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Review

In search of novel highly active mitochondria-targeted antioxidants: Thymoquinone and its cationic derivatives

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ABSTRACT

Since the times of the Bible, an extract of black cumin seeds was used as a medicine to treat many human pathologies. Thymoquinone (2-demethylplastoquinone derivative) was identified as an active antioxidant component of this extract. Recently, it was shown that conjugates of plastoquinone and penetrating cations are potent mitochondria-targeted antioxidants effective in treating a large number of age-related pathologies. This review summarizes new data on the antioxidant and some other properties of membrane-penetrating cationic compounds where 2-demethylplastoquinone substitutes for plastoquinone. It was found that such a substitution significantly increases a window between anti- and prooxidant concentrations of the conjugates. Like the original plastoquinone derivatives, the novel compounds are easily reduced by the respiratory chain, penetrate through model and natural membranes, specifically accumulate in mitochondria in an electrophoretic fashion, and strongly inhibit H_2O_2-induced apoptosis at pico- and nanomolar concentrations in cell cultures. At present, cationic demethylplastoquinone derivatives appear to be the most promising mitochondria-targeted drugs of the quinone series.

1. Quinones as mitochondria-targeted antioxidants

Mitochondria are the only intracellular organelles whose interior is negatively charged relatively to the exterior [1,2]. This fact can be used to specifically address various compounds to mitochondria. To this end, it was suggested to combine the transported compound with a positively charged ion easily penetrating through biomembranes [3,4]. To make an ion permeable for membranes, its ionized atom should be surrounded by bulky hydrophobic residues that delocalize the electric charge of this atom [1,2,5]. Such a principle was employed to construct mitochondria-targeted antioxidants [5–18]. Among them, some quinone derivatives proved to be the most active (Fig. 1). As was found in our group, the antioxidant activity measured in isolated mitochondria treated with Fe^{2+} and ascorbate increases in the series: 10-(6′-ubiquinonyl)decyltriphenylphosphonium (MitoQ) < 3′-demethoxyMitoQ (DMMQ) = (6′-methylplastoquinonyl) decyltriphenylphosphonium

Abbreviations: $\Delta\psi$, transmembrane electric potential difference; BLM, bilayer planar phospholipid membrane; C_{12}R1, dodecyl rhodamine 19; C_{12}TPP, dodecyltriphenylphosphonium; DMMQ, 3′-demethoxyMitoQ; MDA, malondialdehyde; MDR, multidrug resistance; MitoQ, 10-(6′-ubiquinonyl)decyltriphenylphosphonium; ROS, reactive oxygen species; SkQ1, 10-(6′-plastoquinonyl)decyltriphenylphosphonium; SkQ3, (6′-methylplastoquinonyl) decyltriphenylphosphonium; SkQR1, 10-(6′-plastoquinonyl) decylrhodamine 19; SkQT1(p), 10-(6′-toluquinonyl) decyltriphenylphosphonium; SkQT1(m), 10-(5′-toluquinonyl) decyltriphenylphosphonium; SkQT1, a mixture of SkQT1(p) and SkQT1(m) in proportion of 1.4:1; SkQTR1, 10-(6′-toluquinonyl) decylrhodamine 19; TQ, thymoquinone

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(SkQ3) < 10-(6′-plastoquinonyl)decyltriphenylphosphonium (SkQ1) [15,16]. Thus, substitutions of methoxy group by methyl and methyl group by an H atom seem to be favorable for antioxidant activity. It would be interesting to continue this quinone series by substituting one more methyl group in plastoquinone by an H atom, just as it occurs in 2-demethylplastoquinone, an intermediate of plastoquinone biosynthesis [19] and in so-called *thymoquinone* (Fig. 1A), a plant antioxidant responsible for many favorable pharmacological effects of black cumin (see below, Section 3). We studied thymoquinone-like derivatives conjugated with penetrating cations, namely decyltriphenylphosphonium (in SkQT1) and decylrhodamine 19 (in 10-(6′-toluquinonyl) decylrhodamine 19 (SkQTR1)) (Fig. 1B). In the next section, some results of this study are reviewed.

2. Cationic thymoquinone derivatives: effect on model membranes, isolated mitochondria and cell cultures

SkQT1 and SkQTR1 were synthesized in essentially the same way as their plastoquinone analogs, SkQ1 and 10-(6′-plastoquinonyl) decylrhodamine 19 (SkQR1) [15]. SkQT1 samples were a mixture of *p* and *m* isomers (Fig. 1B) in the proportion of 1.4:1. SkQTR1 was purified as *p* isomer, like other SkQs [15].

