



## Review

## An attempt to prevent senescence: A mitochondrial approach

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## ARTICLE INFO

## Article history:

Received 23 September 2008

Received in revised form 16 December 2008

Accepted 18 December 2008

Available online 29 December 2008

## Keywords:

Aging

Senescence

Mitochondria

Reactive oxygen specie

SkQ

Antioxidant

## ABSTRACT

Antioxidants specifically addressed to mitochondria have been studied to determine if they can decelerate senescence of organisms. For this purpose, a project has been established with participation of several research groups from Russia and some other countries. This paper summarizes the first results of the project. A new type of compounds (SkQs) comprising plastoquinone (an antioxidant moiety), a penetrating cation, and a decane or pentane linker has been synthesized. Using planar bilayer phospholipid membrane (BLM), we selected SkQ derivatives with the highest permeability, namely plastoquinonyl-decyl-triphenylphosphonium (SkQ1), plastoquinonyl-decyl-rhodamine 19 (SkQR1), and methylplastoquinonyldecyltriphenylphosphonium (SkQ3). Anti- and prooxidant properties of these substances and also of ubiquinonyl-decyl-triphenylphosphonium (MitoQ) were tested in aqueous solution, detergent micelles, liposomes, BLM, isolated mitochondria, and cell cultures. In mitochondria, micromolar cationic quinone derivatives were found to be prooxidants, but at lower (sub-micromolar) concentrations they displayed antioxidant activity that decreases in the series SkQ1 = SkQR1 > SkQ3 > MitoQ. SkQ1 was reduced by mitochondrial respiratory chain, i.e. it is a rechargeable antioxidant. Nanomolar SkQ1 specifically prevented oxidation of mitochondrial cardiolipin. In cell cultures, SkQR1, a fluorescent SkQ derivative, stained only one type of organelles, namely mitochondria. Extremely low concentrations of SkQ1 or SkQR1 arrested H<sub>2</sub>O<sub>2</sub>-induced apoptosis in human fibroblasts and HeLa cells. Higher concentrations of SkQ are required to block necrosis initiated by reactive oxygen species (ROS). In the fungus *Podospora anserina*, the crustacean *Ceriodaphnia affinis*, *Drosophila*, and mice, SkQ1 prolonged lifespan, being especially effective at early and middle stages of aging. In mammals, the effect of SkQs on aging was accompanied by inhibition of development of such age-related diseases and traits

**Abbreviations:**  $\Delta\psi$ , transmembrane electric potential; BLM, planar bilayer phospholipid membrane; C<sub>12</sub>TPP, dodecyl-triphenylphosphonium; DCF, 2',7'-dichlorodihydrofluorescein diacetate; DMQ, demethoxyMitoQ (MitoQ lacking a methoxy group); DPQ, decyl plastoquinone; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; MitoQ, compound composed of ubiquinone and decyl-triphenylphosphonium; NAC, N-acetyl cysteine; ROS, reactive oxygen species; SkQ, compounds composed of plastoquinone or methylplastoquinone and decyl (or amyl) triphenylphosphonium, rhodamine 19, methylcarnitine, or tributylammonium; SkQ1, compound composed of plastoquinone and decyl-triphenylphosphonium

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as cataract, retinopathy, glaucoma, balding, canities, osteoporosis, involution of the thymus, hypothermia, torpor, peroxidation of lipids and proteins, etc. SkQ1 manifested a strong therapeutic action on some already pronounced retinopathies, in particular, congenital retinal dysplasia. With drops containing 250 nM SkQ1, vision was restored to 67 of 89 animals (dogs, cats, and horses) that became blind because of a retinopathy. Instillation of SkQ1-containing drops prevented the loss of sight in rabbits with experimental uveitis and restored vision to animals that had already become blind. A favorable effect of the same drops was also achieved in experimental glaucoma in rabbits. Moreover, the SkQ1 pretreatment of rats significantly decreased the H<sub>2</sub>O<sub>2</sub> or ischemia-induced arrhythmia of the isolated heart. SkQs strongly reduced the damaged area in myocardial infarction or stroke and prevented the death of animals from kidney ischemia. In p53<sup>-/-</sup> mice, 5 nmol/kg × day SkQ1 decreased the ROS level in the spleen and inhibited appearance of lymphomas to the same degree as million-fold higher concentration of conventional antioxidant NAC. Thus, SkQs look promising as potential tools for treatment of senescence and age-related diseases.

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## 1. Introduction: hypothesis on mitochondria-targeted antioxidants as geroprotectors

The free radical hypothesis of Harman [1] is the most frequently quoted concept on the mechanism of aging. Harman supposed that oxidation of biopolymers by reactive oxygen species (ROS) plays a leading role in weakening of vital functions of organism with age. This hypothesis has been supported by the finding that the oxidation level of DNA, proteins, and lipids increases with aging [2–4]. Such an effect can be a consequence of an increase with age in ROS production or a decrease in the antioxidant defense in old age, or simply a result of a damaging ROS action cumulated proportionally to the organism's age [2,5,6].

A question arises – what is the source of ROS involved in the aging processes? The cell contains a set of enzymes that convert O<sub>2</sub> to the primary forms of ROS: superoxide (O<sub>2</sub><sup>-</sup>) or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [2,7]. However, the activity of all these enzymes is significantly lower than the activity of the mitochondrial respiratory chain. Mitochondria of a human adult take up about 400 l of O<sub>2</sub> per day and convert it to water by four-electron reduction. If even a small portion of this amount of O<sub>2</sub> is reduced through the chemically simpler one-electron pathway, this will produce such a O<sub>2</sub><sup>-</sup> quantity which might greatly exceeds the abilities of all other mechanisms of ROS generation taken together.

In the respiratory chain, O<sub>2</sub><sup>-</sup> is mainly generated in Complexes I and III. The highest rate of superoxide generation in intact mitochondria in the absence of any inhibitors is observed in Complex I carrying out reverse transfer of electrons from CoQH<sub>2</sub> to NAD<sup>+</sup> at the maximal proton potential (i.e. in the absence of ADP). This rate equal to 1 nmol O<sub>2</sub>/mg protein per minute is nearly fivefold higher than the rate of O<sub>2</sub><sup>-</sup> production in Complex III under the same conditions [8]. It is 10% of the respiration rate without ADP (in state 4) and about 1.5% of the maximal respiration rate in the presence of ADP (in state 3). Such an amount of superoxide is impressive if one takes into account the high toxicity of products to which O<sub>2</sub><sup>-</sup> can be converted, i.e. OH<sup>•</sup>, HO<sub>2</sub><sup>•</sup> and ONOO<sup>-</sup>. In fact, we carry in our mitochondria a potential generator of very strong toxins that can kill not only single cells but also an entire organism. Such a catastrophe will be caused not only by the direct toxic action of ROS, but also as a consequence of triggering apoptosis and necrosis, which are easily induced by ROS.

Recently, Lambert et al. [9] reported that the lifetime of mammals and birds is longer the lower is the rate of H<sub>2</sub>O<sub>2</sub> generation by heart mitochondria during the reverse transfer of electrons from succinate through Complex I (animals of 12 species very differently systematically positioned were studied: from mice to cattle and baboons and from quails to pigeons). In fact, this observation confirmed earlier results of Sohail et al. [10] and Barja et al. [4,11] for a smaller number of warm-blooded species (see Discussion for an interesting exception to this). No correlation was shown between lifespan and ROS production during the forward electron transfer in respiratory chain [9]. Moreover, no indications could be found in the available literature that rate

of ROS production by other ROS-generating mechanisms somehow affects the lifespan.

These observations suggest that protection of cells against mitochondrial ROS might arrest or at least decelerate aging. As to the treatment of aging with antioxidants, the literature is rather vast and ambiguous: from the statement of Ames et al. [12,13] that such a drug against age is already found to conclusions of Bjelakovic et al. [14] and Howes [15] about a complete fruitlessness of this approach and, thus, the erroneousness of Harman's hypothesis.

We think that there are three essential omissions in the studies on the treatment of aging with antioxidants. (1) If it is necessary to neutralize just intramitochondrial ROS generated by Complex I protruding into the mitochondrial matrix, antioxidants specifically addressed to mitochondrial interior are required. However, there is not a single example of a successful use of such antioxidants in gerontology, described in the literature. Ames et al. [13] employed *tert*-butylhydroxylamine as geroprotector and stated that this compound is accumulated by mitochondria. However, not a single piece of evidence was reported for such a statement. (2) Commonly used antioxidants (even if they principally can occur, as in the case of vitamin E, in the mitochondrial membrane) are not specifically targeted to this membrane and are also present in other cellular membranes. This may result in unfavorable side effect due to involvement of ROS in certain metabolic regulations. Our goal should be to prevent an age-related increase in the level of *intramitochondrial* ROS rather than to discharge any ROS in cells and tissues. (3) All of them are *natural* substances, and their excess can be cleared by cellular enzymes. We are dealing with just such a situation when vitamin E is used to retard aging. Assuming that aging is programmed [2,5,6,16–20], it is reasonable to suggest that the organism seeks to realize the aging program. Therefore, the coming vitamin E (which potentially might be inhibitory to the aging mechanism) is attacked by cytochrome P450 which is induced in the liver by excess vitamin E [21].

An “ideal” geroprotector operating as an antioxidant has to meet the following criteria: 1) the antioxidant must not remove ROS completely, but only their excess generated inside mitochondria by reverse electron transfer in the respiratory chain; 2) the antioxidant must not induce cytochrome P450 or any other enzymes in the organism which is by all means seeking to terminate its ontogenesis by switching on the aging program [2,5,6,22]<sup>1</sup>.

The problem of mitochondria-targeted antioxidant might be solved based on the phenomenon observed in our group jointly with the group of Dr. E. A. Liberman already in 1969–1970 [23–26]. The case in point is the discovery of so-called penetrating ions, i.e. synthetic hydrophobic compounds that can easily penetrate across the mitochondrial membrane in spite of the presence of ionized atoms

<sup>1</sup> Ref. [22] as well as English versions of our other papers published in Biochemistry (Moscow) and quoted in this review is available in Internet (see <http://protein.bio.msu.ru/biokhimiya>).

in their molecules. Alkyl triphenylphosphonium is a typical representative of such ions. In this cation, the positive charge on the phosphorus atom is strongly displaced by three hydrophobic phenyl residues. As a result, water dipoles cannot be held on the cation and do not form an aqueous shell preventing its penetration into the hydrophobic regions of the membrane. The interior of mitochondria is the only cell compartment negatively charged relative to its environment (i.e. to the cytosol) [26,27]; therefore, on entering the cell, penetrating cations will be accumulated within mitochondria. This accumulation should be described by the Nernst equation (a 10-fold gradient at  $\Delta\psi = 60$  mV). If in the energized mitochondria  $\Delta\psi$  is  $\sim 180$  mV, the penetrating cation concentration in the mitochondrial matrix will be 1000 times higher than in the cytosol.

This consideration led us to an assumption that penetrating cations can be used by mitochondria as “molecular electric locomotives” to accumulate uncharged substances attached to these cations [28]. We used such an idea, trying to explain the role of cationic group of carnitine in the transport of fatty acid residues into mitochondria [27,28]. On being electrophoretically concentrated in the inner leaflet of the inner mitochondrial membrane, fatty acyl and carnitine would be able to act as antioxidant interrupting chain reactions of peroxidation of phospholipids and proteins which constitute this leaflet. Such a suggestion has been confirmed by data obtained in our group by Drs. Yu. N. Antonenko and A. A. Pashkovskaya. They found that palmitoyl carnitine prevented a ROS-induced inactivation of gramicidin channel incorporated into a BLM (the paper in preparation).

At the border of the last two centuries, the principle of “molecular electric locomotives” was successfully used by Dr. M. P. Murphy for the addressed delivery into mitochondria of antioxidants thiobutyl [29], vitamin E [30], and ubiquinone [31]. The so-called MitoQ composed of ubiquinone and decyl-triphenylphosphonium cation seemed to be especially promising. MitoQ is obviously more advantageous than palmitoyl carnitine or cationic derivative of vitamin E because its reduced form oxidized during the antioxidant process can be regenerated by accepting electrons from the respiratory chain. In other words, MitoQ is a *rechargeable* antioxidant [32]. The experiments of Murphy and his group clearly showed that MitoQ was accumulated and regenerated within mitochondria and protected them and also cell cultures against oxidative stress [30–36].

We have confirmed the data of Murphy et al. on the antioxidant activity of MitoQ but have found that this activity turns to prooxidant with a rather small increase in the MitoQ concentration [37] (for other observations about the prooxidant effect of MitoQ, see [33,38,39]). Thus, in rat heart mitochondria, the  $C_{1/2}$  values for the MitoQ antioxidant and prooxidant effects were shown to be 0.3 and 0.5  $\mu\text{M}$ , respectively (see below, Fig. 2B). Addition of 1.5–2.5  $\mu\text{M}$  MitoQ caused such a fast generation of hydrogen peroxide by mitochondria oxidizing NAD-dependent substrates that its rate was higher than all recorded values described in the literature, approximating the respiration rate in state 4 [37]. Such a situation makes risky the use of MitoQ as an antioxidant in practice. Therefore, we decided to start a search for antioxidants stronger than MitoQ and with a broader window between the anti- and prooxidant effects.

## 2. SkQ: synthesis and *in vitro* studies

The project of using penetrating ions in medical practice was started in 2003. It was initiated owing to grants from the “Paritet” charitable foundation (now “Volnoe Delo”) created by Mr. O. V. Deripaska, a member of the Moscow University Board of Trustees.

In the beginning, demethoxyMitoQ (DMQ), a compound where CoQ moiety lacks one of  $\text{CH}_3\text{-O-}$  groups, has been synthesized. This was done since it was already known that the nematode *Caenorhabditis elegans* lives sevenfold longer if it contains a deletion in the genes encoding (i) the enzyme that converts demethoxyCoQ into ubiquinone and (ii) the insulin receptor [40]. It was found that the new

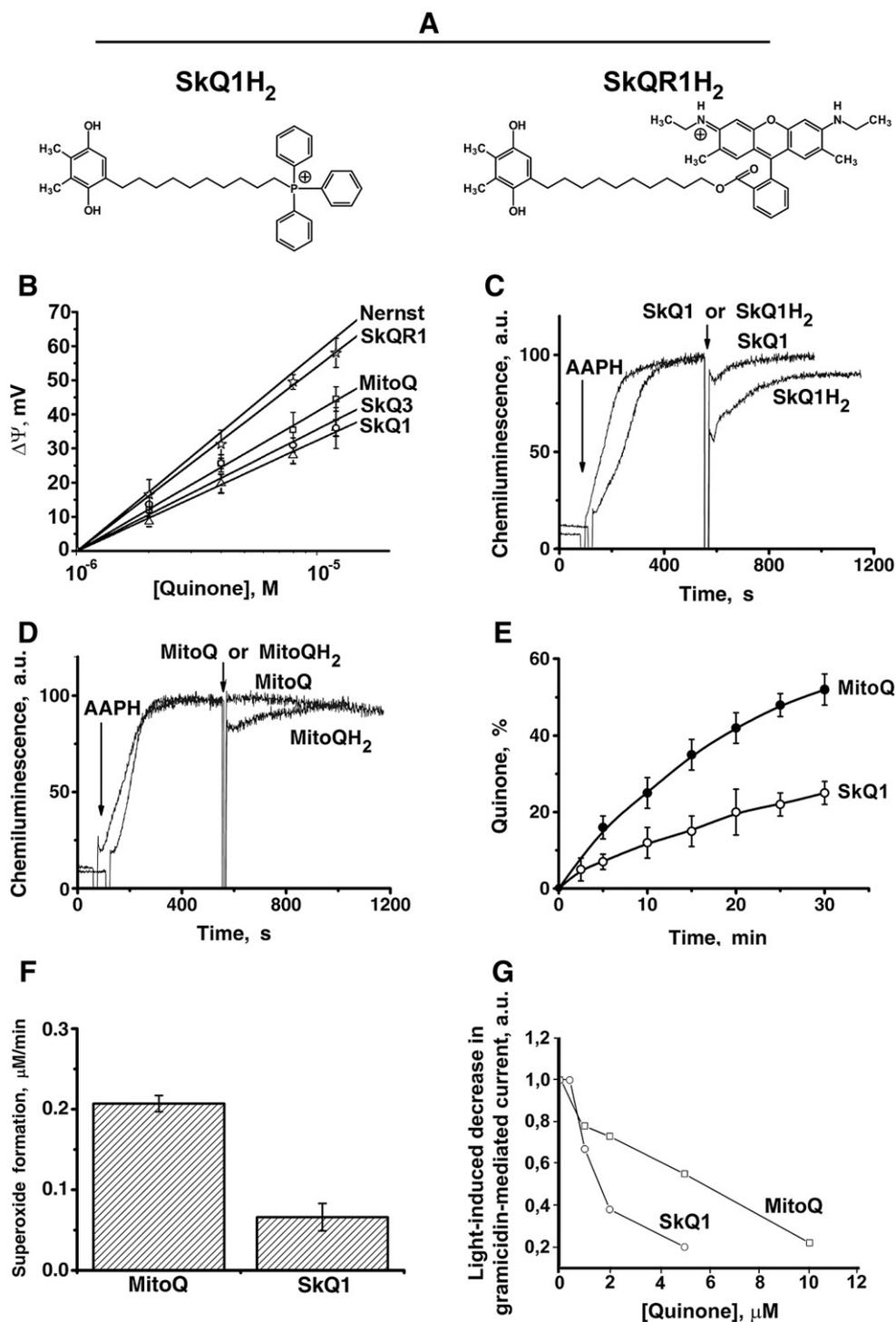
substance displays an increased difference between the anti- and prooxidant concentrations compared to MitoQ, but unfortunately this difference was less pronounced than we would have liked.

We then turned our attention to *plastoquinone*, an electron carrier acting instead of ubiquinone in photosynthetic electron transport chains in chloroplasts of plants and cyanobacteria. Evolution of plants resulted in a situation when ubiquinone involved in the mitochondrial respiratory chain was substituted by plastoquinone in the chloroplast chain of the same plant cell, possibly, just because of the better antioxidant properties of plastoquinone, as shown in chemical experiments on model systems by Kruk et al. [41] and by our group [42]. In fact, an oxygen-generating chloroplast is under conditions of much stronger oxidative stress than a mitochondrion which takes up oxygen, lowering thereby  $[\text{O}_2]$ . As compared with ubiquinone, plastoquinone has methyl groups instead of methoxy groups, whereas the methyl group of ubiquinone is replaced by hydrogen. We have synthesized plastoquinonyl-decyl-triphenylphosphonium, a compound where the ubiquinone moiety was replaced by plastoquinone. The new substance was named SkQ1 where Sk is for penetrating cation (“Skulachev ion”, a term introduced by David Green [43]) and Q is for plastoquinone. Whereas the anti- and prooxidant concentrations of MitoQ differed less than twofold (300 and 500 nM), this difference for SkQ1 was shown to be increased up to 32-fold (25 and 800 nM) [37] (see below, Fig. 2B).

This result showed that we had found a very effective antioxidant specifically addressed to mitochondria and not complicated by danger of prooxidant effect within a large range of concentrations. In this connection, we suggested that Mr. Deripaska change the grant to an investment project aimed at creation of a new type of drugs and biotechnology products on the basis of SkQ. The proposal was accepted, and the investment project was started in the spring of 2005.

