong lowering of rate of the death caused by diseases other than cancer (Fig. **22F**). The most common causes of death were infections, such as pneumonia, hepatitis, nephritis, and colitis, which were a consequence of the fact that the mice were living under non-sterile conditions. The action of SkQ1 was most probably a consequence of a preventive effect of SkQ1 on an age-dependent decline of the immune system. In OXYS rats (see below), SkQ1 decreases the rate of age-dependent involution of thymus and spleen follicles respon-

sible for formation of such crucial components of immunity as T- and B-lymphocytes, respectively [48].

Prevention of decline of immunity was not the only geroprotective effect of SkQ1 on rodents. In particular, it was shown that SkQ1 completely prevented disappearance of regular estrous cycles in the outbred mice (Fig. **22G**). Fig. (**22H**) shows photographs of two 630-day-old female mice, one receiving a minimal dose of SkQ1 (0.5 nmol/kg per day) and the other without SkQ1. It seems obvious that the mouse without SkQ1 is in poor condition compared with the one receiving SkQ1. The control animal had lost whiskers and showed obvious traits of baldness and lordokyphosis. We also found that torpor and body temperature decrease during the last weeks of life observed in mice not treated with SkQ1 were not pronounced in the SkQ1-treated mice. The food and water consumption and body weight did not significantly differ in the studied mice, so the effect of SkQ1 cannot be explained by caloric restriction.

In further experiments with the mice, fibroblasts obtained from the tails of the control and SkQ1-treated animals of various ages were studied in our group by I.M. Spivak. She found that spontaneous apoptosis of fibroblasts from the old control mice occurred three times more often than in young animals. This difference disappeared if mice received 0.5, 5, or 50 nmol SkQ1/kg per day. Fibroblasts from mice receiving SkQ1 retained the ability to activate apoptosis by added H_2O_2, but the effect of H_2O_2 was not as strong as in fibroblasts from untreated mice of the same age. It was also shown that the *in vivo* SkQ1 treatment prevents development of such typical traits of aging as appearance of β-galactosidase activity and phosphorylation of histone H2AX [32, 59].

Post mortem analysis of the influence of SkQ1 on mice showed that SkQ1 was without statistically significant effect on the death of mice caused by cancers other than mammary adenocarcinomas. It seemed that this adenocarcinoma was increased by SkQ1. However, further analysis revealed that this unfavorable effect is a consequence of a favorable one, namely prolongation of estrous cycles by SkQ1, rather than of any carcinogenic activity of SkQ1.

The mammary adenocarcinoma problem was studied with two breeds of mice, i.e. outbred SHR and transgenic HER-2/neu mice. In the latter case, all the mice died due to development of mammary adenocarcinomas before the age of one year. SkQ1 was without measurable effect on longevity of the HER-2/neu mice and parameters of carcinogenesis. However, SkQ1 completely prevented disappearance of regular estrous cycles in the outbred SHR mice (see above, Fig. **22G**). Thus, most probably mammary gland adenocarcinoma could not develop in the great majority of the control mice receiving no SkQ1 due to age-dependent mammary gland involution [65].

To study possible anti-oncogenic potential of SkQ1, B.P. Kopnin and his group investigated effect of diet supplementation with various SkQ1 doses on tumor appearance in $p53^{-/-}$ mice. Such mice show increased levels of ROS [66] and are highly predisposed to the spontaneous development of a variety of neoplasms starting at about three months of age. The predominant types of malignancy are T- and B-cell lymphomas (70-90%) [67, 68]. We found that 5 nmol SkQ1/kg per day caused about twofold decrease in intracellular level of ROS in mouse tissues, in particular in spleen [69]. In mice receiving this dose of SkQ1, the development of tumor was delayed, and the lifetime of the animals was increased by approximately 30%. In fact, fifty percent death for $p53^{-/-}$ mice treated and not treated with SkQ1 occurred on days 250 or 180, respectively (Fig. **22I**). Effect of SkQ1 on early death was especially demonstrative. On day 185, about 60% of the $p53^{-/-}$ mice without SkQ1 had died, whereas not a single animal had died among those who obtained SkQ1. This effect of extremely low dose of SkQ1 was comparable with the effect of very much (more than 1,000,000-fold!) higher dose of the conventional antioxidant NAC (6 mmol/kg per day) used in the previous studies [66, 67] and confirmed in the experiment shown in Fig. (**22I**). Lower (0.5 nmol/kg per day) and higher (50 nmol/kg per day) SkQ1 dosages were less effective in tumor prevention. Similar effect of SkQ1 was observed on survival of athymic mice with xenografts of human uterine cervix carcinoma SiHa [69, 32] (Fig. **22J**).