In the first series of experiments, generation of diffusion potential of SkQT1 on bilayer planar phospholipid membranes (BLM) was demonstrated. Like other penetrating cations [2,4], the concentration gradient of SkQT1 was found to generate an electric potential difference, the compartment with lower [SkQT1] being positively charged due to downhill transmembrane

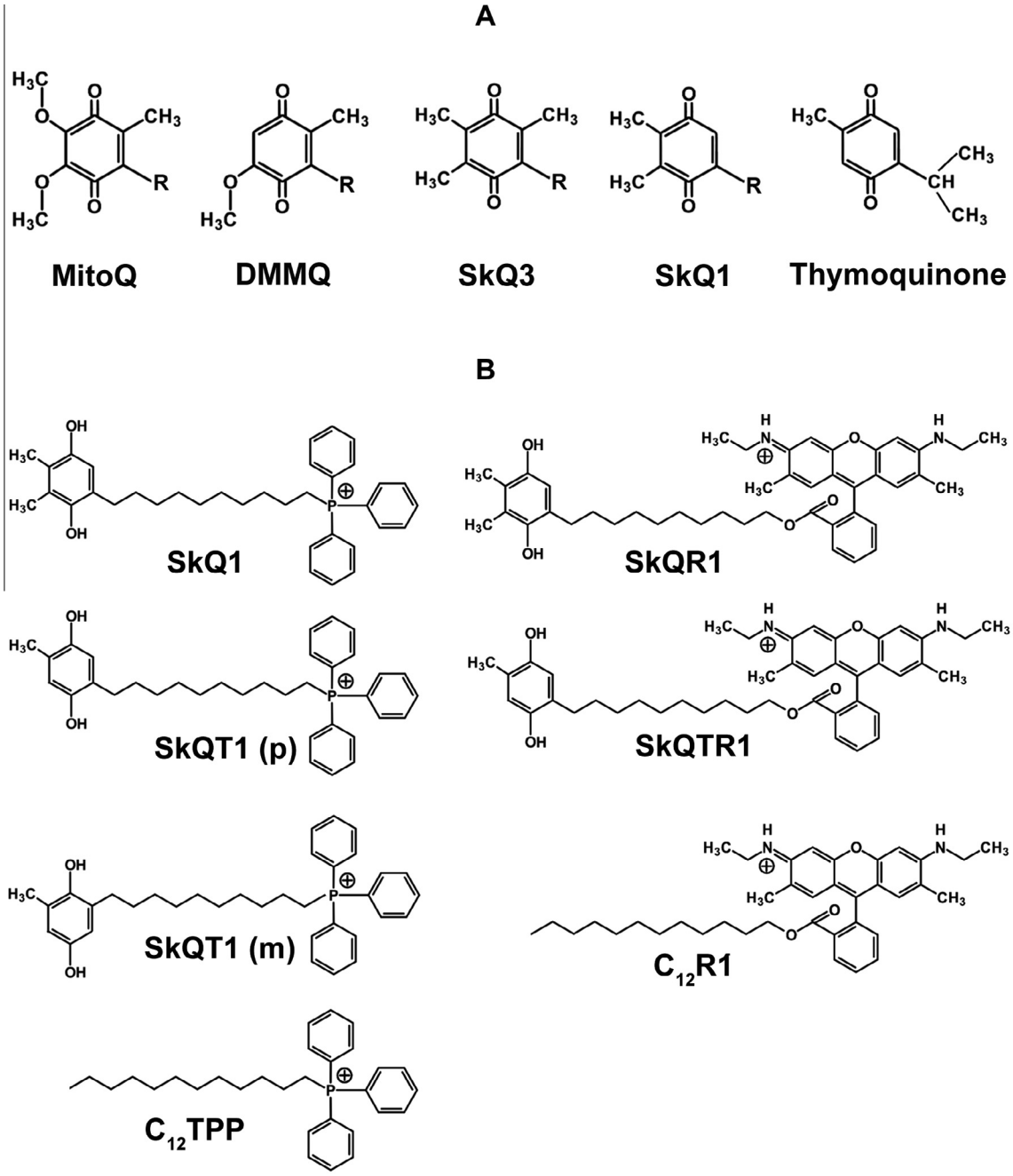

Fig. 1. Formulas of certain compounds considered in this review. (A) Mitochondria-targeted cationic quinone derivatives and thymoquinone. R, decyltriphenylphosphonium. (B) Cationic quinol derivatives of SkQ series and their analogs lacking quinol residue.

diffusion of this cation. The magnitude of transmembrane electric potential difference ($\Delta\psi$) reached the theoretical value (about 60 mV per 10-fold concentration gradient of the penetrating cation, Fig. 2A).