On searching for the best antioxidant among cationic derivatives of quinones, a number of substances were synthesized by our chemists Drs. G. A. Korshunova, N. V. Sumbatyan, and L. S. Yaguzhinsky. It was taken into account that (1), in contrast to ubiquinone (CoQ), plastoquinone and another chloroplast electron carrier vitamin  $\text{K}_1$ , as well as the “professional antioxidant” vitamin E, contain no methoxy groups, and (2) vitamin  $\text{K}_1$ , vitamin E, and CoQ have in the fifth position of the quinone ring a methyl group, which is absent in plastoquinone. As a result, the majority of our quinone derivatives contained either plastoquinone (SkQ1, SkQ2M, SkQ4, SkQ5, SkQR1) or methylplastoquinone (SkQ3). For comparison, MitoQ and DMQ containing, respectively, ubiquinone and demethoxyubiquinone, were also synthesized. As cations, first of all alkyl triphenylphosphonium derivatives were used with the alkyl residue linking the cation with the quinone being either decyl (SkQ1, see Fig. 1A; SkQ3; SkQ4; DMQ and MitoQ) or amyl (SkQ5). In some cases, a different cation was used. Instead of phosphonium, compounds with an ionized nitrogen atom were tried: methylcarnitine (SkQ2M), tributylammonium (SkQ4), or Rhodamine 19 (SkQR1, see Fig. 1A). Moreover, a substance containing two methylene groups instead of plastoquinone (dodecyltriphenylphosphonium,  $\text{C}_{12}\text{TPP}$ ) was synthesized [37].

The new compounds were first tested for their ability to penetrate the model membranes. As experiments on BLMs showed, the gradient of SkQR1 concentration generated diffusion potential close to Nernstian (60 mV per tenfold gradient, the “plus” sign in the compartment with the lower concentration of the cation) in the range  $10^{-6}$ – $10^{-5}$  M SkQR1. The same concentrations of SkQ1, SkQ3, and MitoQ generated  $\Delta\psi$  of the right direction but with lower values than Nernstian (Fig. 1B). The  $\Delta\psi$  values for SkQ2M, SkQ4, and SkQ5 were much lower than Nernstian [37]. These relationships proved to be a result of different permeabilities of the studied cation across a BLM. Direct measurement the rate of transport of the cations from one half-membrane BLM leaflet to the other showed that this rate decreases as follows: SkQR1 > SkQ1 > SkQ3 > MitoQ [44]. With a cation of low permeability, there was a risk that the cation diffusion potential will



**Fig. 1.** Penetrating and anti- and prooxidant properties of SkQs and MitoQ. (A) Formulas of SkQ1H<sub>2</sub> and SkQR1H<sub>2</sub>. (B) Generation of diffusion electric potential on a BLM by SkQs and MitoQ. In the right compartment, the concentration of the studied compound was  $1 \times 10^{-6}$  M. In the left compartment, this concentration is indicated by the abscissa. Incubation mixture, 10 mM Tris-HCl, 10 mM MES, 10 mM KCl, pH 4.0. (C and D) Quenching of AAPH-induced chemiluminescence of luminal by SkQ1H<sub>2</sub>, SkQ1, MitoQH<sub>2</sub> or MitoQ. (E and F) Autoxidation of SkQ1H<sub>2</sub> and MitoQH<sub>2</sub> by O<sub>2</sub> in aqueous solution. E, Rates of SkQ1 and MitoQ formations from SkQ1H<sub>2</sub> and MitoQH<sub>2</sub>, respectively. F, Rates of O<sub>2</sub><sup>-</sup> formation, TIRON (sodium 4,5-dihydrobenzene-1,3-disulfonate) being a spin trap. 5 μM SkQ1H<sub>2</sub> and MitoQH<sub>2</sub>. (G) SkQ1 and MitoQ defend gramicidin channels in a BLM against photoinactivation in the presence of Methylene Blue. Here and in B, the BLM was formed from diphytanoyl phosphatidyl glycerol dissolved in decane. Incubation medium, 100 mM KCl, 10 mM TRIS, 10 mM MES (pH 7.0), 0.5 μM Methylene Blue. (From Antonenko et al. [37]).

be shunted by fluxes of other ions, an effect which should decrease the  $\Delta\psi$  value below that predicted by the Nernst equation. In principle, such a difficulty might be overcome by increasing concentration of the penetrating cation. However, at high concentrations all of them show a detergent activity resulting in damage to the BLM. The problem can be solved by substituting thick planar phospholipid membrane for BLM.

In this case, [SkQ1] could be increased up to  $5 \times 10^{-4}$  M. Within the range  $5 \times 10^{-5}$ – $5 \times 10^{-4}$  M, SkQ1 was shown to generate Nernstian potential [37]. Based on these findings, SkQ1 and SkQR1 were chosen for the majority of further studies.

Anti- and prooxidant activities of the SkQs were tested in model system, i.e. aqueous solutions, micelles, liposomes and BLM. To

measure the ability of SkQ1 and MitoQ to quench  $\text{OH}^\cdot$  in an aqueous solution, we studied the effect of these quinones on luminal-mediated chemiluminescence induced by an azo-initiator, 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) [45,46]. It is seen (Fig. 1C) that  $0.5 \mu\text{M}$  SkQ1H<sub>2</sub> (the reduced form of SkQ1) strongly decreases chemiluminescence. As for MitoQH<sub>2</sub>, it showed lower efficiency than SkQH<sub>2</sub> (Fig. 1D).

Prooxidant activities of SkQH<sub>2</sub> and MitoQH<sub>2</sub> in an aqueous solution are compared in Fig. 1E and F. As measures of such activities, the rates formation of quinones from corresponding quinols or of reduction  $\text{O}_2$  to  $\text{O}_2^\cdot^-$  were chosen (Fig. 1E, F). Both methods clearly showed significantly higher prooxidant activity of MitoQH<sub>2</sub> compared to the same concentration of SkQ1H<sub>2</sub>.

Antioxidant activity of the studied compounds was also analyzed using an unsaturated fatty acid in detergent micelles. In this case, peroxidation of methyl ester of linoleate (ML) in Triton X-100 micelles was followed by measuring  $\text{O}_2$  consumption with a Clark oxygen electrode. Antioxidant activity of SkQ1H<sub>2</sub> and MitoQH<sub>2</sub> was characterized by the rate constant  $k_1$  for the reaction between the peroxy radical of oxygenated linoleate residue ( $\text{LO}_2^\cdot$ ) and a quinol ( $\text{QH}_2$ ):



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



The experiment was started with the measuring the rate of non-inhibited oxidation of ML,  $R_0$ . The addition of SkQ1H<sub>2</sub> or MitoQH<sub>2</sub> resulted in a strong decrease in the rate of oxygen consumption and the appearance of a pronounced induction period. Importantly, the oxidized forms of SkQ1 and MitoQ were inactive in this system. As the antioxidant was consumed, the rate of inhibited oxidation ( $R$ ) increased progressively with time, finally reaching the level of  $R_0$ . The time course of changes in  $R$  followed the Eq. (3):

$$F = \ln \frac{1 + R/R_0}{1 - R/R_0} - \frac{R_0}{R} = \frac{k_1 R_0}{k_2 [\text{ML}]} t + \text{const}. \quad (3)$$

The  $k_1/k_2$  value was determined as a slope of an  $F$  versus  $t$  plot (in more detail, see [46,47]).

The absolute values of  $k_1$  were calculated from  $k_1/k_2$ , assuming  $k_2 = 60 \text{ M}^{-1} \text{ s}^{-1}$ . The values of  $k_1$  for SkQ1H<sub>2</sub> and MitoQH<sub>2</sub> were found to be  $2.2 \cdot 10^5$  and  $0.58 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$ , respectively. Thus, the reactivity of SkQ1H<sub>2</sub> towards  $\text{LO}_2^\cdot$  was almost four times higher than that of MitoQH<sub>2</sub>. This is in line with data obtained for simpler analogs of SkQ1H<sub>2</sub> and MitoQH<sub>2</sub>, 2,3,5-trimethyl-1,4-hydroquinone and 2,3-dimethoxy-5-methyl-1,4-hydroquinone [41,47].

In a further series of experiments, we examined the antioxidant action of SkQ1 and MitoQ in model lipid membranes. First, we used one of the most conventional methods to assess lipid peroxidation, i.e. the thiobarbituric acid-reactive substances (TBARS) assay. Lipid peroxidation in asolectin liposomes was induced by the addition of  $100 \mu\text{M}$   $\text{FeSO}_4$ ,  $1 \text{ mM}$  ascorbate, and  $30 \text{ mM}$  *tert*-butyl-hydroperoxide (tBOOH). Under these conditions, TBARS formation was mainly due to production of malondialdehyde. This process was found to be substantially slowed in the presence of  $10 \mu\text{M}$  SkQ1H<sub>2</sub> [37].

To directly follow the kinetics of oxidative destruction of lipid, we used C11-BODIPY(581/591), a fluorescent lipid peroxidation probe [48,49]. It was revealed that the addition of  $5 \mu\text{M}$   $\text{Fe}^{2+}$  led to a very strong inhibition of the BODIPY fluorescence in asolectin liposomes. The  $\text{Fe}^{2+}$ -induced fluorescence changes were almost completely prevented in the presence of  $0.5 \mu\text{M}$  SkQ1H<sub>2</sub>. Oxidized SkQ1 was without any effect [37].

To model a ROS-caused damage to a membrane protein, a new method was developed, i.e. ROS sensitivity of the BLM conductance created by gramicidin D [49]. Tryptophan residues of this channel-forming peptide were shown to be attacked by ROS, resulting in the

inactivation of the channels. Light and Methylene Blue were employed as a ROS-generating system (Fig. 1G). It is seen that the ROS-induced inactivation of the gramicidin-mediated current could be prevented by SkQ1 or MitoQ, SkQ1 being more effective. Similar relationships were revealed when ascorbate,  $\text{FeSO}_4$ , and tBOOH were used to generate  $\text{OH}^\cdot$  [37,49].

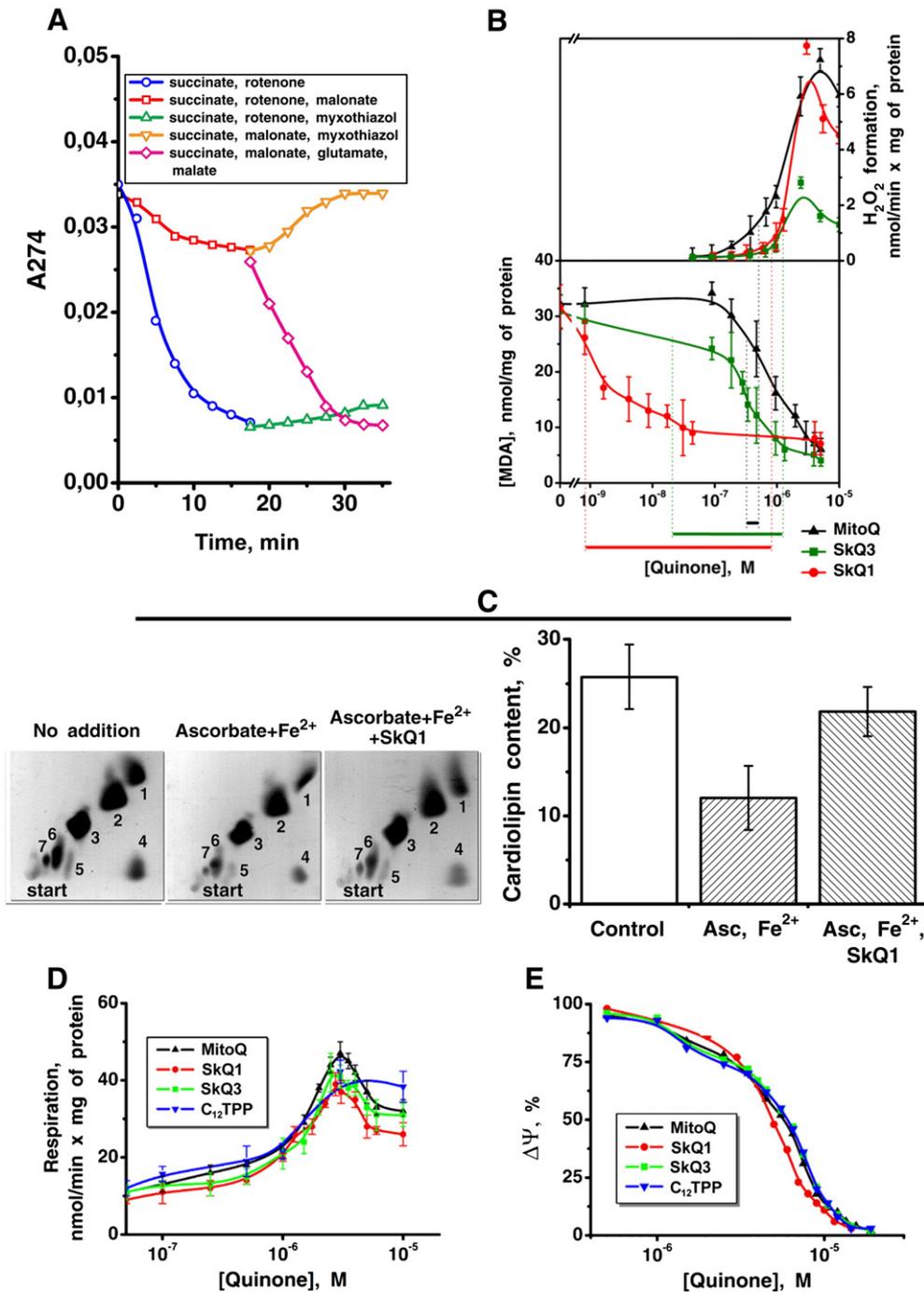
Uptake of SkQs by mitochondria was monitored with a hydrophobic cation-sensitive electrode. Addition of a  $\Delta\psi$ -discharging uncoupler initiated partial release of SkQs to the medium, the effect being larger with SkQ5 than with the more hydrophobic SkQ1 [37].

Next we tested whether SkQ1 and other plastoquinone derivatives could be reduced by the animal mitochondrial respiratory chain (Fig. 2A). Either NAD-linked substrates or succinate were found to be used as electron donors. In both cases, reduction of SkQ1 was completely arrested by antimycin A, myxothiazol being without effect. The relationships may be explained assuming that SkQ1, like plastoquinone [50–53], is reduced by endogenous  $\text{CoQH}_2$  bound in center *i* of Complex III, localized in the inner leaflet of the inner membrane. Oxidation of SkQ1H<sub>2</sub> was found to be enzymatic (by myxothiazol-sensitive Complex III of the respiratory chain) or non-enzymatic (by either lipid radicals or  $\text{O}_2$ ). The overall rate of SkQ1H<sub>2</sub> 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 toward  $\text{LO}_2^\cdot$  (see above, Eq. (1)) [37].

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  $\text{Fe}^{2+}$  and ascorbate in rat heart mitochondria. As prooxidant action, we measured stimulation by the same compounds of the  $\text{H}_2\text{O}_2$  production in mitochondria oxidizing glutamate and malate in State 4. Fig. 2B shows that antioxidant activity of SkQ1 is measurable at much lower concentration of this substance than that of SkQ3 and especially of MitoQ, whereas prooxidant SkQ1 activity 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 very much larger for SkQ1 than for MitoQ, SkQ3 occupying an intermediate position. SkQR1 proved to be as active as SkQ1. Decylplastoquinone lacking a cation was even less active than MitoQ, whereas  $\text{C}_{12}\text{TPP}$  lacking a quinone residue was quite inactive at the studied concentrations [37]. The prooxidant effect was much smaller with  $\text{C}_{12}\text{TPP}$ . Uncouplers as well as respiratory chain inhibitors (rotenone and myxothiazol as well as cyanide) strongly lowered the rate of  $\text{H}_2\text{O}_2$  formation in the presence of micromolar concentrations of cationic quinones [37].

As further analysis revealed, it is cardiolipin that is first oxidized under conditions of  $\text{OH}^\cdot$  generation by  $\text{Fe}^{2+}$  + ascorbate in heart mitochondria. The amount of cardiolipin was strongly decreased after  $\text{Fe}^{2+}$  + ascorbate treatment, the effect being significantly lowered by  $100 \text{ nM}$  SkQ1 (Fig. 2C) [37].

This observation deserves special attention. As a matter of fact, cardiolipin peroxidation appears to be a key event in mitochondrial oxidative stress. (1) Cardiolipin is the most sensitive constituent of the inner mitochondrial membrane to the ROS-induced peroxidation (see above). This means that its peroxidation initiates chain reaction "setting on fire" other membrane constituents. (2) Cardiolipin operates as an anchor for cytochrome *c* in the mitochondrial membrane while oxidized cardiolipin fails to perform such a function [54]. As a result, cytochrome *c* releases to the mitochondrial intermembrane space and acquires cardiolipin-peroxidase activity, initiating a vicious cycle of further cardiolipin peroxidation [55]. (4) The loss of cardiolipin inactivates all the respiratory chain complexes,  $\text{H}^+$ -ATP-synthase, ATP/ATP-antiporter, etc., induces an increase in permeability of the inner mitochondrial membrane and, as a consequence, the collapse of  $\Delta\psi$ , swelling of matrix, disruption of the outer mitochondrial membrane, and release of cytochrome *c* and



**Fig. 2.** SkQ effects on isolated rat heart mitochondria. (A) Reduction of SkQ1 and oxidation of formed SkQ1H<sub>2</sub> by isolated rat heart mitochondria. The reduction and oxidation were measured by a decrease and an increase in optical density at 274 nm, respectively. Where indicated, 2.5 mM succinate with or without 2.5 mM malonate was added at zero time. 1 μM myxothiazol or 4 mM glutamate and 1 mM malate were added after 18 min incubation. Rotenone (2 μM) was added at zero time in all samples except that with glutamate and malate. 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) Anti- and prooxidant activities of SkQ1, SkQ3, and MitoQ in isolated heart muscle mitochondria. Antioxidant activity was measured as inhibition of MDA formation initiated by adding 10 mM ascorbate and 100 μM FeSO<sub>4</sub> to mitochondria oxidizing 4 mM glutamate and 1 mM malate (other components as in A). Prooxidant activity was estimated as stimulation of H<sub>2</sub>O<sub>2</sub> formation under similar conditions but without ascorbate and FeSO<sub>4</sub>. Colored bars below abscissa indicate windows between concentrations of given quinone derivative causing 20% anti- and 20% prooxidant effects. (C) SkQ1 specifically protects mitochondrial cardiolipin from damage by OH<sup>•</sup>. A silicagel-60 chromatogram of mitochondrial phospholipids. Elution, chloroform:methanol:water, 65:25:4 (horizontal); chloroform:acetone:methanol:acetic acid:water, 6:8:2:2:1 (vertical). 1, cardiolipin; 2, phosphatidyl ethanolamine; 3, phosphatidyl choline; 4, phosphatidic acid; 5, phosphatidyl serine; 6, phosphatidyl inositol; 7, lysophosphatidyl inositol. Where indicated, 100 nM SkQ1 was added. Other conditions as in B. (D and E) Effects of SkQ1, SkQ3, MitoQ, and C<sub>12</sub>TPP on State 4 respiration (D) and membrane potential (E) of mitochondria. For conditions, see B. (From Antonenko et al. [37]).

other intermembrane proapoptotic proteins into the cytosol. The latter effect initiates apoptosis [56]. Thus, it is not surprising that the prevention of cardiolipin peroxidation by SkQs results in strong protective effects under conditions of oxidative stress inducing apoptosis and necrosis (see below).