Effects of SkQ1 on Progeria

We studied three different animal model systems of accelerated aging (progeria). Special attention was given to age-related retinal pathologies. The retina is a tissue with especially high risk of ROS-induced damage since (i) it contains a high level of polyunsaturated fatty acids, (ii) it is exposed to the light producing ROS such as singlet oxygen, and (iii) oxygen concentration in the retina is near-arterial, i.e., much higher than in the great majority of other tissues (in spite of the fact that retina is a tissue of very high respiratory activity) [70, 71]. There are numerous indications of a crucial role of ROS in the main age-related ocular pathologies, i.e. retinopathies such as macular dystrophy [72, 73, 74], retinitis pigmentosa [75, 76], hereditary optic neuropathy [77]), as well as glaucoma [78, 79], cataract [72, 80, 81], and uveitis [82, 83]. Polyunsaturated fatty acids in mitochondrial cardiolipin are first of all attacked by mitochondria-produced ROS that are quenched by SkQs (see above). This is why the mentioned diseases attracted our attention as a possible field of therapeutic application of SkQs.

We investigated the therapeutic SkQ1effect on cataract and retinopathies using OXYS rats, a strain suffering from increased sensitivity to oxidative stress. In these rats, cataract and retinopathies appear as early as at 2.5-3 month age [84, 85]. Experiments showed [48, 32] that addition to the food of 250 nmol SkQ1/kg per day completely prevented development of cataract and retinopathy in OXYS rats up to the age of two years (Figs. **23A, B**, Table **3**).

Table 3. Effect of SkQ1 Added to Food (250 nmol/kg per day) on the *b*-wave Magnitude of the Rat Electroretinogram. Standard Errors are indicated. (From Neroev *et al*. [48])

Strain	OXYS			Wistar		
Age (months)	3	24	24	3	24	24
SkQ1	-	-	+	-	-	+
b-wave, μV	56±3	19±13	42±9	54±11	37±9	55±12