When SkQTR1 was used instead of SkQT1, the BLM responses were more complicated. In this case, the theoretical value of the diffusion potential could be observed only at acidic pH, the maximal $\Delta\psi$ being obtained at pH 4.0 (see Supplemental information, Fig. S1A and B). The effect was biphasic since $\Delta\psi$ decayed with time. If a pH gradient was increased across BLM, an H^+ diffusion potential was generated (Fig. 2B). Such an effect was observed even without adding fatty acids which were required when SkQ1 substituted for SkQTR1 [20]. These relationships can be explained by the fact that rhodamine 19 (the cationic group of SkQTR1) per se can operate as a protonophore, being a weak base (protonophorous properties of SkQR1 were already described elsewhere [21]). Protonophorous effect of SkQTR1 disappeared at acidic pH when the concentration of deprotonated SkQTR1 became rate-limiting. This effect increased in time after creation of the transmembrane SkQTR1 gradient, most probably due to slow saturation by SkQTR1 of the half-membrane leaflet facing the compartment of low [SkQTR1].

In the next experiments, SkQT1 and related compounds were studied in isolated mitochondria in vitro. SkQT1, like SkQ1 [15,16], was found to be a rechargeable antioxidant. Fig. 3A shows that the oxidized form of SkQT1 is reduced by the respiratory chain in an antimycin- and myxothiazol-sensitive manner. Similar properties are inherent in thymoquinone (Fig. 3B) and SkQ1 [15,16,22].

As is shown in Fig. 3C, SkQT1 prevents formation of malondialdehyde (MDA) in mitochondria treated with Fe^{2+} and ascorbate, being almost 10- and 100-fold more active than SkQ1 and thymoquinone, respectively.

Fig. 3D demonstrates the inhibiting effect of SkQT1 and SkQ1 on H_2O_2 production by isolated rat heart mitochondria during the reverse electron transfer from succinate to NAD^+. It is seen that SkQ1 lowers this production, in line with our previous observations [17], depicting a pronounced optimum since an inhibition observed at low SkQ1 concentrations changes to activation when the concentration increases. Experiments on SkQT1 also revealed such a biphasic action, the window between inhibiting and activating concentrations being much larger than that for SkQ1 (600 folds for SkQT1 *vs* 20 folds for SkQ1).

High (micromolar) levels of both SkQ1 and SkQT1 decrease membrane potential in animal (Fig. 3E) and yeast (not shown) mitochondria. Moreover, both compounds strongly potentiated a $\Delta\psi$ decrease caused by palmitate (Fig. 3F), an effect accounted for by facilitating palmitate anion translocation by these penetrating cations [20].

It was also found that SkQT1 stimulated State 4 respiration (Fig. S2A), which can be explained by discharge of $\Delta\psi$ with electrophoretic movement of SkQT1 and cycling of endogenous fatty acids [15,20]. At high concentrations, SkQ1 and SkQT1 inhibited State 4 and State 3 respiration of mitochondria utilizing both NAD^+ substrates and α-glycerophosphate (Fig. S2A–C).

In Fig. 4, effects of SkQT1 and SkQTR1 on cultures of human cells were tested. As shown in Fig. 4A, SkQTR1 specifically stains mitochondria in intact human fibroblasts, resembling in this respect previously studied SkQR1 [15–17]. In Fig. 4B, antiapoptotic effects of SkQT1, SkQTR1, SkQ1, SkQR1, C_{12}TPP and dodecyl rhodamine 19 (C_{12}R1) were compared. It is seen that the SkQTs are more efficient than the SkQs in preventing H_2O_2-induced apoptosis. They inhibited apoptosis at lower concentrations and suppressed this process more strongly than SkQs. Like other SkQs, SkQTs prevented fragmentation of elongated mitochondria ("thread → grain transition"), accompanying apoptosis (not shown).

In the next series of experiments, we examined a novel effect of our antioxidants, i.e., inhibition of growth of human sarcoma cells. One can see in Fig. 4C that low concentrations of SkQ1, SkQR1 and SkQT1 are inhibitory for a culture of human rhabdomyosarcoma. Effective concentrations were 0.02 nM, 0.2 nM and 2 nM for SkQ1, SkQR1 and SkQT1, respectively. Further study revealed that non-targeted antioxidants can substitute for SkQ but at about 1 million-fold higher concentrations (Trolox, 0.1 mM; N-acetyl cysteine, 1 mM). The uncoupler FCCP (1 μM) completely abolished the SkQ inhibition. Dodecyltriphenylphosphonium (C_{12}TPP) and C_{12}R1, penetrating cations lacking antioxidant (quinone) residue, were inactive up to micromolar concentrations. Fibrosarcoma and osteosarcoma cells were also antioxidant-sensitive to some degree, while human fibroblasts, cardiomyocites H9C2 and myoblasts C2C12 appeared to be resistant. The mechanism of growth inhibition consisted in (i) activation of apoptosis sensitive to zVADfmk and (ii) arrest of the cell cycle at G2/M stage (not shown in figures).