In experiments on isolated mitochondria, it was also found that the hydrophobic cations used (SkQ1, SkQ3, MitoQ, and C<sub>12</sub>TPP) possess some uncoupling activity. At concentrations  $5 \times 10^{-7}$ – $5 \times 10^{-6}$  M, they stimulate State 4 respiration and (at slightly higher concentration) lower Δψ (Fig. 2D and E, respectively). The mechanism of such

an effect was found to consist in an increase by our cations of specific  $H^+$  conductance of the membrane, which was mediated by free fatty acids. In BLMs, it was shown that the above cations induce high  $H^+$  conductance provided a fatty acid is added [Severina, I. I. et al., in preparation]. Most probably, we deal here with the same phenomenon as was previously described for mitochondria treated with tetraphenylphosphonium which was shown to potentiate uncoupling by fatty acids [57]. The mechanism was assumed to consist in operation of a hydrophobic cation as a carrier for fatty acid anions, which otherwise are of low penetrating ability. This effect together with diffusion of protonated fatty acids (that penetrate well) in the opposite direction can organize in a membrane a futile  $H^+$  cycle.

It should be stressed that uncoupling activity of SkQ can also be involved in its antioxidant effect, especially if we deal with ROS produced in the Complex III center of facing the intermembrane space of the mitochondrion. Such ROS, in contrast to those produced by Complex I inside mitochondria, are hardly quenched by SkQ1 concentrated in the inner leaflet of the inner mitochondrial membrane. However, SkQ: fatty acid anion/SkQ antiporter between the inner and the outer leaflets, resulting in a  $\Delta\psi$  decrease, can strongly lower the rate of ROS generation not only in Complex I but also in Complex III due to very steep dependence of this rate upon  $\Delta\psi$ . Thus, a small  $\Delta\psi$  decrease (“mild uncoupling” [58]) was shown to induce manifold decrease in ROS production [8]. One might suggest that the uncoupling effect of hydrophobic cations mimics the mild uncoupling by uncoupling proteins (UCPs), as well as by ATP/ADP, antiporter, also mediated by fatty acids [59].

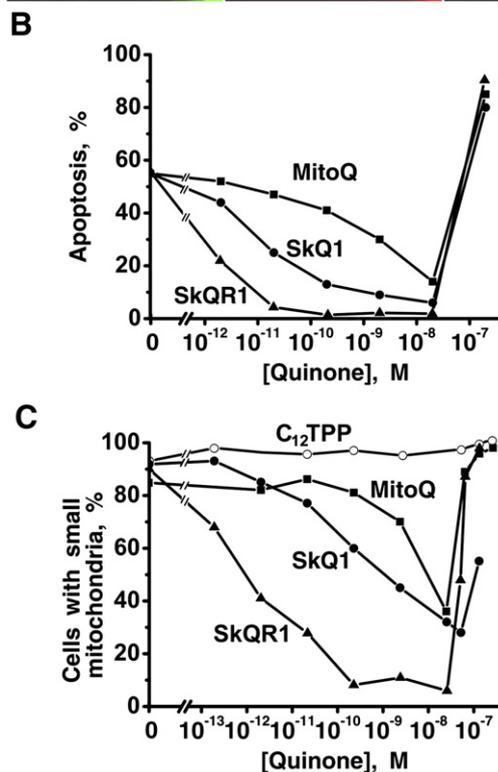
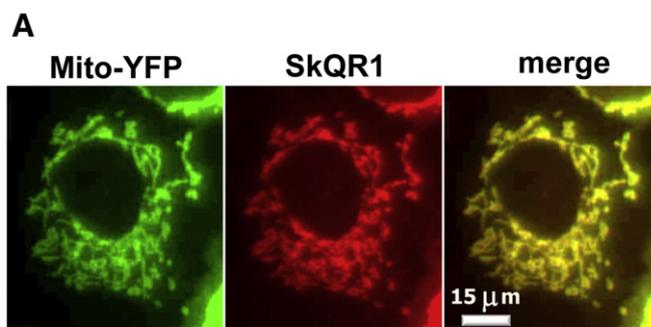
In this context, one may mention of another possible function of minor UCPs (UCP2, 3, etc.) postulated by Dr. F. Goglia and one of us (V. P.S.) [60], namely translocation of fatty acid peroxides from the inner to the outer leaflet of the inner mitochondrial membrane, where they are further metabolized by cytochrome *c* and some other enzymes. In this way, mitochondrial DNA and other intramitochondrial systems of great importance can be preserved under conditions when ROS are produced by Complex I. It was found in our group [61] that fatty acid peroxides are of much lower uncoupling activity than intact fatty acids. This is most probably due to low permeability of the membranes even to their protonated forms [60]. Such an assumption explains why fatty acid peroxides cannot organize a protonophoric cycle although their anions are translocated by UCPs. If SkQ and  $C_{12}TPP$  are also competent in translocation of fatty peroxide anions, this should result in irreversible extrusion of such anions from the mitochondrial interior. These relationships are summarized in Table 1.

In the next part of the study, cell cultures were investigated. Here we started with an experiment showing intracellular localization of SkQ. To this end, SkQR1, a fluorescent SkQ derivative, was applied. SkQR1 was found to be specifically accumulated by mitochondria of HeLa cells, showing the same intracellular localization as mitochondria-targeted jellyfish yellow fluorescent protein, YFP (Fig. 3A) or MitoTracker Green (not shown). The SkQR1 staining of mitochondria inside the living cell completed during 1 h, and subsequent incubation of the cells in a medium without SkQR1 resulted in slow SkQR1 release ( $t_{1/2} = 2.5$  h).

**Table 1**

Four possible antioxidant effects of SkQ in mitochondria

N	Effect	Process	Active compounds	Source of ROS
1	Reduction of inner $LO_2$	$LO_2^- + SkQH_2 \rightarrow LOOH + SkQ^-$	SkQH <sub>2</sub>	Complex I
2	Oxidation of inner $O_2^-$	$O_2^- + SkQ \rightarrow O_2 + SkQ^-$	SkQ	Complex I
3	Mild uncoupling by fatty acid cycling	$(RCOO^-)_{in} SkQ (RCOO^-)_{out}$	SkQ, SkQH <sub>2</sub> , $C_{12}TPP$	Complexes I and III
4	Extrusion of inner fatty acid peroxides (LOOH)	$(LOOH)_{in} SkQ (LOOH)_{out}$	SkQ, SkQH <sub>2</sub> , $C_{12}TPP$	Any



**Fig. 3.** Effects of SkQ1, SkQR1, and MitoQ on human cell cultures. (A) Co-localization of SkQR1 (a fluorescent SkQ derivative) and mitochondria-targeted jellyfish yellow fluorescent protein fused with the leader sequence of cytochrome oxidase subunit VIII (MitoYFP). HeLa cells were transfected with Mito-YFP (Clontech) and incubated for 15 min with 100 nM SkQR1. Confocal microscopy. (B) SkQ1 and SkQR1 are more efficient than MitoQ in arresting  $H_2O_2$ -induced apoptosis in human fibroblasts. Fibroblasts were grown for 7 days with or without cationic quinones and then treated with 400  $\mu M$   $H_2O_2$  for 24 h. (C) 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  $\mu M$   $H_2O_2$  for 3 h. (From Antonenko et al. [37]).

Uncoupler FCCP prevented the SkQR1 staining and stimulated its efflux if added to non-stained and stained cells, respectively [37].

In further study, we asked whether SkQs possess antiapoptotic and antinecrotic effects when cell death is induced by ROS. As the experiments showed, SkQs prevented 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 Saretsky et al. [35] for MitoQ). In this case, 0.2 nM SkQ1 (Fig. 3B) almost completely abolished apoptosis of human fibroblasts, induced by 400  $\mu M$   $H_2O_2$ . Dissipation of  $\Delta\psi$  by the uncoupler FCCP prevented the antiapoptotic effect of such a low SkQ1 concentration. The protective effect of small amounts of SkQ1 could be overcome by increasing  $[H_2O_2]$  to 500  $\mu M$  (not shown) or  $[SkQ1]$  to  $2 \times 10^{-7}$  M. High  $[SkQ1]$  stimulated the proapoptotic effect of  $H_2O_2$  (Fig. 3B). Moreover, it was found that low  $[SkQ1]$  prevented migration of

proapoptotic protein BAX to mitochondria and mitochondrial cytochrome *c* release to cytosol in response to  $H_2O_2$ . Neither decylplastoquinone nor  $C_{12}TPP$  at nanomolar concentrations could substitute for SkQ1 [37]. In this context, it should be noted that prevention by cationic quinones of endogenous oxidative stress in cell cultures was first observed by Murphy et al. on fibroblasts from patients with Friedreich's ataxia [36]. This disease is associated with a disorder in production of the mitochondrial protein frataxin, which results in an increase in the content of iron ions in the matrix and, as a consequence, in oxidative stress. Such fibroblasts can survive in culture only in the presence of antioxidants, in particular, decylubiquinone ( $C_{1/2} = 3 \cdot 10^{-8}$  M). MitoQ was much more effective than decylubiquinone ( $C_{1/2} = 5 \cdot 10^{-10}$  M; cf. in our experiments on normal fibroblasts, inhibition of  $H_2O_2$ -induced apoptosis by SkQ1,  $C_{1/2} = 8 \cdot 10^{-12}$  M [37]). In Murphy's experiments, an excess of MitoQ ( $5 \cdot 10^{-7}$  M and more) could not protect the ataxia cells against oxidative stress, whereas an excess of decylubiquinone ( $1 \cdot 10^{-4}$  M) was as efficient as its lower quantities [36].

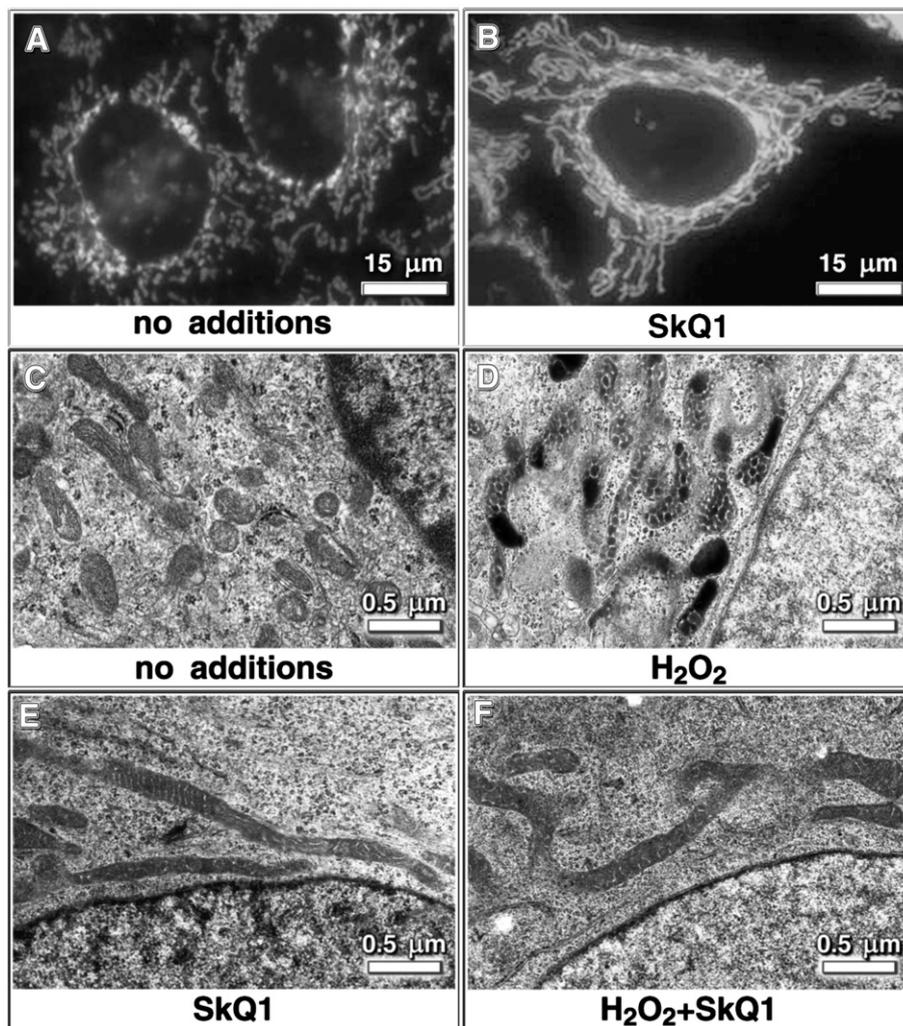
Addition of small amount of  $H_2O_2$  to HeLa cells was found to induce a burst in endogenous ROS formation. This phenomenon described by one of us (D.B.Z.) as a ROS-induced ROS release [62] was completely abolished by pre-treatment of the cells with 20 nM SkQ1 [37].

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 [63]). In this case, preincubation of fibroblasts with the cationic quinones for 2 h was sufficient to observe the protective effect (Figs. 3C, 4C–F), which increased as follows: MitoQ < SkQ1 < SkQR1. Measurable activity was observed at  $2 \times 10^{-13}$  M SkQR1. An increase in the quinone concentrations to  $3 \times 10^{-8}$  M MitoQ,  $4 \times 10^{-8}$  M SkQR1, and  $1 \times 10^{-7}$  M SkQ1 abolished the protective action (Fig. 3C).

Without  $H_2O_2$ , low SkQ1 concentrations stimulated formation of the mitochondrial network (Fig. 4A,B). Local damage to this network by a very narrow laser beam resulted in a  $\Delta\psi$  collapse over the entire mitochondrial reticulum, whereas without SkQ mitochondrial  $\Delta\psi$  was found to be discharged mitochondria only in a small part of the cell [37]. This might be explained assuming that the SkQ1-induced decrease in the level of endogenous ROS stimulates fusion of the majority of mitochondria into an electrically-united mitochondrial reticulum [63].

In other experiments, pre-treatment of HeLa cell with 1  $\mu$ M SkQ1 for 1 h arrested necrosis induced by illumination of cells stained by MitoTracker Red as a photosensitizer. The  $C_{1/2}$  for this effect was between 200 and 500 nM. Under the same conditions, a mixture of 1  $\mu$ M TPP and 1  $\mu$ M decylplastoquinone was inefficient. NAC and Trolox could substitute for SkQ1 but at very much higher concentrations (20 mM and 1 mM, respectively). The data of this section suggest that SkQs effectively operate as antioxidants at the cell level, preventing ROS-induced apoptosis and necrosis [37].



**Fig. 4.** Effect of SkQ1 on mitochondrial morphology in HeLa cells. (A–F) Confocal and electron microscopy, respectively. (A, C and D) Without SkQ1; (B, E and F) After 7 day treatment with 20 nM SkQ1. After 7 days, the cells were kept without (A–C, E) or with (D, F) 100  $\mu$ M  $H_2O_2$  for 6 h. A, B, Mitochondria were visualized with MitoTracker Green. (From Antonenko et al. [37]).

**Table 2**

Effect of *in vivo* SkQ1 treatment on stability of cardiac rhythm after 40 min perfusion of isolated heart with 0.1 mM H<sub>2</sub>O<sub>2</sub> (from Bakeeva et al. [68])

SkQ1 dose, nmol/kg × day	Number of hearts studied	Hearts with normal rhythm		Hearts with fibrillation		Arrhythmia index
		Number	%	Number	%	
0 (control)	25	9	36	11	44	3.6 ± 0.5
0.02	11	6	55	1	10	1.2 ± 0.5*
0.2	22	16	72	4	18	0.8 ± 0.5**
2.0	11	7	64	1	10	2.7 ± 0.9
250	10	3	30	4	40	4.6 ± 1.1

Asterisks, statistically significant (\**p* < 0.05; \*\**p* < 0.01) difference between the SkQ1 and control groups.

In another series of experiments, the level of reduced glutathione was measured in HeLa cells. It was found that this level is lowered by adding H<sub>2</sub>O<sub>2</sub>, which pointed to development of oxidative stress. This effect was prevented by SkQ1 and SkQR1, the C<sub>1/2</sub> being about 2 × 10<sup>-12</sup> M [37].

The efficiency of extremely low concentrations of SkQ1 and SkQR1 as antioxidants and antiapoptotic agents in experiments on cell cultures is most probably due to cooperation of two factors. First, the coefficient of their distribution between the hydrophobic and aqueous phases is very high: in the octanol/water system, it is 13,000 for SkQ1 [37]. Then, we gain three orders of magnitude more due to the effect of Δψ on the inner mitochondrial membrane (~180 mV) and one order more from the Δψ on the outer cell membrane (~60 mV). In total, the SkQ1 concentration gradient between the extracellular medium and inner half-membrane layer of the inner mitochondrial membrane is immense: 13,000 × 1000 × 10 = 1.3 × 10<sup>8</sup>. This means that at the SkQ1 concentration in the medium of 1 · 10<sup>-12</sup> M, its concentration in the inner half-membrane layer of mitochondria will be 1 · 10<sup>-12</sup> · 1.3 · 10<sup>8</sup> = 1.3 · 10<sup>-4</sup> M. On the way to the inner mitochondrial membrane, SkQ1 crosses the outer cellular membrane, endoplasmic reticulum membranes, and the outer mitochondrial membrane, being at first accumulated within them due to high lipid/water distribution coefficient. In the next step, SkQ1 goes to the inner mitochondrial membrane to be distributed according to the Nernst potential [64].

Experiments on simple model systems, isolated mitochondria, and living cells revealed some advantages of SkQs as antioxidants over MitoQ. This was more apparent on mitochondria and especially cells than on 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 manifold more active as antioxidant compared to MitoQ. The properties in question are the following.

- (1) In aqueous solution, SkQ1 quenches OH<sup>·</sup> several times better than MitoQ (Fig. 1C, D).
- (2) In aqueous solution, SkQ1H<sub>2</sub> is oxidized by O<sub>2</sub> to form O<sub>2</sub><sup>-·</sup> three times slower than MitoQ (Fig. 1E, F).
- (3) SkQ1 inhibits methyl linoleate peroxidation in micelles four times stronger than MitoQ [37].
- (4) In BLMs, SkQ1 protects gramicidin from being attacked by ROS at three times lower concentrations than MitoQ (Fig. 1G).
- (5) The affinity of mitochondrial cardiolipin for SkQ1 was shown to be 2.5 times higher than for MitoQ [37].
- (6) SkQ1 is four times more hydrophobic than MitoQ [37].
- (7) SkQR1 and SkQ1 cross the phospholipid bilayer ten and two times faster than MitoQ, respectively [44].