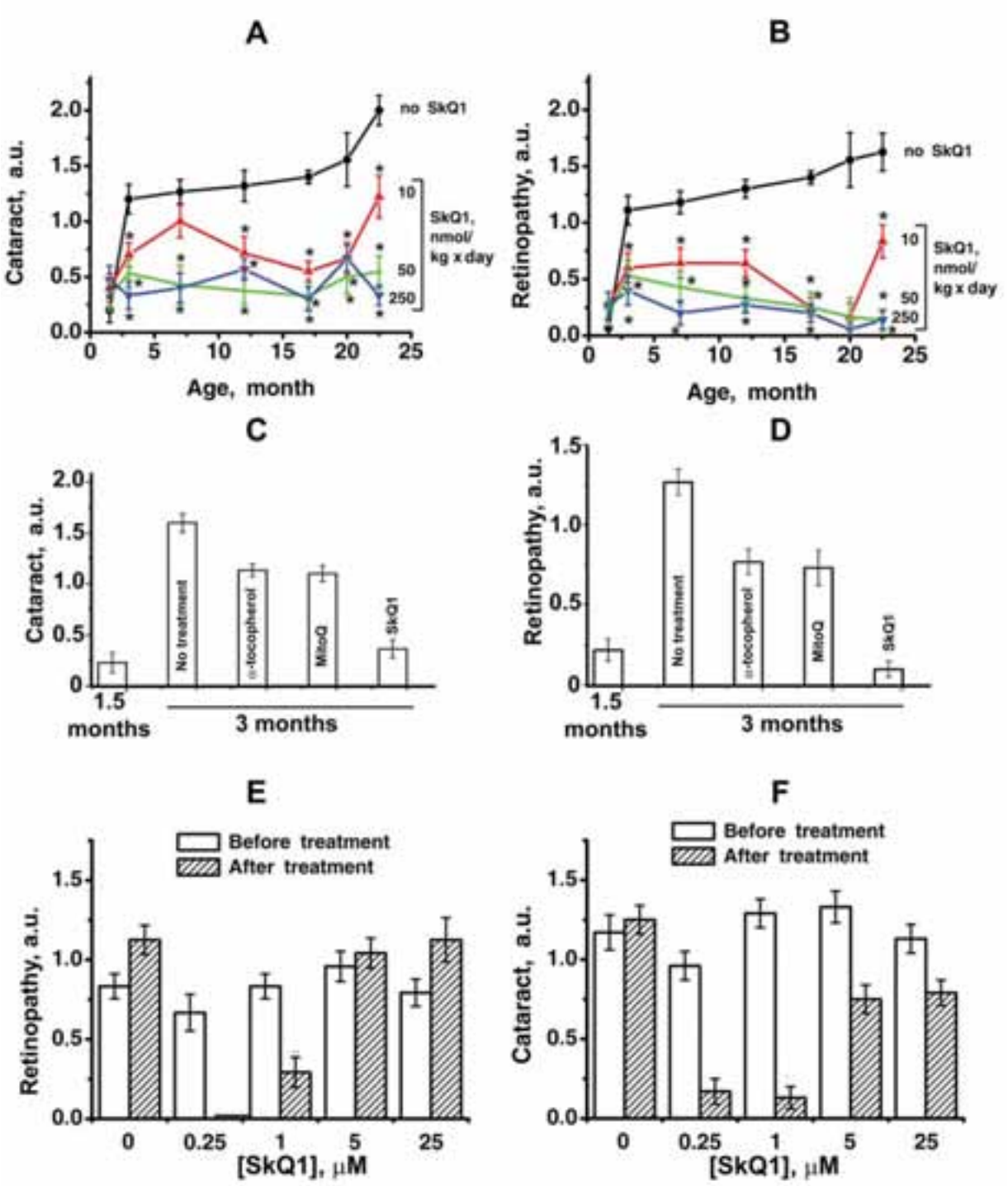

Fig. (23). Effects of SkQ1 on cataract and retinopathies in OXYS rats. **A-D,** Prophylaxis of diseases; **E, F,** Therapy of already developed diseases. **C, D,** α-Tocopherol dose, 670 μmol/kg per day; MitoQ and SkQ1 dose 250 nmol/kg per day; the treatment by α-tocopherol, MitoQ or SkQ1, the last 1.5 month. (*From Neroev et al. [48]) and Kolosova et al. in preparation*). Bars, standard errors. In A and B, ∗, $p < 0.05$ as compared to control.

The conclusion concerning preventive effect of SkQ1 against ocular pathologies on 24 month old OXYS rats was confirmed by histological analysis of sections across the retina. In old OXYS rats without SkQ1 treatment, the photoreceptor layer was absent, whereas OXYS rats receiving SkQ during all their life retained this layer. In old Wistar rats the photoreceptor layer was present even without SkQ. These results are consistent with our observations that the electroretinogram showed very low activity in the majority of 24-month-old OXYS rats but was retained in OXYS rats treated with SkQ1 as well as in Wistar rats (Table **3**).

Vitamin E (α-tocopherol) was much less efficient than SkQ1 against ocular pathologies. Even 500 μmol vitamin E/kg per day (i.e. 10,000-fold higher dose than SkQ1) decreased the cataract and retinopathy levels far less than SkQ1

(Fig. **23C, D**). It is remarkable that the effects of SkQ1 were not accompanied by any induction of cytochromes P450 in liver, in contrast to those of vitamin E [48]. MitoQ was also much less effective than SkQ1 (Figs. **23C, D**). This might explain the results of Vlachantoni *et al*. [86] who failed to obtain any positive effect on attempts to treat inherent retinopathies in rodents by MitoQ.