It should be stressed that the series of antioxidant active concentrations SkQs ≫ Trolox > N-acetyl cysteine is in line with our

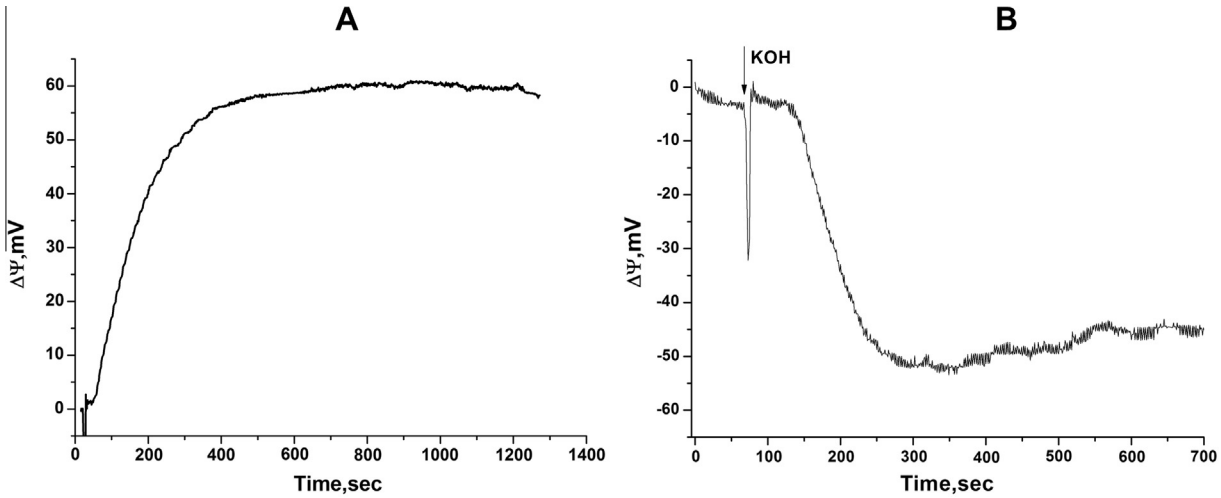

Fig. 2. Responses of SkQT1 and SkQTR1 in BLM. To form BLM, synthetic diphythanoylphosphatidyl chlorine dissolved in decane was used. (A) Diffusion potential generated by 10-fold SkQT1 gradient. The response was initiated by adding 2 μM SkQT1 to one of compartments separated by BLM. Initial SkQT1 concentrations in both compartments was 0.2 μM. Incubation mixture, 1 mM KCl, 1 mM Tris-Mes, pH 6.5. (B) Protonophorous activity of SkQTR1. Incubation mixture, 300 nM SkQTR1, 1 mM KCl, 1 mM Tris, pH 8.5. Addition of KOH to one of the compartments shifted pH to 9.5. For details of the methods, see [15,64].