### 3. Favorable effects of SkQs shown on heart arrhythmia, heart infarction, stroke, and kidney ischemia

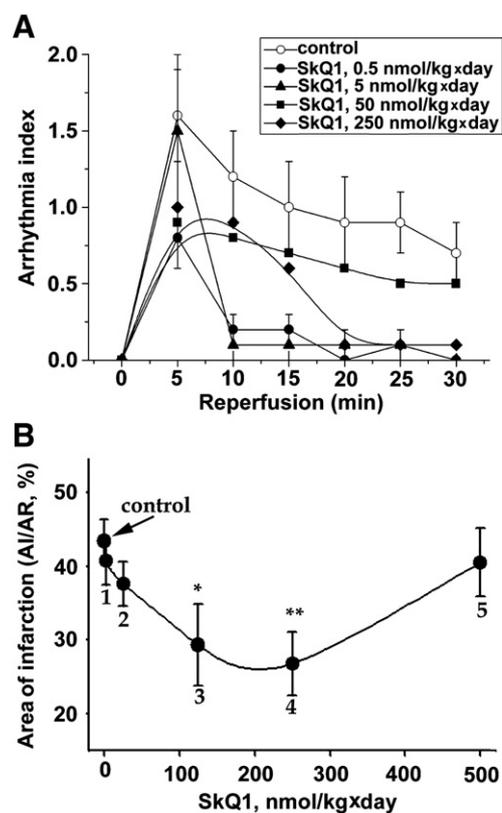
In further experiments, effects of *in vivo* SkQ treatments on some common age-related pathologies of heart, brain, and kidney were

studied. All of them are shown to be accompanied by strong increase in the ROS level [65–68].

In particular, the SkQ1 effect was tested on isolated rat heart in which an arrhythmia was induced by addition of H<sub>2</sub>O<sub>2</sub> into the perfusate or by ischemia/reperfusion. The arrhythmia was significantly less pronounced and fibrillation was much less frequent if the hearts had been obtained from rats pretreated for two weeks with SkQ1 (2 · 10<sup>-11</sup> mol/kg × day) (Table 2 and Fig. 5A). Increasing [SkQ1] to 2.5 × 10<sup>-7</sup> M abolished the favorable effect in the case of the H<sub>2</sub>O<sub>2</sub>-induced arrhythmia (Table 2). As to that induced by ischemia/reperfusion, the maximal favorable effect of SkQ1 was observed at 5 × 10<sup>-10</sup> M, it decreased at 5 × 10<sup>-8</sup> M, and increased again at 2.5 × 10<sup>-7</sup> M SkQ1 (Fig. 5A) [69].

A similar pretreatment of rats was employed in an *in vivo* model of myocardial infarction. SkQ1 (2.5 · 10<sup>-7</sup> mol/kg × day) decreased by 40% the damaged zone of the heart muscle. This SkQ1 concentration was much lower than that of MitoQ used by Murphy's group to normalize contractile function and mitochondrial structure in heart after ischemia/reperfusion [70]. Lower (<1 × 10<sup>-7</sup> mol) and higher (5 × 10<sup>-7</sup> mol) SkQ1 doses were without measurable effect (Fig. 5B).

Activities of plasma lactate dehydrogenase (LDH) and creatine kinase MB isoenzyme (CK-MB) activities did not significantly differ between the controls and SkQ1-fed groups in the steady state, a fact indicating that SkQ1 supplementation at a dose of 250 nmol/kg × day for two weeks did not damage the outer cell membranes. Myocardial infarction was associated with a profound increase in both plasma LDH and CK-MB activities at the end of reperfusion. These activities were markedly reduced when rats were fed with SkQ1. Moreover, the *in vivo* pretreatment with SkQ1 was found to partially prevent effects



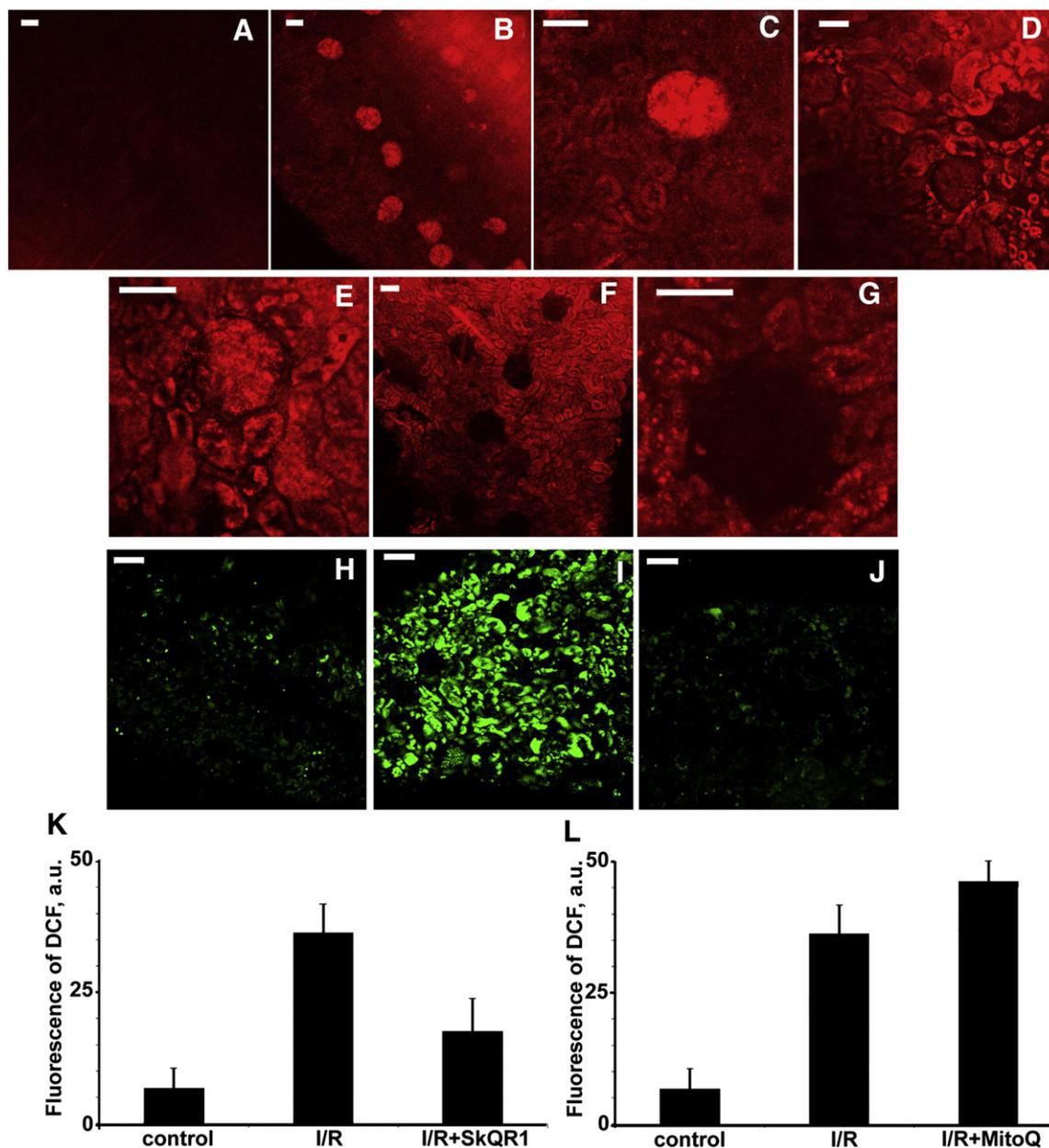
**Fig. 5.** Preventive effects of SkQs on ischemia/reperfusion-induced damage to rat heart. (A) Feeding of rats with SkQ1 during 3 weeks decreases the ischemia/reperfusion-induced arrhythmia in isolated rat heart. (B) SkQ1 reduces myocardial infarction area in rats *in vivo*. Control, 250 nmol NaBr/kg × day for two or three weeks (*n* = 24); NaBr was used since SkQ1 was a bromide salt; 1, 2.5 nmol SkQ1/kg × day for three weeks (*n* = 12); 2, 25 nmol SkQ1/kg × day for two weeks (*n* = 11); 3, 125 nmol SkQ1/kg × day for two or three weeks (*n* = 9); 4, 250 nmol SkQ1/kg × day for three weeks (*n* = 11); 5, 500 nmol SkQ1/kg × day for two weeks (*n* = 10). \**p* < 0.05; \*\**p* < 0.02 versus control. (From Bakeeva et al. [69]).

of ischemia/reperfusion on content of adenine nucleotides, creatine phosphate, and lactate as well as on the structural parameters of cardiomyocytes (actomyosin fibrils, Z-discs, and mitochondria) in the infarction area. In numerous mitochondria, continuity of both the outer and inner membranes disappeared, other mitochondria were swollen, and matrix became transparent after ischemia/reperfusion. These and some other pathological changes did not occur in hearts of the SkQ-fed rats. In particular, no disrupted mitochondrial profiles are seen. In fact, the only obvious effect of ischemia/reperfusion consisted in this case in a decrease in the electron density of the mitochondrial matrix space [69].

It should be mentioned that amount of SkQ1 effective in treatment of arrhythmia was much lower than those of infarction (0.02–0.5 and 100–250 nmol/kg×day, respectively). Such a difference might be explained by different localization and concentration of mitochondria

in pacemaker cells and cardiomyocytes that are the most probable targets for SkQ1 in cases of arrhythmia and infarction, respectively. In the former case, we are dealing with small cells and rather rare mitochondria more or less equally distributed in various cell parts. In the latter case, numerous mitochondria are concentrated below sarcolemma of these very large cells, whereas the rest are localized between actomyosin fibrils, the cell being filled with much water-insoluble material (fibrils, Z-discs, sarcoplasmic reticulum, etc.) which might adsorb SkQs.

In other experiments, the effect of SkQs on kidney ischemia has been studied. At first, a culture of kidney epithelial cells was applied as a model. It was revealed that preincubation with SkQ1 increases survival of these cells after 24 h anoxia followed by 24 h reoxygenation. Optimal concentrations of SkQ1 were between 10 and 250 nM. It was also shown that SkQ1 prevented fission of elongated



**Fig. 6.** Effects of SkQR1 on rat kidney. A–J, Pharmacokinetics of SkQR1 distribution over kidney compartments after its intravenous injection. A, 10 min after injection of 300  $\mu$ l of 1 mM SkQR1 to the inferior vena cava; B, C, 60 min after injection of 200  $\mu$ l 1 mM SkQR1; D, E, 120 min after injection of 200  $\mu$ l 0.1 mM SkQR1; F, G, 3 days after injection of 200  $\mu$ l 0.1 mM SkQR1. Bar, 50  $\mu$ m. H–L, ROS generation in kidney slices detected by DCF fluorescence. H, control isolated kidney; I, isolated kidney after 40 min ischemia followed by 10 min reperfusion; J, kidney from a rat that received a single injection of 1  $\mu$ mol SkQR1/kg×day before ischemia; K, average ROS production evaluated from 6 different experiments; L as K but instead of SkQR1 the same concentration of MitoQ was injected. I–J, bar, 100  $\mu$ m. I/R, ischemia/reperfusion. (From Bakeeva et al. [69]).

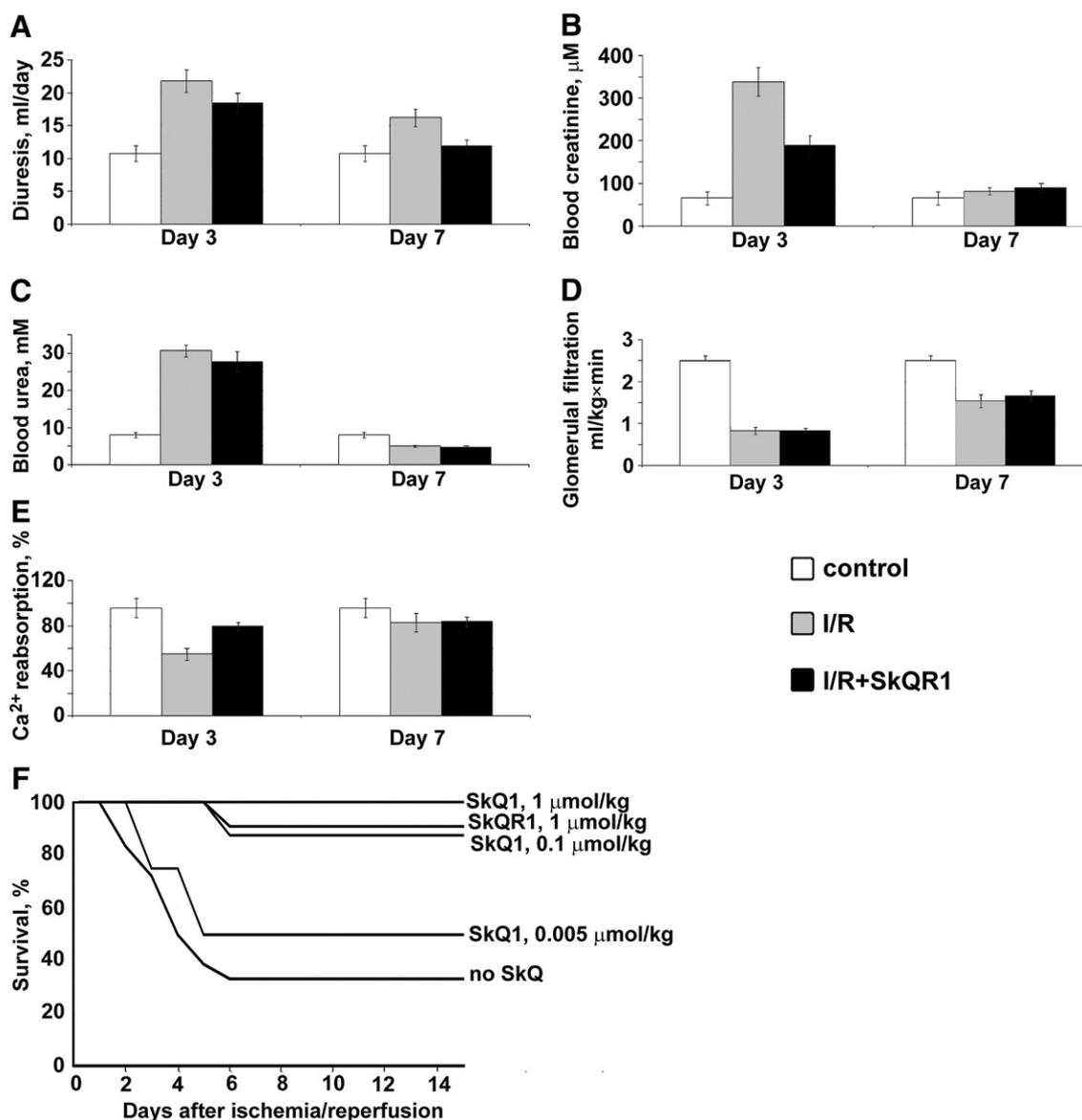
mitochondrial filaments to small roundish mitochondria, induced by the anoxia/reoxygenation procedure [69,71].

In the next study, we followed the fate of SkQR1 in kidney slices after either intravenous or intraperitoneal injection of this compound into rats. Fluorescence was clearly seen in glomerular zones of the kidney 60 min after injection into the vena cava inferior (cf. Fig. 6A–C). After 2 h, fluorescence decreased in these regions with parallel appearance of the fluorescence in the kidney tubular epithelial cells (Fig. 6D, E). In three days, further accumulation of SkQR1 in epithelium and complete loss of fluorescence in glomerular zones took place (Fig. 6F, 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 co-localization of the two dyes in the kidney cells [69].

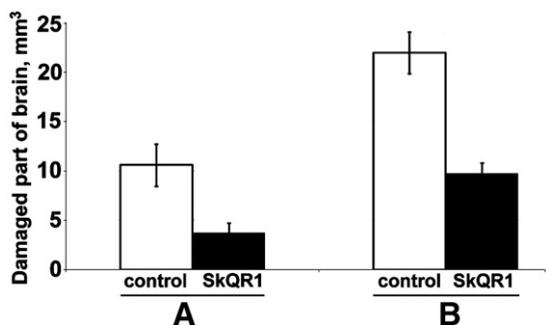
On isolated kidney, a 40 min ischemia followed by reoxygenation resulted in a strong increase in the ROS level detected by DCF fluorescence in kidney slices. Earlier we demonstrated that this elevated DCF fluorescence originated from mitochondria [72]. A single intraperitoneal injection of SkQR1 (1  $\mu\text{mol/kg}$ ) to the animal a day before ischemia partially normalized the ROS level (Fig. 6). Injection of

the same amount of MitoQ did not decrease the ROS level. SkQ1 caused some decrease which, however, was statistically insignificant [69].

Some markers reporting on the distortion of the kidney function after ischemia/reoxygenation are presented in Fig. 7A–E. These experiments were done on animals having a single kidney (the other has been removed before the ischemia). This model recently applied, e.g., by Serviddio et al. [73], was chosen to put higher load on the kidney function since the single-kidney ischemia in animals carrying two kidneys was shown to be without lethal effect on the organism. In the case of single kidney experiments, there was an obvious beneficial action of SkQR1. Partial normalization of diuresis, blood [creatinine], and  $\text{Ca}^{2+}$  reabsorption took place, facts showing that this compound protects to a certain degree the kidney tissue from damage induced by ischemia/reperfusion. A single-kidney ischemia (90 min) followed by reoxygenation was extremely traumatic to the whole organism. The majority of rats died during the first six days after this kind of treatment. However, a preventive single injection of SkQR1 or SkQ1 a day before ischemia almost totally abolished the death of the experimental animals (Fig. 7F). The rats stayed alive for at least half a year after ischemia [69].



**Fig. 7.** Effects of SkQR1 (1  $\mu\text{mol/kg}$ , intraperitoneally injected a day before 90 min ischemia) on kidney functioning and survival of rats after kidney ischemia in single-kidney rats. A–E, parameters of kidney functioning. A, diuresis (ml/day); B, blood creatinine ( $\mu\text{M}$ ); C, blood urea (mM); D, glomerular filtration rate (ml/min $\times$ kg); E,  $\text{Ca}^{2+}$  reabsorption (%). F, Survival of single-kidney rats exposed to 90 min kidney ischemia followed by reoxygenation. SkQ1 or SkQR1 were intraperitoneally injected a day before the ischemia. (From Bakeeva et al. [69]).



**Fig. 8.** Protective effect of SkQR1 against brain damage induced by compressive brain ischemia. SkQR1 treatment, single intraperitoneal injection of 1  $\mu\text{mol}$  SkQR1/kg one day before the experiment. (A and B) parietal and frontal parts of the rat brain, respectively. Numbers of animals in A and B, 19 and 13, respectively. (From Bakeeva et al. [69]).

To obtain brain ischemia, we used a compression model of hemorrhagic insult by application of a load to a certain brain area [74]. This model simulates the situation in the brain when the outflow blood is released into the brain cavity as a result of a hemorrhagic insult, causing the pressure on the certain areas of the brain and, as a consequence, an ischemia. Analysis of serial 100  $\mu\text{m}$  brain sections revealed severe damage to the tissue that could be decreased by intraperitoneal injection of SkQR1 (0.5 or 1  $\mu\text{mol}/\text{kg}$ ) a day before compressive ischemia (Fig. 8). Treatment with 0.5  $\mu\text{mol}/\text{kg}$  failed to substitute for SkQR1 [69]. One may speculate that SkQR1 as better penetrant than SkQ1 more easily reaches brain.