We also found that instillations of drops of nanomolar SkQ1 significantly reverse pathological changes in middle-age OXYS rats (Figs. **23E, F**). This effect was also observed in Wistar rats suffering from cataract. In very old (24 months) OXYS rats, cataract and retinopathy were reversed by SkQ1 only slightly (although SkQ1 still effectively prevented the diseases, see Figs. **23A, B**).

Reversal of an already developed retinopathy by SkQ1 drops was confirmed by electron microscopy. Retinopathy in 11-month-old OXYS rats results in obliteration of choriocapillaries. This parameter is at least partially normalized after a 1.5-month course of instillations of 250 nM SkQ1 (one drop per eye daily). Reappearance of choriocapillaries on the treatment with SkQ1 was accompanied by normalization of some other morphological features, i.e., distribution of lipofuscin granules in retinal pigmented epithelial cells and disappearance of hernias formed due to disruption of Bruch's membrane [48, 109].

Favorable effects of SkQ1 can disappear when it is added in excess. The optimal dose of SkQ1 is tissue-dependent [48]. In skeletal muscles of OXYS rats, 50 nmol SkQ1/kg per day decreased the level of lipid peroxidation and protein carbonylation. The effect disappeared at 250 nmol SkQ1/kg per day. In bones of the same rats, 250 nmol SkQ1 was still as effective as 50 nmol in preventing age-dependent osteoporosis. In eyes of OXYS rats, drops of 250 nM to 1 μM SkQ1 were effective in reversal of cataract and retinopathy, 5 μM being ineffective or much less effective (Figs. **23E, F**). In Wistar rats, even 25 μM SkQ1 was still favorable in treatment of cataract. These relationships can be explained assuming that (i) disappearance of therapeutic action of SkQ1 is due to prooxidant activity of its high concentrations [15] in OXYS rats suffering from oxidative stress, and (ii) the intrinsic antioxidant status of Wistar rats is much better compared with OXYS rats, so the Wistar rats are better defended against prooxidant effect of high SkQ1 doses [48, 32].

Finally, treatment with SkQ1 was tested in veterinarian practice when conventional drugs had failed. A total of 304