Favorable effect of SkQR1 on neurons could be related to inhibition of the ROS-induced apoptosis by this antioxidant. It was found that SkQR1 protects primary culture of rat cortical neurons against  $\text{H}_2\text{O}_2$ -induced apoptosis. The optimal SkQR1 concentration was 100 nM [69].

#### 4. SkQ1 inhibits tumor development in p53-deficient mice

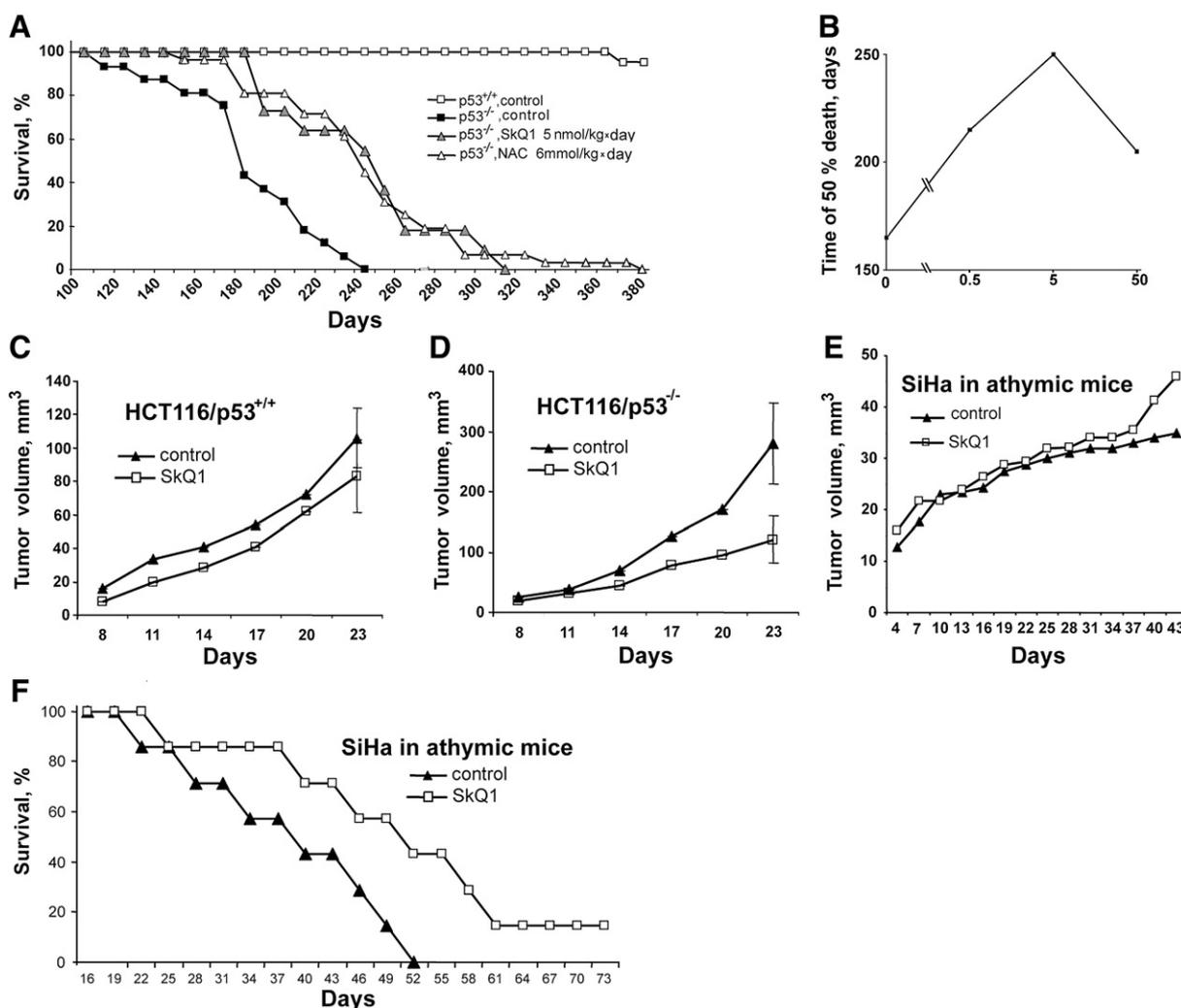
As discussed above, an increase in oxidative stress is a key factor of the aging process. Moreover, it is known that elevation of ROS plays an important role in development of tumors (reviewed in [75,76]). Such relationships may be at least partially responsible for strong increase with age in occurrence of cancer. In fact, moderate rise of the ROS content, which *per se* does not kill a cell, leads to enhanced DNA oxidation and, as a result, to genetic instability and accumulation of oncogenic mutations in proto-oncogenes, tumor suppressor genes, and some other genes, causing neoplastic cell transformation and/or further tumor progression [75,76,79]. In addition, changes in level of ROS which function as secondary messengers in many signal transduction pathways affect cell proliferation, migration, and viability that also contribute to tumorigenesis [77,78,80–82]. It is noteworthy that 1.5–3-fold increase in intracellular ROS concentration represents an important component of oncogenic effects of such genomic alterations typical for human neoplasms as inactivating mutations of the tumor suppressor p53 or activating mutations of RAS and MYC proto-oncogenes [79,82–87]. NAC and some others antioxidants were shown to (i) mitigate genetic instability caused by the p53, Ras, and Myc malfunctioning [83–85]; (ii) decrease growth rate of Ras-transformed cells [86]; (iii) diminish the intensity of Ras-induced morphological transformation and cell motility [82]; (iv) inhibit ROS-dependent tumor angiogenesis induced by Ras and p53 dysfunctions [87,88] or by some other oncogenic events [78,89]. As a result, development of tumors (in particular, in transgenic mice with p53 gene knockout (p53<sup>-/-</sup>) [83,90] or with Myc protein super-production [91]) was shown to significantly delay.

In this part of the study, we asked whether mitochondria-targeted antioxidant SkQ1 affects tumor development. For this purpose, we used two approaches: (i) analysis of the effect of diet supplementation with SkQ1 on spontaneous development of tumors (prophylactic

activity), and, (ii) a study on the influence of SkQ1 on progression of already formed tumors (therapeutic activity). In the first case, we used mice with homozygous p53 gene knockout [92,93] resulting in ROS up-regulation [83]. In the second part of the work, we studied the growth of xenografts of a set of human carcinomas with different status of p53 tumor suppressor. Investigations of various p53 abnormalities was done because (i) a p53 malfunctioning represents the most universal molecular alteration in various human tumors (reviewed in [90,94–96]) whose oncogenic effect is at least partially dependent on attenuation of antioxidant defense [83,90,96]; (ii) several types of p53 abnormalities are characteristic for human tumors, including missense-mutations leading to synthesis of proteins which fail to perform the wild-type p53 functions but acquire quite other activities [94,95]. In particular, we studied xenografts of HCT116 colon carcinomas (a) expressing wild-type p53, (b) expressing p53 proteins with amino acid substitutions in positions 248 and 273 which represent the hot-spots of mutations in colorectal cancers, or (c) showing loss of p53 expression due to homozygous p53 gene knockout. In addition, we analyzed xenografts of SiHa human HPV-16-associated cervical carcinoma in which p53 functioning is mitigated due to its interaction with E6 viral oncoprotein. Finally, we studied SkQ1 effects on tumor cells cultured *in vitro* to understand possible mechanisms of inhibitory influence of SkQ1 on growth of some types of tumors [97].

To study possible anti-oncogenic potential of SkQ1 we, first, studied effect of diet supplementation with various SkQ1 doses on tumor appearance in the p53<sup>-/-</sup> mice. Such mice show increased levels of ROS [83] 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%) [90,91]. We found that 5 nmol SkQ1/kg  $\times$  day caused about twofold decrease in intracellular level of ROS in mouse tissues, in particular in spleen cells [97]. In mice receiving this dose of SkQ1, the tumor development was delayed and the lifetime of animals was increased by approximately 30–40% (Fig. 9A). In fact, 50% death for p53<sup>-/-</sup> mice obtaining or not obtaining SkQ1 occurred on days 250 or 180, respectively. Effect of SkQ1 on the early death was especially demonstrative. On day 185, about 60% of the p53<sup>-/-</sup> mice without SkQ1 had died, whereas not a single animal died among those who obtained SkQ1 (Fig. 9A). Such effect of extremely low dose of SkQ1 was comparable with the effect of very much (more than 1,000,000-fold!) higher dose of conventional antioxidant NAC (6 mmol/kg  $\times$  day) used in our previous studies [83,90] (confirmed in the experiment shown in Fig. 9A). Lower (0.5 nmol/kg  $\times$  day) and higher (50 nmol/kg  $\times$  day) SkQ1 dosages were less effective in tumor prevention (Fig. 9B).

In the next series of experiments, we studied the effect of SkQ1 on growth of xenografts of human tumors in immunodeficient athymic mice. For this purpose, we used tumor cells differing in the p53 status. In particular, we studied a set of derivatives of HCT116 colon carcinoma mimicking all situations observed in human colon cancers, i.e. the cells expressing wild-type p53 protein, cells with simultaneous expression of wild-type and dominant-negative mutant p53 proteins, and full loss of p53 expression. All the HCT116 cell derivatives with abnormal p53 expression/function showed some increase in ROS levels as compared with the original HCT116 cell line expressing wild-type p53 protein. Supplementation of the diet with 5 nmol SkQ1/kg  $\times$  day caused certain, but statistically non-significant, inhibition of the growth of HCT116 tumors expressing the wild-type (Fig. 9C) or mutant (not shown) p53 proteins. The inhibitory effect of the used SkQ1 dosage on the growth of HCT116/p53<sup>-/-</sup> xenografts was more prominent and statistically significant. This case is shown in Fig. 9D. Surprisingly, the xenografts of HCT116/p53<sup>-/-</sup> derivative which expressed additionally the R248W mutant p53 protein showed lower sensitivity to growth-suppressive effect of SkQ1, although such cells practically did not differ from original HCT116/p53<sup>-/-</sup> cells in intracellular ROS level (not



**Fig. 9.** Effect of SkQ1 on the lifespan of p53<sup>-/-</sup> mice (A, B) or athymic mice with SiHa xenografts (F), on growth of the HCT116/p53<sup>+/+</sup> (C) and HCT116/p53<sup>-/-</sup> (D) human colon carcinoma xenografts or the SiHa human cervical carcinoma xenografts (E) in athymic mice. SkQ1 was added to the drinking water. A, B, Control p53<sup>+/+</sup> mice (30 animals), control p53<sup>-/-</sup> mice (17 animals), and p53<sup>-/-</sup> mice received 0.5 nmol SkQ1/kg×day (32 animals), 5 nmol SkQ1/kg×day (11 animals), 50 nmol SkQ1/kg×day (19 animals), or 6 mmol NAC/kg×day (26 animals). A log-rank test comparing the group of non-treated p53<sup>-/-</sup> mice and the groups treated with 5 nmol SkQ1/kg×day or 6 mmol NAC/kg×day showed two-sided distribution,  $p < 0.005$ . (From Agapova et al. [97]).

shown). These relationships were apparently due to different activity of the multidrug resistance pump in cells of different p53 status. As shown in experiments with fluorescing SkQR1, this cation is extruded from the cell by the pump, the process being more effective in p53 R248W mutant cells than in wild type or p53<sup>-/-</sup> [97].

In addition, we found that the lifespan of the SkQ1-treated tumor-bearing immunodeficient mice increased as compared with control SkQ1-untreated animals even in those cases when the treatment with SkQ1 showed no significant effect on the growth rate of tumor xenografts. For example, diet supplementation with 50 nmol SkQ1/kg×day did not inhibit at all the increase in volumes of SiHa human cervical carcinoma xenografts but significantly delayed the death of such animals (Fig. 9E, F).

As it was found earlier in our group, antioxidant NAC lowered the growth rate of HCT116 xenografts mainly through the inhibition of tumor angiogenesis (tumors treated with NAC showed about twofold decrease in the number of typical blood microvessels and 2–3-fold increase in the number of underdeveloped vessels without lumens) [88]. This is why we decided to study effect of SkQ1 on tumor angiogenesis. We found that SkQ1 inhibited angiogenesis *in vivo* in a model of subcutaneous Matrigel implants. When injected intraperitoneally, SkQ1 significantly decreased the load of implants with blood as well as the content of small blood vessels in Matrigel,

the effect being observed only at highest dose of SkQ1 used (10 μmol/kg×day, 10 days). The content of relatively large (>40 μm) vessels was strongly decreased even at 0.1 μmol SkQ1/kg×day. Angiogenesis in Matrigel implants depends on invasion of the endothelial cells (or their precursors) and further development of the blood vessels. The latter process could be strongly controlled by transcription factor HIF-1α which was activated (stabilized) under hypoxic conditions [98]. It was recently shown by Chandel, Murphy et al. that stabilization of HIF-1α under hypoxia is mediated by ROS produced in mitochondria of endothelial cells since this effect is inhibited by MitoQ [99]. Our data suggest that *in vivo* maturation of the vessels is suppressed by low doses of SkQ1 while their invasion was less sensitive to our antioxidant.

Immunohistochemical analysis of HCT116/p53<sup>-/-</sup> tumor xenografts has shown that, similar to treatment with NAC, supplementing diet with 5 nmol SkQ1/kg×day caused about twofold increase in the number of underdeveloped lumen-free blood vessels but, unlike NAC, caused only minor, statistically non-significant decrease in the number of typical vessels with lumens.

To learn more about possible mechanisms responsible for SkQ1-induced inhibition of growth of xenografts, we studied how SkQ1 affects the cell culture *in vitro*. First, it was found that SkQ1 is able to inhibit the rate of the tumor cell proliferation. In fact, the HCT116/

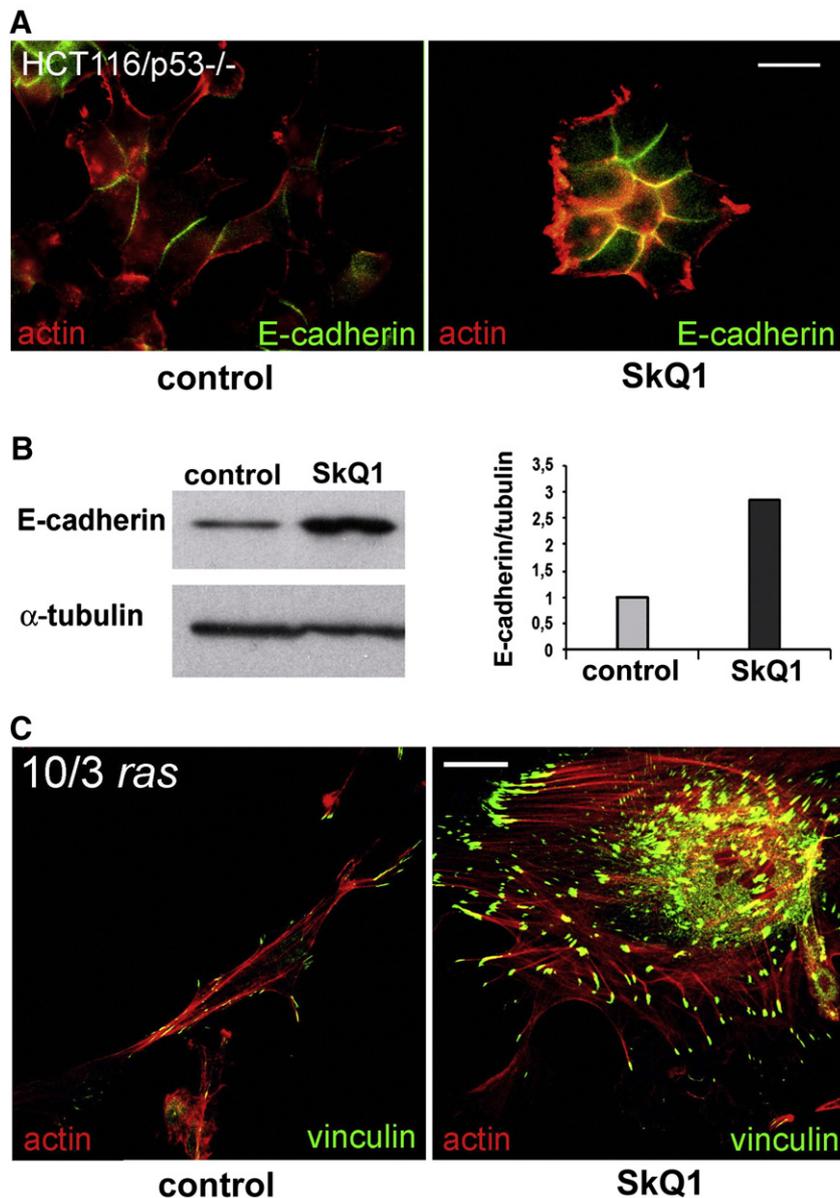
p53<sup>-/-</sup> cells showed some deceleration of the cell growth in the culture after incubation with 5–50 nM of SkQ1 for 5–7 days. A similar effect was observed in other tested cell cultures, in particular in SiHa cells [97].

The HCT116/p53<sup>-/-</sup> cells do not show normal epithelial type of cytoskeletal organization. Treatment of these cells with SkQ1 caused restoration of epithelial features (Fig. 10A). Actin cytoskeleton became more organized and filaments at the cell periphery and at cell–cell contacts were well pronounced. Immunostaining of E-cadherin revealed formation of prolonged E-cadherin-positive contacts. As a result, the cells treated with SkQ1 formed typical epithelial islets in culture, which were absent in untreated cells.

We also studied several epithelioid cell lines originated from carcinoma of uterine cervix (CaSki, SiHa, C4I, HeLa). These cells contain the wild-type p53 but show deregulated p53-dependent signaling due to infection with papilloma viruses HPV16 or 18 and interaction of viral E6 oncoprotein with p53. One of these cell lines, SiHa (contains integrated HPV16), was studied in detail. These cells

showed disorganized system of actin filaments and the absence of organized intercellular E-cadherin-positive contacts. Treatment with SkQ1 increased the total amount of E-cadherin (Fig. 10B) and restored actin bundles and well-organized intercellular contacts of SiHa cells. Morphology of these cells and their islets became almost indistinguishable from normal keratinocytes. The morphology of non-transformed keratinocyte line HaCat was not significantly affected by SkQ1. Phenotype reversion (restoration of the normal epithelial-like morphology) by SkQ1 treatment was observed also with other carcinoma cell lines (CaSki, C4I, and HeLa). Of note, the well known carcinoma HeLa, which is strongly dedifferentiated and has lost almost all epithelial features, became organized in a more epithelial fashion after treatment with SkQ1 [97]. Our data are in line with previously published results on NAC-induced apical–basolateral differentiation of colon and ovary carcinoma cell lines [100].

The effect of SkQ1 on transformed fibroblasts was studied in mouse fibroblast cell line 10(3) with deleted p53. These cells can be considered as minimally transformed cells. They are non-tumorigenic



**Fig. 10.** Actin filaments and E-cadherin-positive intercellular contacts in HCT116/p53<sup>-/-</sup> (A), and amount of E-cadherin in SiHa (B) before and after treatment with SkQ1 (40 nM for 7 days). (A) immunostaining with specific antibodies; (B) Western blot analysis. (C) Reversal of the Ras-induced morphological transformation in 10/3 mouse fibroblasts incubated with 20 nM SkQ1 for 7 days. Immunostaining of actin filaments and vinculin-positive focal adhesion contacts. Bar, 20 μm. (From Agapova et al. [97]).

and retain basic morphological features of normal fibroblasts [101]. The cells were dramatically changed upon transformation by oncogene N-Ras<sup>Asp13</sup>. Transformation was accompanied by great decrease in the cell area. A major feature of cell transformation during malignization consists in the loss and/or reorganization of actin structures, which leads to enhanced cell motility and invasion [102]. Ras induced dissipation of actin stress fibers and related vinculin-positive focal adhesions. This effect can be caused, at least in part, by inactivation of the Rho-ROCK-LIMK signaling pathway mediating cofilin phosphorylation and stress fiber formation [103]. We recently showed that ROS up-regulation is critical for Ras-induced modulation of cofilin activities responsible for reorganization of cell architecture and actin cytoskeleton [82]. Treatment of the Ras-transformed cells with SkQ1 caused restoration of normal fibroblastic morphology, i.e., re-appearance of the stress fibers and vinculin focal adhesion contacts, and strong enlargement of cell area (Fig. 10C). Similar phenotypic reversion was observed in the SkQ1-treated SV40-transformed human fibroblasts MRC5-V2 [97].

Concluding this section, the data presented above clearly demonstrate that SkQs do not possess a cancerogenic activity. Even more, SkQ1 showed an anticancer effect at least in cells deficient in p53, i.e., in a situation typical for many kinds of the human cancerogenesis. In fact, we found that SkQ1 suppressed spontaneous tumorigenesis in p53<sup>-/-</sup> mice as well as HCT116/p53<sup>-/-</sup> tumor xenograft growth in athymic mice. These relationships can be accounted for assuming that mitochondrial ROS-mediated apoptosis, which is specifically inhibited by SkQs does not participate in the anticancer defense. Rather, mitochondrial ROS are cancerogenic, so their scavenging by SkQ1 decreases probability of malignization.

### 5. SkQ treatment of eye diseases. SkQ1 returns vision to blind animals

Retina is in fact a tissue with the highest risk of ROS-induced damage since (i) it contains a high level of polyunsaturated fatty acids, a very good target for ROS, (ii) it is exposed to light producing such kind of ROS as singlet oxygen and (iii) oxygen concentration in 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) [104]. There are numerous indications of the crucial role of ROS in the main age-related ocular pathologies, i.e. retinopathies (such as maculodystrophy [105,106], retinitis pigmentosa [107,108], hereditary optic neuropathy [109]), glaucoma [110,111], cataract [112,113], and uveitis [114,115]. Polyunsaturated fatty acids in mitochondrial cardiolipin are first of all attacked by mitochondria-produced ROS that are quenched by SkQs [37]. This is why the above-listed pathologies attracted our attention as a possible field of therapeutic application of SkQs. Below we shall describe some results obtained when animals of various ages, suffering from retinopathies, cataract, uveitis, glaucoma, and some other eye diseases were treated with SkQ1 [116].

We studied the therapeutic effect of SkQ1 on cataract and retinopathies, using OXYS rats, a strain suffering from constant oxidative stress. In these rats, cataract and retinopathy appear as early as at 2.5–3 months age [117,118].

In the first series of experiments, it was found that levels of lipid peroxidation (estimated by measuring MDA) and oxidation (carbonylation) of proteins are higher in skeletal muscles of one year old Wistar rats than in three month old ones. The effect of age was even larger in OXYS rats. Feeding with very low amounts of SkQ1 (50 nmol/kg × day) resulted in a decrease in the lipid and protein oxidation levels. The mineral mass levels in vertebra and extremities were lower in OXYS than in Wistar rats due to senile osteoporosis. Again, SkQ1 feeding was favorable, increasing the mineral mass in OXYS rats [116].

Thus, the above data showed that SkQ1 is competent in preventing some consequences of oxidative stress in OXYS rats. Then an attempt was undertaken to treat eye diseases in these animals with SkQ1. As

experiments showed, addition of the above-mentioned SkQ1 amounts to the food completely prevented development of cataract and retinopathy in OXYS rats up to age of two years (Fig. 11A–C, Table 3).

The above conclusion concerning preventive effect of SkQ1 on 24 month old OXYS rats was confirmed by histological analysis of sections across the retina (Fig. 11D–F). The figures show that in an old OXYS rat without SkQ1 treatment, the photoreceptor layer is absent, whereas an OXYS rat receiving SkQ during all its life retained this layer. In old Wistar rats the photoreceptor layer was present even without SkQ. These results are in line with our observations that the electroretinogram disappeared in the majority of the 24 month old OXYS rats but was retained in OXYS rats with SkQ1 as well as in Wistar rats (Fig. 11C, Table 3).

Vitamin E was much less efficient than SkQ1. Even 500 μmol vitamin E/kg × day (i.e. 10,000-fold higher than SkQ1) decreased the cataract and retinopathy levels far less than SkQ1. It is remarkable that the SkQ1 effects were not accompanied by any induction of cytochromes P450 in liver, in contrast to those of vitamin E [116].

It was also found that instillations of drops of nanomolar SkQ1 significantly reverse pathological changes in middle-age OXYS animals (Fig. 11G, H). The latter effect was also observed in Wistar rats suffering from cataract. In very old (24 months) rats, neither cataract nor retinopathy was reversed by SkQ1 (although SkQ1 still effectively prevented the diseases, see Fig. 11A–F).

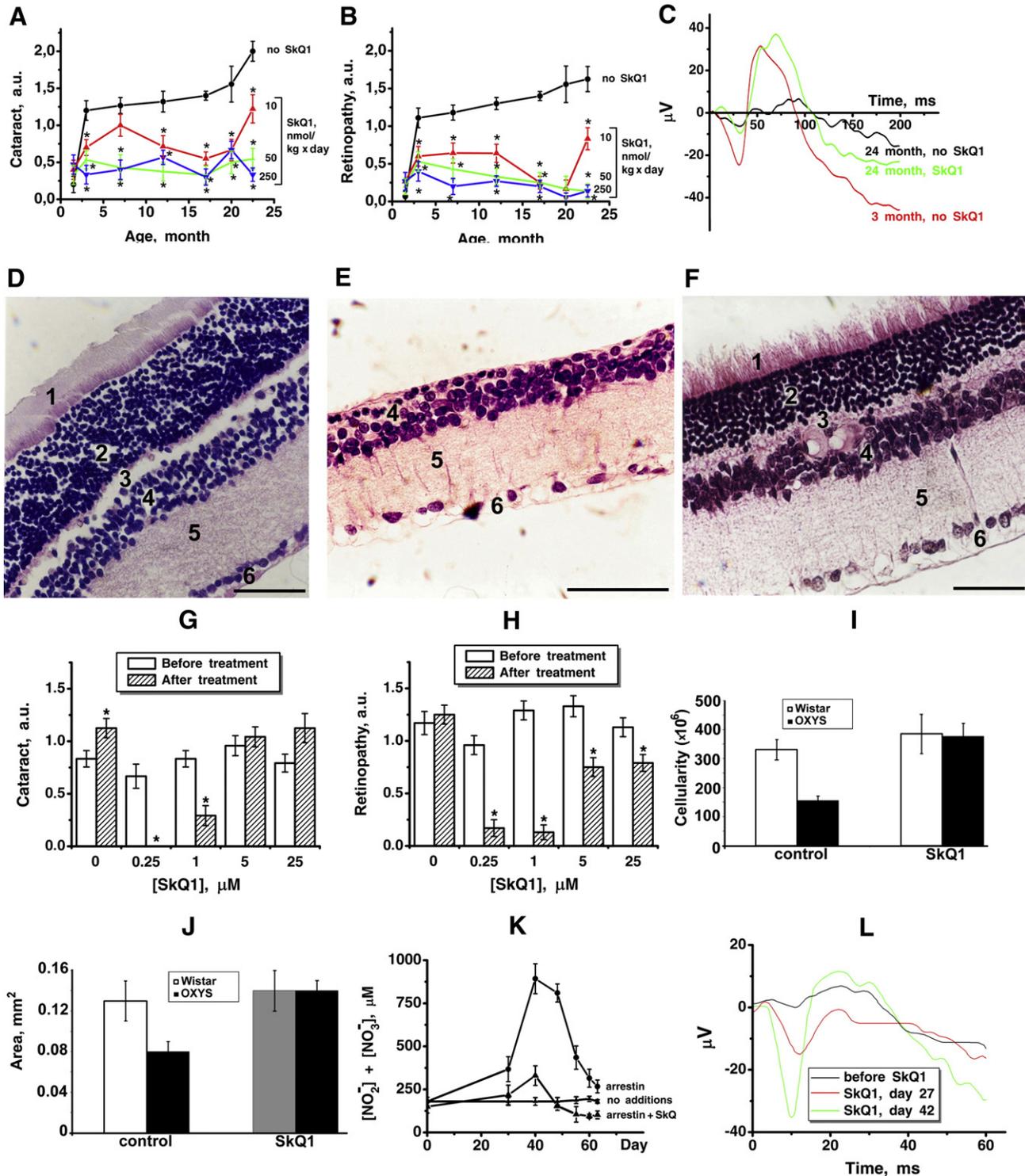
Reversal of an already developed retinopathy by SkQ1 drops was confirmed by electron microscopy. It was shown that retinopathy in 11-month-old OXYS rats results in obliteration of choriocapillaries. This parameter is at least partially normalized after a 1.5-month course of instillations of 250 nM SkQ1 (one drop daily). Reappearance of choriocapillaries in the presence of 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 [116].

Favorable effects of SkQ1 can disappear when it is added in excess. In skeletal muscles of OXYS rats, this occurred at 250 nmol SkQ1/kg × day. In bones of the same rats, 250 nmol SkQ1 was still as effective as 50 nmol. In eyes of OXYS rats, drops of 10 nM–1 μM SkQ were effective in reversal of cataract and retinopathy, 5 μM being ineffective (Fig. 11G,H). As to eyes of Wistar rats, even 25 μM SkQ was still of favorable activity. These relationships can be explained assuming that (i) disappearance of therapeutic action of SkQ is due to prooxidant activity of its high concentrations [37] in OXYS rats suffering from oxidative stress, and (ii) the intrinsic antioxidant status of Wistar rats is much better compared with OXYS rats, so these rats are better defended against prooxidant effect of high [SkQ1].

In the same OXYS rats, it was found that SkQ1 feeding prevents a premature age-dependent decline of the immune system, namely involution of the thymus and a decrease in the area occupied by primary follicles in spleen (Fig. 11I and J, respectively).

In another series of experiments, uveitis was induced by immunization of a rabbit with a photoreceptor-specific protein, arrestin, which resulted in blindness. This effect was prevented and reversed by SkQ1 instillations (four drops of 250 nM SkQ1 per day) [116]. It was also found (Fig. 11K) that the same SkQ1 treatment strongly inhibited formation of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> in eyes of the uveitis-suffering animals (under uveitis, these processes are known to be initiated by interaction of NO and O<sub>2</sub><sup>-</sup>, which are superproduced in the retina of the uveitis-suffering animals [119]).

Experimental glaucoma was also studied in rabbits. The disease was induced by a series of the instillations of 2% hydroxypropyl methyl cellulose to the eye anterior sector. This resulted in appearance of such typical glaucoma features as increase in intraocular pressure (P<sub>o</sub>), a strong decrease in the aqueous humor outflow (C), as well as in humor production (F), a rise of Bekker's coefficient (BC), and some increase in the lens thickness. Analysis of



**Fig. 11.** Effects of SkQ1 on animal eye diseases (A–H and K, L; from Neroev et al. [116]) and on age-dependent involution of thymus (I, J) (from Obukhova et al., in preparation). SkQ1 prevents development of cataract (A) and retinopathies (B, D–F) in OXYS rats. C, electroretinograms of three OXYS rats: 3 months, no SkQ1; 24 months, no SkQ1 and 24 months, 250 nmol SkQ1/kg × day with food. Flash, 3 cd · s/m<sup>2</sup>. G, H, Therapeutic effect of various SkQ1 concentrations upon already developed cataract and retinopathies in OXYS rats. The treatment (one drop of SkQ1 solution daily) was started when rats were 9 months old. Drops of SkQ1 were instilled during 52 days. In each group, 24 eyes of 12 animals were studied. I and J, SkQ1 prevents an age-dependent decrease in cellularity of the right lobe of the thymus and in area occupied by the spleen follicles, respectively. When indicated, 3.5-month-old rats received 250 nmol SkQ1/kg × day with food. #, *p*, 0.05. A histological study of sections across the retina of 24-month-old Wistar (D) and OXYS (E, F) rats. F, OXYS rats obtained SkQ1 with food from age 1.5 months (250 nmol/kg × day). Staining with hematoxylin and eosin. D, Wistar rats; normal retina structure is well seen, i.e. (1) rods, (2) outer nuclear layer, (3) outer plexiform layer, (4) inner nuclear layer, (5) inner plexiform layer, and (6) ganglionic cell layer. E, OXYS rats without SkQ1; there is no photoreceptor compartment (rods as well as outer nuclear and plexiform layers are absent). F, OXYS rats with SkQ1; all the layers are present. D–F, bar, 50 µm. K, SkQ1 prevents an experimental uveitis-induced increase in the nitrite and nitrate levels in the aqueous humor of the eye interior sector. Twenty eyes (10 rabbits) were studied, namely, 6 eyes without and 14 eyes with SkQ1 (four drops of 250 nM SkQ1 were instilled daily during 33 days). L, Electroretinograms of the left eye of a dog suffering from an inherited retinal dysplasia. Where indicated, one drop of 250 nM SkQ1 was instilled daily during 27 or 42 days. The dog was blind before the SkQ1 treatment. After the treatment, vision returned.

**Table 3**

Effect of SkQ1 added to food (250 nmol/kg × day) on the b-wave magnitude of the rat electroretinogram

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

From Neroev et al. [116].

Standard errors are indicated.

photographs of the fundus revealed excavation of the optic disk. Pathologic changes of all these parameters were prevented by instillation of drops of 5  $\mu$ M SkQ1 solution. Higher SkQ1 (25  $\mu$ M) was less efficient than 5  $\mu$ M SkQ1 (Table 4).

Finally, an attempt was undertaken to apply SkQ1 in cases of veterinarian practice when conventional medical treatments failed. A total of 135 animals (dogs, cats, and horses) suffering from various retinopathies were treated daily with drops of 250 nM SkQ1. In 89 cases, the animals were completely blind before the treatment. Vision was returned to 67 of them (Table 5). There was not a single case when SkQ1 had an unfavorable effect or its efficiency declined in the course of the treatment time.

Electroretinograms of a dog whose visual function was recovered by means of 250 nM SkQ1 instillations are shown in Fig. 11L. The dog was blind because of inherited retinal dysplasia. As the figure shows, before the SkQ1 treatment there was practically no electric response to light. After 27 days of SkQ1 instillations, visual function was partially recovered and some electric response appeared. Even larger response was revealed on day 42 of the treatment, which was accompanied with further improvement of vision [116].

Among dogs, cats, and horses suffering from retinopathies were those with inherited retinal dysplasia (degeneration), progressing retinal degeneration, or a secondary retinal degeneration. The best results of SkQ1 treatment were obtained with inherited dysplasia (a positive effect in 67% cases) and secondary degeneration (in 54% cases). As to progressing degeneration, SkQ1 helped in 29% cases. Moreover, SkQ1 was effective in some cases of dry eye syndrome as well as for treatment of uveitis and some other autoimmune eye diseases, conjunctivitis, and certain corneal diseases (Table 6). Drops of SkQ1 were without effect on neuro-ophthalmological pathologies.

It should be mentioned in this context that an attempt to treat inherited retinopathies by a mitochondria-targeted antioxidant was recently undertaken by Wright et al. [120]. They used MitoQ and failed to obtain any positive result. The reason for this might be small size of the window between anti- and prooxidant concentrations of MitoQ [37].

## 6. SkQ1 prolongs lifespan and retards senescence

In the final series of experiments, we tried to inhibit by SkQ1 the senescence program of different organisms. We started with short-lived species, namely the mycelial fungus *Podospora anserina*, a

**Table 4**

Drops of 5  $\mu$ M SkQ1 prevent development of experimental glaucoma in rabbits

Group	Parameter				Lens thickness, mm
	P <sub>0</sub>	C	F	BC	
Control	17.4 ± 0.7	0.16 ± 0.02	1.28 ± 0.26	108 ± 24.2	7.3 ± 0.08
Glaucoma	19.6 ± 0.8*	0.05 ± 0.01*	0.38 ± 0.13*	387 ± 47.4*	7.9 ± 0.14*
Glaucoma + 5 $\mu$ M SkQ1	16.5 ± 0.8	0.14 ± 0.04	2.5 ± 0.49*	117.2 ± 27.6	7.5 ± 0.08
Glaucoma + 25 $\mu$ M SkQ1	22.5 ± 1.0*	0.10 ± 0.04	2.0 ± 0.43	224.6 ± 39.2*	7.8 ± 0.21*

From Neroev et al. [116].

\*  $p < 0.05$  for the eye with experimental glaucoma vs. normal eye.

**Table 5**

Therapeutic effect of instillations of Vetomitin (drops of SkQ1) on retinopathies

Animal species	Number of animals					
	Before treatment			After treatment		
	Blind	Partial loss of vision	Total	Vision was returned to blind animal	Vision was improved	Vision was not improved
Dog	58	19	77	46	19	12
Cat	27	9	36	17	5	14
Horse	4	18	22	4	18	0
Total	89	46	135	67	42	26

From Neroev et al. [116].

crustacean *Ceriodaphnia affinis*, and an insect *Drosophila melanogaster* [121].

During the last decade, the fungus *P. anserina* has become the subject of interesting experiments on regulation of lifespan. In particular, this parameter can be significantly changed experimentally by affecting mitochondrial respiration, morphology, and ROS formation [122,123]. This is why we started the gerontological part of the project with studies on *P. anserina*.

As experiments showed (Fig. 12A), low (100 nM) SkQ1 added to the agar medium increases the lifespan of *P. anserina*. SkQ1 was especially effective at early stages of aging, e.g., on day 21, 70% of the fungi die without SkQ1 and only 25% with SkQ1. The median lifespan was increased by 50% by SkQ1. The difference decreased with age so that lifespan of the last 10% of fungi was increased by only 20%. Higher (1  $\mu$ M) and lower (20 nM) SkQ1 concentrations were less effective than 100 nM. The effect of SkQ1 was accompanied by retardation of development of such traits of mycelial aging as appearance of brown color and disappearance of protrusions of mycelium to the air phase (aerial mycelium) [121].

The crustacean *C. 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 not a problem. One more advantage is that the concentration of SkQ1 affecting the living subject could be exactly estimated since *C. affinis* can live in aqueous solutions of SkQ1 of known dilution. Fig. 12B shows that 5.5 and 0.55 nM SkQ1 increase the *C. affinis* lifespan, whereas 55 nM SkQ1 has an opposite effect. The median lifespan at the optimal (0.55 nM) SkQ1 concentration was doubled compared with the samples without SkQ1 [121].