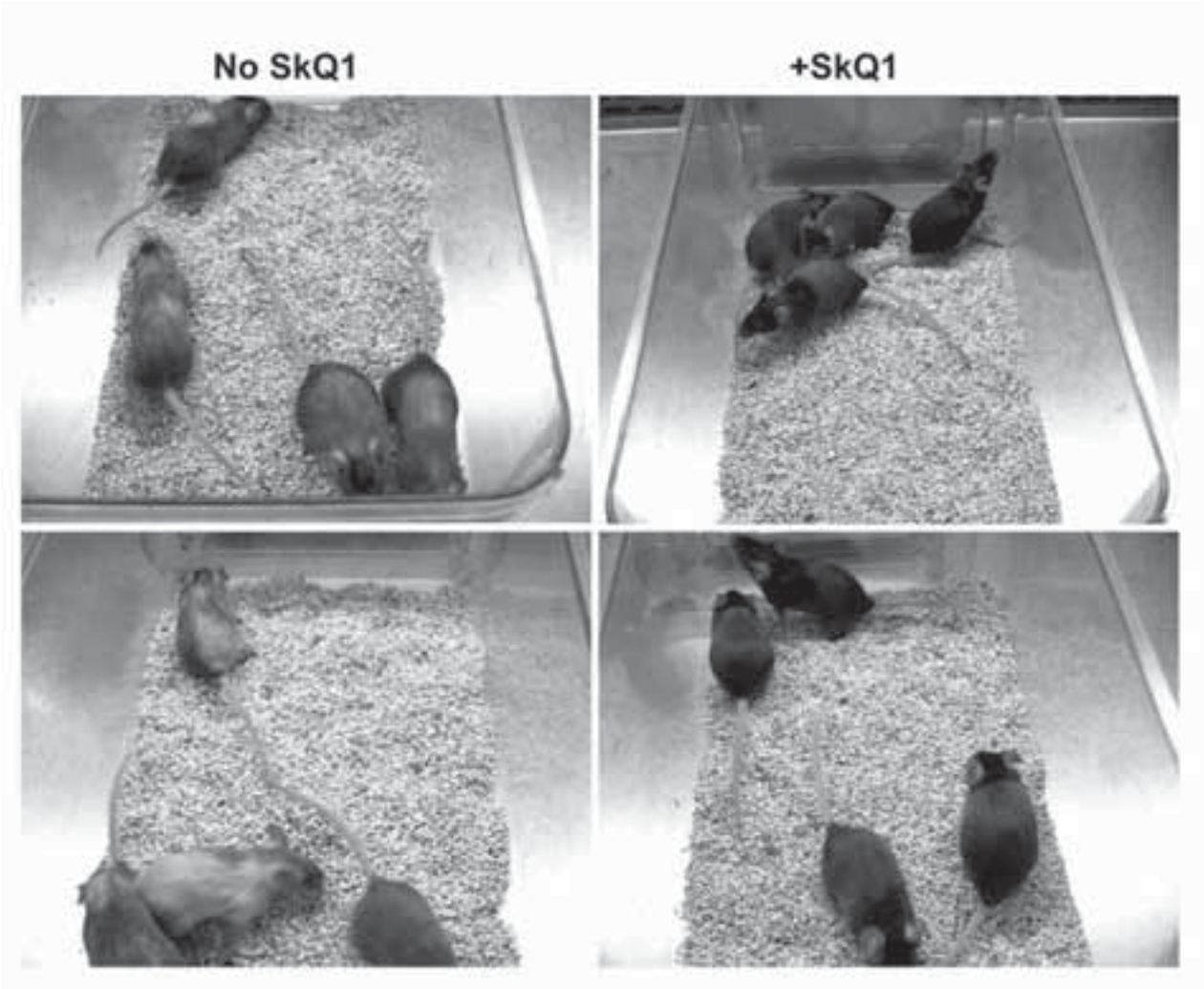

Fig. (24). SkQ1 hinders hairs graying in black mice treated with γ-irradiation. (*For explanation, see the text; from A.G. Ryazanov, in preparation*).

animals (dogs, cats, and horses) suffering from various retinopathies were treated daily with drops of 250 nM SkQ1. In 269 cases, obvious improvement was obtained. In 96 cases, the animals were completely blind before the treatment. Vision was returned to 67 of these animals. There was not a single case when SkQ1 had an unfavorable effect or its efficiency declined in the course of the treatment time [48, 32 and Sotnikova *et al*., in preparation]. At present, clinical trials of SkQ1 drops are carried out in three Moscow hospitals.

Besides the progeria in OXYS rats, two other models of accelerated aging were studied. Demonstrative data were obtained by Dr. A.G. Ryazanov from the R.W. Johnson Medical Institute, Piscataway, New Jersey, who followed development of some traits of senescence after γ-ray (7.8 Gy) treatment of black female mice C 57BL/6. Among them, the most obvious was change of the hair color. Three month after the treatment, the mice became gray due to achromotrichia. This effect was strongly diminished if the mice received 5 mmol SkQ1/kg per day for 1.5 - 3 months before irradiation and continue to receive it after this treatment (Fig. **24**). It is important that protective effect of SkQ1 on irradiation-induced hair graying was not due to an unspecific radioprotective action, which is not inherent in SkQ1. SkQ1 could not save animals from death due to hematopoietic syndrome or gastrointestinal problems caused by γ-irradiation. It did not affect apoptosis in intestinal stem cells and did not increase survival of two studied cell lines irradiated in cultures.

One more progeric model was studied in the Wenner-Gren Institute (Stockholm), where B. Cannon, J. Nedergaard, I.G. Shabalina, and coworkers studied progeric mtDNA-mutator mice with the D257A mutation in the proof-reading domain of mitochondrial DNA polymerase γ [87]. This mutant polymerase can synthesize DNA but cannot recognize and repair its own errors during synthesis. As a result, the frequency of mutations in mitochondrial DNA increases, strongly shortening the lifespan and causing premature development of numerous traits of senescence [87, 88]. It was found that SkQ1 prevents or retards appearance of many senescence traits and increases the lifespan. It should be noted that these experiments were done on animals living in a specific pathogen free animal house (Shabalina *et al*., in preparation).