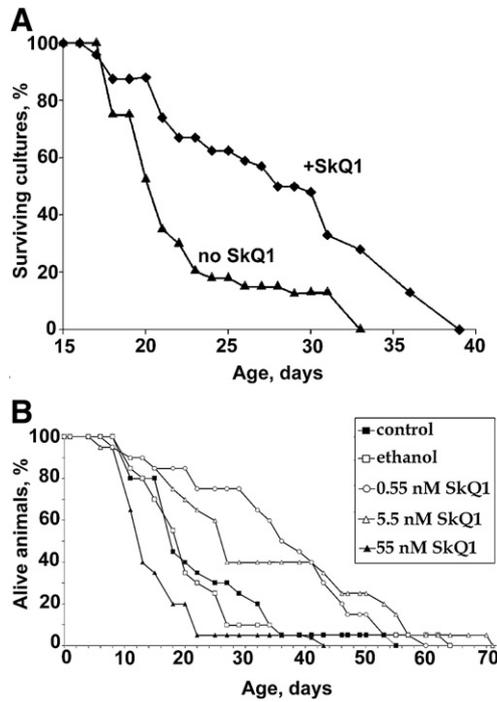
*D. melanogaster* is an effective model system for gerontological studies due to its reasonably short lifespan and the possibility to use genetically well characterized lines. Our experiments showed that SkQ1 prolonged lifespan of flies, though the effect was not as strong as in *C. affinis*. The food with supplemented SkQ1 (three drops of 20  $\mu$ M SkQ1 solution coated to the food surface weekly) proved to be effective

**Table 6**

Therapeutic effect of instillation of Vetomitin (drops of 250 nM SkQ1) on uveitis, conjunctivitis and cornea diseases

Animal species	Total	Positive effect	No change
<i>Uveitis</i>			
Dog	26	23	3
Cat	8	8	0
Horse	19	19	0
Total	53	50	3
<i>Conjunctivitis</i>			
Dog	32	32	0
Cat	15	15	0
Horse	5	5	0
Total	52	52	0
<i>Cornea diseases</i>			
Dog	31	31	0

From Neroev et al. [116].



**Fig. 12.** Effect of SkQ1 on lifespan of fungus *Podospora anserina* (A) and of crustacean *Ceriodaphnia affinis* (B). In A, 100 nM SkQ1 was used. In B, ethanol solutions of different SkQ1 concentrations were added to the growth medium, ethanol being diluted by factor of 1,000. Addition of the same amount of ethanol without SkQ1 did not affect lifespan. (From Anisimov et al. [121]).

in both females and males. Maximal effect was revealed between 20 pM and 20 nM SkQ1.

Detailed analysis of the effect of SkQ1 on lifespan of females revealed that it is more pronounced at early stages of life. As seen in Fig. 13A, mortality during the first 10 days of life was almost completely abolished by 20 pM SkQ1, whereas during the first 20 days SkQ1 decreased mortality by only 30%. At higher ages, the effect of SkQ1 became even smaller. This phenomenon is also illustrated by Fig. 13B where mortality rate is presented in half-logarithmic coordinates as a function of age. It is evident that SkQ1 effect was at its maximum for the first 10 days, when mortality was decreased by one order of magnitude. A mixture of 20 pM solution of TPP and PQ, two constituents of SkQ1, was completely ineffective [121].

Fig. 13C–E summarizes results of experiments when the SkQ1 treatment was carried out during certain stages of life rather than during the entire lifespan. Fig. 13C shows that the survival curve of flies fed with SkQ1 during the first week of life did not differ from the survival curve of flies constantly obtaining SkQ1 till death ( $p = 0.75$ ), whereas both were significantly different from the control curve ( $p = 0.0004$  and  $p < 0.0001$ ). Such a short-term SkQ1 treatment was ineffective when started on the 30<sup>th</sup> day (Fig. 13D). However, constant SkQ1 feeding from the 30<sup>th</sup> day till the end of life was effective (Fig. 13E,  $p = 0.0025$ ) [121].

20 pM  $C_{12}$ TPP, a compound containing two additional methylene groups instead of plastoquinone in SkQs, was also found to increase

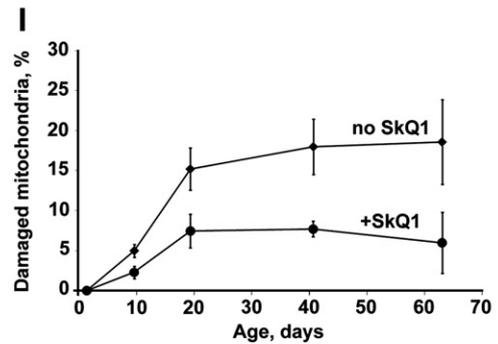
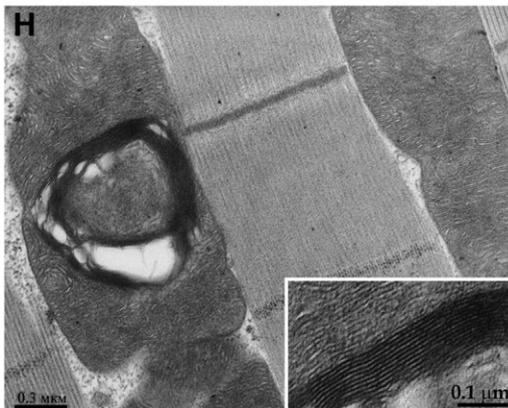
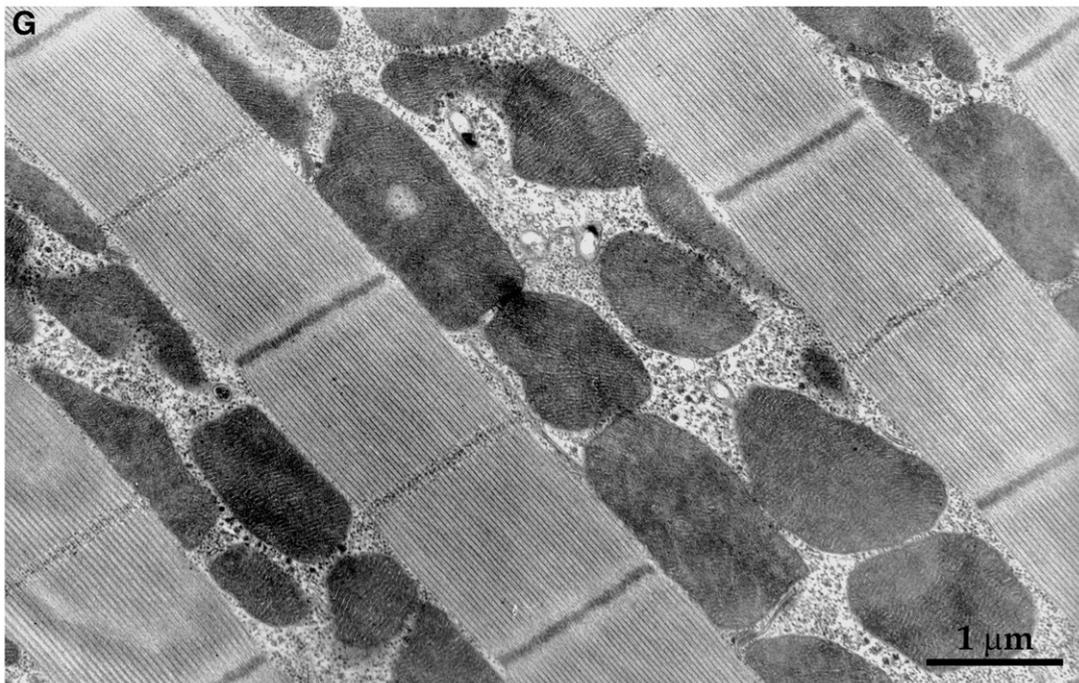
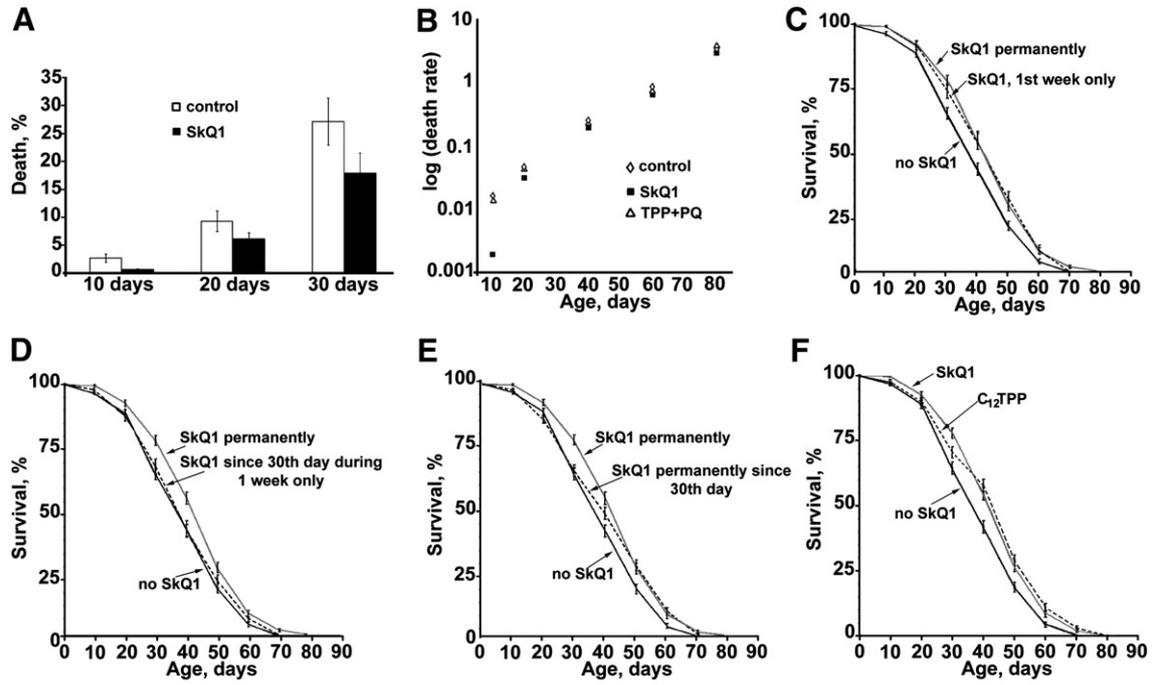
the lifespan of flies. However, substantial difference in effects of SkQ1 and  $C_{12}$ TPP was revealed. SkQ1, as already mentioned, primarily affected young females, whereas  $C_{12}$ TPP was practically ineffective in young flies but was effective later in life. As a result, the  $C_{12}$ TPP survival curve followed the control curve during the first 20 days, while during the last 40 days it followed the SkQ1 curve (Fig. 13F), being significantly different from control curve ( $p < 0.0001$ ) [121]. As was recently found in our group (see above), hydrophobic cations such as SkQs and  $C_{12}$ TPP can operate as carriers of fatty acid anions, mediating in this way mild uncoupling of oxidative phosphorylation by these acids. This, in turn, should decrease the ROS production not only by Complex I but also by Complex III [37].

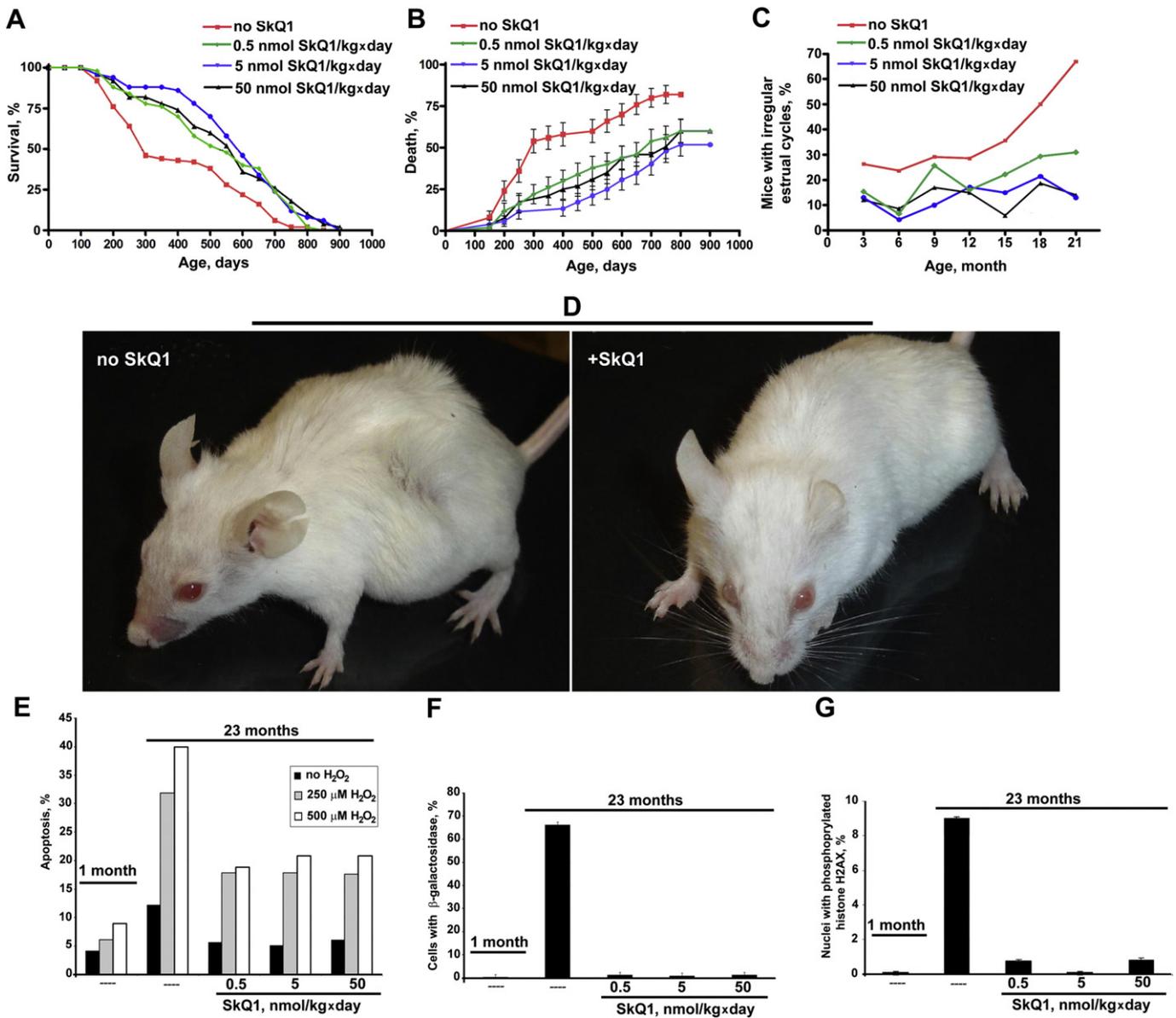
Fig. 13G–I show the effect of SkQ1 on mitochondrial ultrastructure in flight muscles of *D. melanogaster* of different ages. In line with data of Walker et al. [124], we found that aging is accompanied by appearance of organelles of dramatically changed structure (membrane multilayers resembling myelin). Such structures called “mitochondrial swirls” [124] were absent from very young (1.5 days) flies but appeared as early as on the 10<sup>th</sup> day of life. The mitochondrial swirls were usually integrated into chains of mitochondria localized between bundles of actomyosin filaments. In the SkQ1-fed flies, damaged organelles were still very rare even on the 65<sup>th</sup> day (Fig. 13I).

In this context, it should be noted that the idea concerning mitochondrially-targeted quinones as geroprotectors was introduced by Murphy et al. [125] and simultaneously by one of us (V.P.S.) [126]. In Murphy et al. [127], an attempt was undertaken 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 showed several traits of progeria. We also tried 20 pM MitoQ and observed some geroprotective effect even in wild type flies. The effect was smaller than with SkQ1 and could be revealed only on early ages. As to the median lifespan, it 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 the amount of damaged muscle mitochondria was measured [121].

Two long-term experiments were performed to reveal 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 $\times$ day. The results of both experiments are summarized in Fig. 14A. One can see that all the SkQ1 concentrations used significantly prolonged the lifespan, the middle (5 nmol) concentration being optimal. Moreover, like 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, the median lifespan was found to double. For mammals, such a strong shift of the survival curve to the right is a very rare case. On day 300, more than 50% of control mice had already died, whereas in the group of 5 nmol SkQ1 almost all animals were still alive [121].

**Fig. 13.** SkQ1 increases the lifespan of *Drosophila melanogaster*. A and B, SkQ1 affects lifespan mainly at early stages of life (cumulative percent of death and mortality rate, respectively). A total of 425 females per treatment were analyzed, 20 pM SkQ1 or mixture of 20 pM TPP and 20 pM PQ being employed. C–E, SkQ1 fed during the first week of life (C) or constantly, starting from the 30<sup>th</sup> day (E) increases lifespan of females as does SkQ1 fed during entire life, whereas SkQ1 fed during one week starting from the 30<sup>th</sup> day is ineffective (D). From 225 to 425 females per treatment were analyzed. On C and E, survival curves of SkQ1-treated females are significantly different from control curves ( $p < 0.0001$ ,  $p = 0.0025$ , correspondingly). [SkQ1], 20 pM. F, 20 pM  $C_{12}$ TPP, a SkQ analog lacking the quinone part, increases lifespan of *D. melanogaster* females. A total of 425 females per treatment were analyzed.  $C_{12}$ TPP and SkQ1 survival curves are significantly different from the control curve ( $p < 0.0001$  in both cases). G–I, Effect of SkQ1 on mitochondrial age-related ultrastructural changes in the *D. melanogaster* flight muscles. G, 1.5-day-old fly. H, 52-day-old fly. Insert shows a multimembrane structure at high magnification. I, SkQ1 prevents appearance of age-dependent damage to mitochondria (“mitochondrial swirls”). Males; SkQ1, 1.85 nM. (From Anisimov et al. [121]).





**Fig. 14.** SkQ1 increases lifespan and prevents appearance of traits of senescence in mice. (A) 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. (B) Age-dependent mortality of mice for reasons other than tumors. (C) SkQ1 prevents disappearance with age of regular estrous cycles. (D) 630-day-old mice receiving (right photo) and not receiving (left photo) 0.5 nmol SkQ1/kg × day. (E–G) Some properties of fibroblast obtained from tails of mice receiving or not receiving SkQ1. (E) spontaneous and H<sub>2</sub>O<sub>2</sub>-induced apoptosis. (F) percentage of β-galactosidase-positive cells. (G) percentage of cells with phosphorylated histone H2AX. (From Anisimov et al. [121]).

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. 14B). Most of these 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. This made it possible to take into account probable preventive effect of SkQ on an age-dependent decline of the immune system (for preventive effect of SkQ1 on involution of thymus and spleen in OXYS rats, see above, Fig. 11 I, J).

Prevention of decline of immunity proved to be 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 SHR mice (Fig. 14C). In Fig. 14D, photographs of two 630-day-old female mice, one receiving a minimal dose of SkQ1 (0.5 nmol/kg × day) and another without SkQ1, are presented. It seems obvious that the mouse without SkQ1 is in bad shape compared

with that with SkQ1. The animal has lost whiskers and showed obvious traits of baldness and lordokyphosis. We also found that torpor and a body temperature decrease during the last weeks of life observed in mice not treated with SkQ1 were not pronounced in the SkQ1-treated mice. As to food and water consumption and body weight, they did not significantly differ in all the mouse groups studied, so the SkQ effect cannot be explained by a caloric restriction.