CONCLUSIONS

The main results of SkQ studies are summarized in Table **4**. The striking feature of SkQs is the great multiplicity of their effects on both living creatures and their functions. SkQs prolong life of such quite different organisms as the fungus *P. anserine*, the crustacean *C. affinis*, the insect *D. melanogaster*, the fish *N. furzeri*, and mammals (mice). They help animals to survive after kidney ischemia, stroke, and heart attack. They decelerate development of many age-related diseases and traits, including cataract and some other eye diseases, balding, achromotrichia, lordokyphosis, myeloid shift of the blood, etc. The simplest explanation of the mechanism of such a multiplicity of effects is the assumption that mitochondria-targeted antioxidants "clean the dirtiest place in the cell", i.e. the mitochondrial interior, where large amounts of ROS are produced [61]. However, such an explanation fails to account for the fact that SkQs suppresses development of many traits specific for natural senescence (see Table **4**, items 1-7, 11-31). These relationships become quite understandable if one assumes that SkQs arrest or at least partially suppress operation of a program responsible for senescence.

The finding that programs encoded in the cell genome are responsible for many cases of the cell death is one of the most important discoveries of recent decades. These programs actuate different types of apoptosis and necrosis or their combinations (reviews [91-93]). Sometimes, such events include formation of mitochondrial ROS. It is already clear that the ROS-dependent cell death programs are required for ontogenesis, anticancer defense or defense against pathogens of multicellular organisms [93, 93a]. However, functionally similar programs are found in bacteria [94] and unicellular eukaryotes [95]. Based on these phenomena, we conclude that at least unicellular organisms possess mechanisms for self-elimination. We have coined this phenomenon as "phenoptosis" [92]. There is no doubt that phenoptosis is also inherent in multicellular organisms [93, 96, 97, 99], but its molecular mechanisms are still to be elucidated. The proof of the genetically programmed death of individuals has markedly reinforced positions of a few gerontologists who, following the great biologists of the second half of the XIX century—Darwin [98], Wallace, and Weismann [99]—are inclined to consider aging and death of an organism as a final stage of ontogenesis and not a simple consequence of accumulation of accidental errors.

In 1964, Hamilton published a series of two articles entitled "The Genetic Evolution of Social Behavior" [100]. In 1976, the book "The Selfish Gene" [101] was published, where the author Richard Dawkins developed and popularized the idea of that not a species, group, or even an individual was the main unit of natural selection, but that the gene was this unit. In essence, here we are dealing not with the wellbeing of a community but with dictatorship of the genome (the only self-reproducing biological structure) and, hence, its retention, development, and expansion have priority compared to the wellbeing of an individual or a group of individuals. According to this concept, an organism is only a construction, a machine, serving interests of the genome. Some years ago one of us (VPS) formulated the so-called "Samurai Law of Biology—"It is better to die than to be wrong", or in more extended form, "Complex biological systems (organelles and higher) are equipped with programs of self-elimination which are actuated when the system becomes dangerous for any other system of higher position in the biological hierarchy" [93]. Combined with the concept of genome dictatorship, this principle means that any critical state of the organism, when it is unable to ensure the safety of its genome and therefore in the future can produce offspring with significantly altered genome, has to be a signal for the organism's self-elimination, i.e. phenoptosis. As spoke Monsieur Bahys, the good-for-nothing physician in Moliere's comedy "L'Amour Medecin" ("Love as a Healer")—"Il vaut mieux mourir selon les regles, que de rechapper contre les regles" ("It is better to die according to the rules than to recover against the rules"). It is likely that mechanisms of rapid phenoptosis ("the Bahys principle") and slow phenoptosis (senescence) are mediated by intramitochondrial ROS at an early stage of the process (for