In the last series of experiments on the SHR mice, fibroblasts obtained from the tails of the control and SkQ1-fed animals of various ages were studied. It was found that spontaneous apoptosis of fibroblasts from old control mice occurs three times more often than in young animals. This difference disappeared if mice received 0.5, 5, or 50 nmol SkQ/kg × day. Fibroblasts from mice which received SkQ1 retained the ability to activate apoptosis by added H<sub>2</sub>O<sub>2</sub>, but the effect of H<sub>2</sub>O<sub>2</sub> was not as strong as in fibroblasts from untreated mice of the same age (Fig. 14E). It was also shown that the *in vivo* SkQ1 treatment

prevents development of such typical traits of aging as appearance of  $\beta$ -galactosidase activity and phosphorylated histone H2AX (Fig. 14F and G, respectively).

## 7. Discussion: mitochondria-targeted plastoquinone derivatives as potential tools to prolong youth

Experiments on model systems, mitochondria, and living cells *in vitro* have confirmed the suggestion that conjugates of penetrating cations with plastoquinone (SkQs) are much better antioxidants than those with ubiquinone (MitoQ) [37]. As a result, the window between anti- and prooxidant effects of SkQs is much broader than that of MitoQ. Experiments on rats showed that *in vivo* SkQ treatment prevents development of pathological changes of several age-related diseases. It strongly lowers ischemia/reoxygenation-induced arrhythmia and prevents fibrillation of hearts isolated from animals, decreases damaged area of heart after infarction and of brain stroke, and prevents death after kidney ischemia [69]. In p53<sup>-/-</sup> mice, SkQ1 or NAC increase the lifespan, SkQ1 being effective at 10<sup>6</sup> times lower concentration than NAC [97]. On rats, rabbits, dogs, cats, and horses, instillation of SkQ drops into eyes has favorable effect on retinopathies, cataract, glaucoma, and uveitis, the major kinds of aging-induced eye diseases [116]. Nano- or even picomolar concentrations of SkQ1 increased lifespan of fungus *P. anserina*, crustacean *C. affinis*, insect *D. melanogaster*, and mice. In the latter case, 5 nmol SkQ/kg×day doubled the median lifespan. This was accompanied with disappearance of numerous typical features of senescence, i.e. the loss of estrous cycles and whiskers of females, baldness, decrease in body temperature, torpor, lordokyphosis, osteoporosis, etc. Some aspects of the age-dependent decline of the immune system did not occur [121]. Moreover, our experiments suggest that sexual motivation of males, long-term memory, high rate of wound healing, and normal blood pressure are retained in old animals receiving SkQ1 (Kolosova, N. Medvedeva, N. and Skulachev, V., in preparation). SkQ1 prevented development of canities which accompanies an X-ray-induced progeria in black mice (Ryazanov, A., in preparation). Moreover, SkQ1 abolished age-dependent decrease in lymphocytes and increase in neutrophils in mice (Shipunova, I. et al., in preparation). At the biochemical level, it was revealed that SkQ1 prevents such age-induced processes as peroxidation of lipids and proteins, stimulation of apoptosis, appearance of  $\beta$ -galactosidase activity, and phosphorylation of histone H2AX [121].

The geroprotective effects of SkQ1 could be accounted for within the framework of alternative concepts considering aging as a result of (i) accumulation with age of occasional injuries and (ii) operation of a specific genetic program of slow suicide (for reviews, see [2,6]). In the former case, SkQ1 simply “cleans the dirtiest place in the cell”, removing from the mitochondrial interior those ROS that are responsible for age-related injuries [126]. In the latter case, SkQ1 prevents execution of an aging program which slowly kills organisms by means of intramitochondrial ROS, the level of which increases with age in a program-controlled fashion. At present, we cannot discriminate between these two possibilities. However, there are several indirect but independent pieces of evidence making the program scheme more probable.

(1) In experiments with *D. melanogaster*, we found that SkQ1 treatment during the first week of life has the same geroprotective effect as treatment during the whole life (Fig. 13C). This result is, in fact, in line with the observations that (i) two days caloric restriction of the fruit fly prolongs the lifespan of the fly as effectively as caloric restriction during the whole life [128] and (ii) just odor of the food decreased the caloric restriction effect upon lifespan of *D. melanogaster* [129]. These finding can be explained much more easily by a reset of the “ontogenetic clock” controlling the aging program than by the assumption that SkQ1 or caloric restriction directly prevent accumu-

lation of ROS-induced (or any other) injuries. In this context, one might suggest that the age-controlling “ontogenetic clock” system operates in a mitochondrial ROS-dependent fashion so that the target of the geroprotective action of SkQ1 is, in fact, mitochondria of the clock cells or of any other cells involved in execution of the clock signals.

(2) ROS-induced injuries are known to be accumulated in especially large quantities at late age. Therefore, one might expect that SkQ1 would be more effective toward the end of life. In our experiments, we observed, however, quite opposite relationships. SkQ1 prevented age-dependent death at early and middle stages of aging, being only slightly efficient in increasing the maximal lifespan (see Figs. 12–14). This observation fits the hypothesis that the aging program is a mechanism increasing “evolvability” (the rate of evolution), assuming that aging allows natural selection to recognize small useful traits that are insufficient for a strong young organism but become significant when its functions decline with age [2,7,20,22]<sup>2</sup>. Very old organisms (i) compose a small part of population and (ii) their reproductive potential is already lowered so that their contribution to the evolvability process should be limited. This means that in such organisms, the aging program becomes unnecessary for evolution and therefore hardly contributes to the senescence process.

(3) The last but not the least, one should take into account the suggestion concerning SkQ1 as a geroprotector proved to be a consequence of a hypothesis on programmed aging. In such a complicated science as biology, the very fact that hypothesis predicts a set of experimental results is always an argument in favor of its validity.

Within the framework of the concept considering aging as an evolvability-increasing invention of living organisms, it seems obvious that *many* functions of vital importance should decline with age. As a result, evolution of all of them can be accelerated by aging. This is why there are numerous senescence processes which, after all, cause the death of the organism and why effect of SkQs is so pleiotropic. As to non-aging organisms, they may die due to a single reason, and their life is not accompanied by decline of many physiological functions.

As already mentioned above, Brand et al. [9] reported that the lifetime of mammals and birds is longer the lower is the rate of H<sub>2</sub>O<sub>2</sub> generation inside heart mitochondria during the reverse electrons transfer through Complex I. The longest-lived African rodent, the naked mole-rat, was the only exception to this rule. This creature of mouse size is famous for its lifespan of about 28 years instead of the mouse's 2.5–4 years. Cancer, atherosclerosis, immunodeficiency and some kinds of pain are unknown for this animal. The queen and her several sexual partners, the only reproducing members of the large community composed of 200–300 soldiers, have, in fact, no enemies. In the laboratory, naked mole-rats die at age of about 25–28 years for an unknown reason. *Their mortality rate does not depend on age* [130], as if the aging program is absent in this animal. The program seemed to be switched off somewhere downstream of ROS because both the rate of ROS generation on the reverse electrons transfer [9] and the level of biopolymer oxidation [131,132] in naked mole-rats are *higher* than in mice. The latter finding is not surprising since activities of superoxide dismutase and catalase in these two species are of the same order of magnitude, while the third main antioxidant enzyme, glutathione peroxidase, is 70-fold less active in naked mole-rats than in mice [133]. The key for understanding of this paradoxical situation seems to consist in

<sup>2</sup> Imagine two hares, a clever one and a stupid one, being chased by a fox. If the hares are young, their muscles are strong, and they will both easily escape the fox. When the hares become older, they will be affected by muscle wasting as a sign of aging, and they will run slower. However, the clever hare will probably start running before the stupid one, and has more chances of surviving and producing clever leverets. This is how aging may be advantageous for natural selection [2].

that even very high concentrations of H<sub>2</sub>O<sub>2</sub> failed to induce apoptosis in cultured arteria of naked mole-rat [134]<sup>3</sup>. In this respect, the mole-rat is reminiscent of long-lived mice with mutations in genes encoding the protein p66shc [137], the enzyme of CoQ synthesis mClk1 [138], or elongation factor 2 kinase (A.G. Ryazanov et al., in preparation), whose cells are also resistant to apoptogenic action of hydrogen peroxide or the prooxidant menadione.

A possible mechanism explaining how a function that became unnecessary for naked mole-rats could disappear during evolution was recently described by Park et al. [139]. It was found that these rodents lack an 11 amino acid peptide called substance P, which operates in other animals as mediator of certain kinds of the pain signal (in particular, of the pain caused by capsaicin, a compound from capsicum). Capsaicin, like several other pain-inducing factors, does not induce a pain response in the naked mole-rat. The sensitivity to capsaicin was restored when a herpes-containing DNA construct encoding the substance P-synthesizing enzyme was injected into a leg of the animal. The treatment did not affect pain sensitivity of the untreated legs. Most probably, the naked mole-rat queen and her mates are so perfectly defended by the army of soldiers in the center of their underground labyrinth that external pain-causing events are practically excluded. As a result, a mutation making impossible transmission of the pain signal proved to be functionally neutral and therefore avoided elimination by natural selection. The same apparently happened with the program of aging which, according to our hypothesis, requires enemies to operate as an evolution-accelerating mechanism.

It is of importance that SkQ1 concentrations increasing lifespan seem to abolish the aging-induced stimulation of apoptosis rather than completely arrest any ROS-linked apoptotic events (Fig. 14E). This is apparently why doses of SkQ1 used in our experiments on mice did not decrease antitumor defense, which includes apoptosis. In fact, SkQ1, like another antioxidant, NAC, prolonged lifespan of a mouse strain prone to lymphomas [97].

It is remarkable that SkQ1 strongly decreases the death risk for reasons other than cancer (Fig. 14B). The reasons in question were numerous infections inevitable in the non-sterile vivarium where the experiments were carried out. This allowed us to reveal SkQ1 as a tool arresting development of such a typical feature of aging as decline of the immune defense of the organism<sup>4</sup>. Such a decline is due to numerous factors including decrease in number of the antigen-presenting dendrite cells (DC), impairment of chemotaxis and phagocytosis of these cells and neutrophils, failure of DC to properly stimulate naive CD4<sup>+</sup> T cells, some functional injuries in monocytes, decrease in the production and proliferation of normal killer cell, etc. (for review, see [140]). Importantly, a decline in the immune system represents one of the earliest traits of aging, starting in humans at an age before 20 [141].

<sup>3</sup> Quite recently, a paper by Salmon et al. appeared [135]. The authors reported that very low proapoptotic activity of H<sub>2</sub>O<sub>2</sub> of naked mole-rat cells may be a tissue-specific feature. An H<sub>2</sub>O<sub>2</sub> resistance observed in cultured arteria is not the case for culture of isolated fibroblasts that are, in fact, more H<sub>2</sub>O<sub>2</sub>-sensitive in mole-rat than in mouse. Alternatively, it seems possible that the low H<sub>2</sub>O<sub>2</sub> sensitivity might be detected at the level of a naked mole-rat tissue rather than isolated cells. As was quite recently shown in our group [137] and B.V. Chernyak et al., in preparation), addition of H<sub>2</sub>O<sub>2</sub> to a cell culture results in a burst of production of endogenous H<sub>2</sub>O<sub>2</sub> in few hours after the addition. This H<sub>2</sub>O<sub>2</sub> can be released from the cell to induce apoptosis in bystander cells. The latter effect is prevented by adding catalase [136] or by release of catalase from damaged cells. Such a damage may be less frequent for mole rat fibroblasts that are more stable to many injuries [135]. In line with the above explanation, sensitivity to prooxidant paraquat proved to be lower in fibroblasts from mole-rat than from mouse [135].

<sup>4</sup> Quite recently, Dr. K.A. Rogovin in our group started studying the effect of SkQ1 on lifespan of rodents that are kept in cages outdoors. This is done to model conditions of the wild nature which are not only non-sterile but also include large temperature fluctuations (the main reason of death of small wild rodents in Russia is pneumonia in the winter).

One of the reasons for decline of the immune system during aging is related to age-dependent involution of the thymus, the major source of T-cells. Progeria in OXYS rats is accompanied with more rapid decrease of the mass, volume, and cellularity of the thymus than in normal rats (Obukhova, L.A. et al., in preparation). The right lobe of the thymus in OXYS rats contains two times less cells than in Wistar rats at 3.5 months of age. Treatment with 250 nmol SkQ1/kg × day resulted in 2.5-fold increase of this amount in OXYS, while in Wistar the increase was only 25%, so this parameter became equal in the two strains of rats. A similar effect was observed when the area of lymphoid follicles in the spleen (the major source of B-cells) was analyzed (Fig. 11I, J).

Above we listed many crucial traits of senescence which are retarded, arrested, and in certain cases even reversed by SkQ1. The question arises why the SkQ-treated living creatures die in spite of obvious deceleration of the senescence program. The simplest explanation is that SkQ1 fails to arrest age-linked decline of at least one physiological function of vital importance. Another possibility is that the aging program does not operate in very old organisms who die due to accumulation of occasional injuries (as was already mentioned, very old organisms are not interesting for evolution since they are too rare and their reproductive systems have declined). Apparently, to live much longer than usually, one needs some “skill”. *P. anserina* represents an interesting example in this context. This fungus seems to employ two scenarios for life, one short-lived (several weeks) and another very long-lived (years). Switching from the short- to long-lived *modus vivendi* can be achieved by substituting a liquid growth medium for a solid one [142]. A similar switch can be observed also in *P. anserina* growing on a solid medium if mutations in respiratory chain or mitochondrial morphology transition machinery takes place [122,123]. The effect of SkQ1 on the short-lived fungus was measurable but much smaller than the switch to the long-lived scenario. In fact, *P. anserina* responded to SkQ1 like three other species tested (*C. affinis*, *D. melanogaster*, and mice), i.e., by 1.5–2-fold increase in the median lifespan and by rectangularization of the survival curve. These relationships can be explained assuming that in *P. anserina* SkQ1 inhibited execution of the aging program but did not induce a switch to the very long-lived *modus vivendi*.

Something similar was recently observed in Dr. B. Cannon's group who tested, within the framework of our project, the action of SkQ1 on progeric Larsson's “mutator mice” with the D257A mutation in the proof-reading domain of mitochondrial DNA polymerase  $\gamma$ . Such a mutant polymerase can synthesize DNA but cannot recognize and repair its own errors in the process of this synthesis. As a result, frequency of mutations in mitochondrial DNA increases, entailing strong shortening in the lifespan and premature development of numerous traits of senescence [143,144]. It was found that SkQ1 prevents or retards appearance of such senescence traits as lordokyphosis, baldness, lowering of body temperature, torpor, cessation of the estrous cycles, etc., and increases the lifespan, but not so strongly as is necessary to return it to the wild type level. Again, as in the above-described wild-type mice, the SkQ1-receiving animals usually die without showing a senescence scenario. The most probable reason for death of the SkQ1-treated “mutator mice” is pathological changes in the colon epithelium, a disease specific for mice with mitochondrial mutations [145]. It should be noted that Cannon's experiments were done on animals living in sterile vivarium. Generally, the effect of SkQ1 under these conditions resembled very much that on wild-type mice in the non-sterile vivarium used in the experiments described in this paper. The only difference was that infections were absent from reasons of death of “mutator mice” (Shabalina, I. G. et al., in preparation).

In conclusion, highly effective, mitochondria-targeted, rechargeable antioxidants composed of plastoquinone, hydrocarbon linker, and a penetrating cation (SkQs) have been synthesized and tested on model membranes, isolated mitochondria, cell cultures, *ex vivo*

organs, and living organisms. It has been shown in experiments on the fungus *Podospora*, invertebrates *Ceriodaphnia* and *Drosophila*, and mice that SkQ1 increases the lifespan at very much lower concentrations than previously known geroprotectors. This effect is accompanied by rectangularization of survival curve and (in mice) disappearance or retardation of many crucial traits of senescence. In mice, an increase in the lifespan is first of all due to prevention of the age-dependent decline of the immune system. It is suggested that SkQ1 is competent in switching off a senescence program responsible for a concerted decline with age of key physiological functions. Thus, this small molecule looks promising for elaborating a drug prolonging youth.

## Acknowledgements

Supported by Mitotechnology LLC, M.V. Lomonosov Moscow State University (MSU), the Vol'noe Delo Foundation (grant N 99F-06), Russian Ministry of Education and Science, grant "Leading Scientific Schools" N 5762.2008.4. We are very grateful to Professor V.A. Sadovnichii, Rector of MSU, for his interest in the project and encouragement. We appreciate participation in this study of L.S. Agapova, L.T. Archipova, M.M. Archipova, A.V. Avetisyan, I.V. Barskov, V. A. Chertkov, L.V. Domnina, V.B. Dugina, A. Yu. Efimenko, P.A. Egormin, M.V. Egorov, E.K. Fetisova, O.F. Filenko, A.Zh. Fursova, E.N. Grigorian, A.Yu. Grishanova, N.K. Isaev, E.F. Isakova, E.N. Imdina, O. Yu. Ivanova, D.S. Izyumov, A.V. Kazachenko, L.S. Khailova, N.V. Khromova, O.V. Kilina, V.I. Kirpatovskiy, E.P. Kopenkin, P.B. Kopnin, M. V. Korotetskaya, S.S. Korshunov, N. A. Kovaleva, S.V. Kozlovskiy, V. L. Lakomkin, A.L. Lukashov, K.G. Lyamzaev, V.N. Manskiikh, V.M. Mikhelson, M.S. Muntyan, O. K. Nepryakhina, Yu.P. Novikova, A.A. Panteleeva, A.A. Pashkovskaya, D.I. Pilipenko, T.S. Piskunova, P.P. Philippov, O.Yu. Pletjushkina, E.Y. Plotnikov, E. N. Popova, I.G. Popovich, A. V. Pustovidko, O.V. Robustova, T.I. Rokitskaya, N.V. Roshchina, O.Yu. Rybina, T.A. Samoylova, V.B. Saprunova, A.V. Semenchenko, L.I. Serebryakova, F.F. Severin, G.S. Shagieva, R.A. Simonyan, I.V. Skulachev, L. F. Sotnikova, N.A. Stefanova, E.V. Stelmashook, E.V. Stepanova, I.M. Studneva, N.V. Sumbatyan, I.V. Sviryaeva, N.K. Tikhomirova, E.V. Titova, O.V. Tskitishvili, E.A. Tsybul'ko, M.L. Tyndyk, J.M. Vassiliev, A.K. Vasilyeva, I.V. Victorov, M.N. Yurova, M.A. Zabezhinsky, A.A. Zamyatnin and R.A. Zinovkin. We are very grateful to Dr. F.O. Kasparinsky for the help in preparing the figures. We would like to express our sincere gratitude to Professors G. Blobel, B. Cannon, K. Lewis, J. Nedergaard, S. Papa, and A.D. Vinogradov for valuable advice. We are very grateful to Dr. R.H. Lozier for editing the English version of the paper. Generous financial support of Mr. O.V. Deripaska, which in fact made possible this study, is greatly appreciated.

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