Table 4. *In vivo* Effects of SkQs

N	Effect	Species	Type of SkQ Treatment	Refs.
1	Increase in the median lifespan	Fungus *Podospora anserina*	Addition SkQ1 to the growth medium	[32, 59]
2	Increase in the median lifespan	Crustacean *Ceriodaphnia affinis*	Life-long addition of SkQ1 to the growth medium	[59]
3	Increase in the median lifespan	Fly *Drosophila melanogaster*	Life-long instillation of SkQ1 on the food surface	[59]
4	Increase in median and maximal lifespan	Fish *Nothobranchius furzeri*	Life-long addition of SkQ1 to aquarium water	this paper
5	Increase in the median lifespan	mice	Life-long drinking of SkQ1 solution	[32, 59]
6	Increase in the median and maximal lifespan	mtDNA mutator mice	Life-long drinking of SkQ1 solution	I. Shabalina *et al*., in preparation
7	Increase in the maximal lifespan	$p53^{-/-}$ mice or athymic mice with human cervical carcinoma SiHa xenografts	Life-long SkQ1 addition to the food	[69]
8	Decrease of peroxide level in blood	Rats	Daily SkQ1 instillation to cheek pouches for 14 days	[56], this paper
9	Decrease in the blood 8-hydroxy-2'-deoxyguanosine	Rats	Daily SkQ1 instillation to cheek pouches for 14 days	[56], this paper
10	Decrease in chromasomal aberrations in corneal epitheliocytes	Rats	Daily SkQ1 instillation to cheek pouches for 14 days	[56], this paper
11	Prevention of lipid peroxidation and protein carbonylation in skeletal muscles	OXYS rats	Life-long eating of SkQ1-supplemented food	[32, 48]
12	Decrease in level of cardiolipin in skeletal muscles and liver	mtDNA mutator mice	Life-long drinking of SkQ1 solution	I. Shabalina *et al*., in preparation
13	Decrease in ratio of unsaturated/ saturated fatty acids in cardiolipin in skeletal muscles and liver	mtDNA mutator mice	Life-long drinking of SkQ1 solution	I. Shabalina *et al*., in preparation
14	Prevention of age-related increase in ROS-induced apoptosis of fibroblasts	Mice	Life-long drinking of SkQ1 solution	[32, 59]
15	Prevention of age-related increase in β-galactosidase in fibroblasts	Mice	Life-long drinking of SkQ1 solution	[32, 59]
16	Prevention of age-related increase in phosphorylation of histone H2AX in fibroblasts	Mice	Life-long drinking of SkQ1 solution	[32, 59]
17	Prevention of age-related myeloid shift in blood cells	Mice	Life-long drinking of SkQ1 solution	[89]
18	Deceleration of age-related damage to hematopoietic system	Mice	Life-long drinking of SkQ1 solution	[89]
19	Strong decrease in infection-induced mortality	Mice	Life-long drinking of SkQ1 solution	[32, 59]
20	Deceleration of age-related involution of thymus and spleen follicles	OXYS rats	Life-long eating of SkQ1-supplemented food	[32, 48, 106]
21	Deceleration of age-related osteoporosis	OXYS rats	Life-long eating of SkQ1-supplemented food	[32, 48]
22	Prevention of age-related lordokyphosis	Mice	Life-long drinking of SkQ1 solution	[32, 59]
23	Prevention of age-related decline in wound healing	Rats	Life-long drinking of SkQ1-supplemented water	[90]
24	Prevention of age-related disappearance of estrous cycles in females	Mice	Life-long drinking of SkQ1-supplemented water	[48]
25	Prevention of age-related decrease in sexual motivation of males	OXYS rats	Life-long eating of SkQ1-supplemented food	[32, 48]
26	Prevention of age-related changes on behavior	Rats	Life-long drinking of SkQ1-supplemented water	[107]
27	Prevention of age-related balding and loss of whiskers	Mice	Life-long drinking of SkQ1 solution	[32, 59]

(Table 4) Contd…..

N	Effect	Species	Type of SkQ Treatment	Refs.
28	Prevention of age-related cataract and retinopathies	OXYS rats	Life-long eating of SkQ1-supplemented food	[32, 48]
29	Reversal of age-related cataract and retinopathies	OXYS rats	SkQ1 addition to the food or instillations of SkQ1 to eye for 1,5 months	[32, 48]
30	Reversal of age-related retinopathies	Dogs, cats, horses	Instillations of SkQ1 to eye for 1-3 moths	[32, 48]
31	Prevention of achromotrichia after γ-irradiation	Black mice	Drinking of SkQ1 solution for 4.5 - 6 months	this paper
32	Prevention of death after kidney ischemia	Single-kidney rats	Single injection of SkQ1 or SkQR1 a day before ischemia	[32, 50, 54]
33	Partial normalization of blood [creatinine], diuresis and Ca^{2+} resorption after kidney ischemia	Single-kidney rats	Single injection of SkQ1 or SkQR1 a day before ischemia	[32, 50, 54]
34	Normalization of kidney functioning in rhabdomyolysis	Rats	SkQR1 injections on hours 1, 12, 24 and 36 after initiation of rhabdomyolysis	[54, 108], this paper
35	Decrease in brain damage and prevention of locomotory dysfunction after brain ischemia	Rats	Single injection of SkQR1 before ischemia	[32, 50, 54]
36	Decrease in heart damage after heart ischemia	Rats	SkQ1 addition to drinking water for 2-3 weeks before ischemia	[32, 50]
37	Decrease in arrhythmia after heart ischemia	Rats	SkQ1 addition to drinking water for 2-3 weeks before ischemia	[32, 50]
38	Prevention or reversal of experimental uveitis	Rabbits	Instillations of SkQ1 to eye for 1-2 moths	[48]
39	Partial prevention of experimental glaucoma	Rabbits	Instillations of SkQ1 to eye for 1-2 moths	[48]

reviews, see [91, 93, 97]). If this hypothesis is correct, the favorable effect of SkQs not only in aging but also in different acute diseases of young organisms (see Table **4**, items 32-40) can be explained by quenching of these ROS.

It is likely that SkQs can be used as a tool in "rise of the machines"—in the attempt of *Homo sapiens* to put an end to genome tyranny and to cancel those of the genome-dictated programs that are useful for the genome but counter-productive for the individual. Acute phenoptosis of a human actuated in a critical state and terminating his existence by biochemical suicide seems to be as harmful an atavism as aging. There might be other genetic programs counterproductive for the organism. Cancellation of all of them would symbolize a conversion of humans to *Homo sapiens liberatus*, which would be the highest achievement of twenty-first century medicine [91].

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ABBREVIATIONS

Δψ = Transmembrane electric potential

AAPH = 2,2'-Azobis(2-amidinopropane) dihydrochloride

BLM = Bilayer planar phospholipid membrane

C_{12}TPP = Dodecyltriphenylphosphonium

C_{12}R1 = Dodecylrhodamine 19

DCF = 2',7'-Dichlorodihydrofluoroscein diacetate

DMQ = 3-Demethoxy ubiquinonyl decyltriphenylphosphonium

DPQ = Decylplastoquinone

MDA = Malondialdehyde

MitoQ = Ubiquinonyl decyltriphenylphosphonium

NAC = *N*-acetyl cysteine

ROS = Reactive oxygen species

SkQs = A family of penetrating cations containing plastoquinone or 5-methylplastoquinone

SkQ1 = Plastoquinonyl decyltriphenylphosphonium

SkQ3 = 5-Methylplastoquinonyl decyltriphenylphosphonium

SkQ2M = Plastoquinonyl decylmethylcarnitine

SkQ4 = Plastoquinonyl decyltributylammonium

SkQ5 = Plastoquinonyl amyltriphenylphosphonium

SkQB = Plastoquinonyl decylberberine

SkQP = Plastoquinonyl decylpalmatine

SkQR1 = Plastoquinonyl decylrhodamine 19

SkQR4 = Plastoquinonyl decylrhodamine B

TPP = Tetraphenylphosphonium

TMRE = Tetramethylrhodamine ethyl ester

FCCP = Carbonyl cyanide p-trifluoromethoxyphenylhydrazone

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