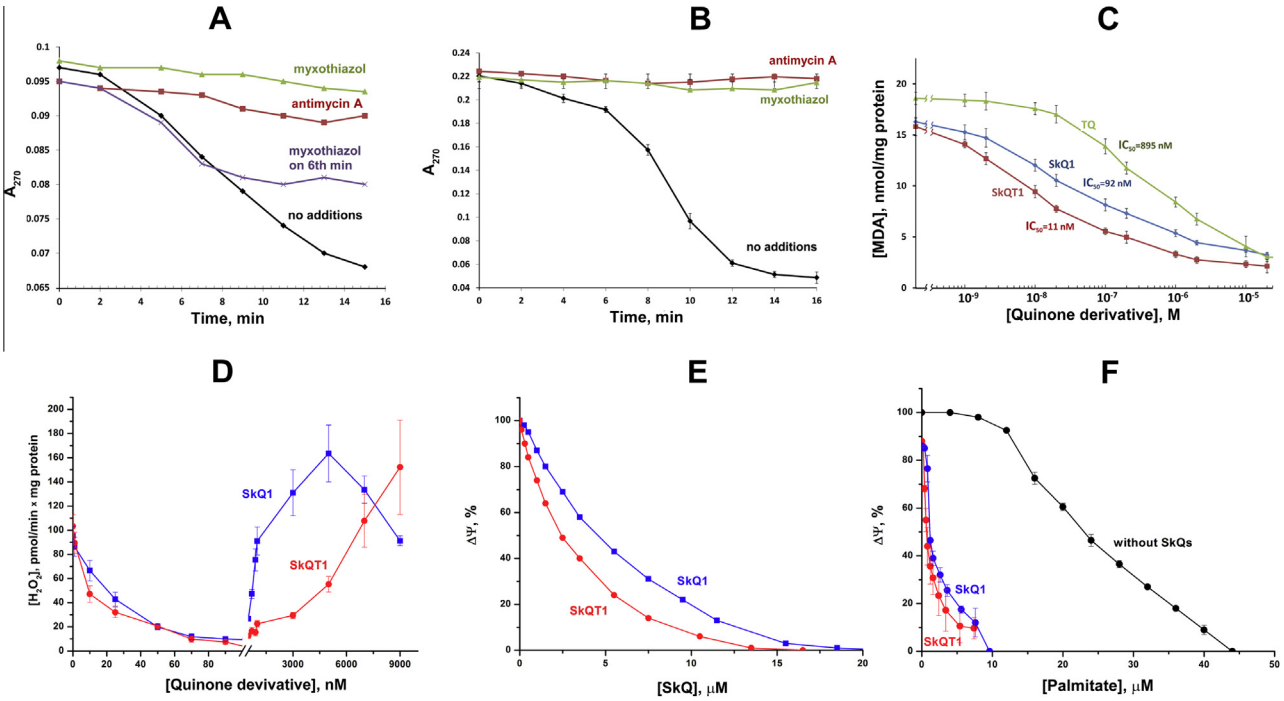

Fig. 3. Effects of quinone derivatives on isolated mitochondria. (A) Reduction of SkQT1 by rat heart mitochondria. Mitochondria were isolated after Palmer et al. [65]. Incubation mixture, 2.5 mM succinate, 2 μM rotenone, 250 mM sucrose, 1 mM EDTA, 10 mM Mops-KOH, pH 7.4, mitochondria, 0.25 mg/ml. Reaction was initiated by adding 10 μM SkQT1 at zero time. Where indicated, 1 μM antimycin A or 1 μM myxothiazol were added. Aminco DW200 was employed to measure light absorbance at 270 nm. (B) Reduction of thymoquinone by mitochondria. Conditions as in (A) but thymoquinone was added instead of SkQT1. (C) Inhibition of malondialdehyde formation by rat heart mitochondria in the presence of Fe^{2+} and ascorbate. Amount of malondialdehyde was measured by means of its reaction with thiobarbituric acid (for procedure, see [15]). The light absorbance was read at 532 nm. (D) Effects of SkQ1 and SkQT1 on H_2O_2 production by rat heart mitochondria in State 4. Rat heart mitochondria (100 μg) were pre-incubated 1 min. at constant stirring at 25 °C in 2 ml solution containing 250 mM sucrose, 10 mM MOPS, pH 7.4, 1 mM EDTA, 5 μM Amplex Red. Then 5 units of horseradish peroxidase were added. Hydrogen peroxide production was initiated by subsequent addition of 10 mM succinate. Data presented as mean ± S.E. (E) Effect of SkQ1 and a mixture of SkQT1(p) and SkQT1(m) in proportion of 1.4:1 (SkQT1) on the membrane potential in rat heart mitochondria. Incubation mixture, 250 mM sucrose, 1 mM EDTA, 10 mM MOPS, 15 μM safranine O, 5 mM succinate and 2 μM rotenone. For other conditions and methods, see [15]. (F) SkQ1 and SkQT1 potentiate ΔΨ decrease caused by palmitate in yeast mitochondria. Incubation mixture, 0.6 M mannitol, 0.5 mM EDTA, 0.2 mM Tris–phosphate (pH 7.2), 20 mM succinate, 20 μM safranine O, and *Yarrowia lipolytica* mitochondria (0.5 mg protein 1 ml). For methods, see [5].

observations obtained in different experimental models [16,17,23]. However, this range inside the SkQ group, i.e., SkQ1 > SkQR1 > SkQTR1, is unusual (Fig. 4C). One of possible explanations might consist in different rate of pumping of these compounds from the cell by multidrug resistance (MDR) ATPase [24]. In fact, it was found (Fig. 4D and Fig. S3A and B) that SkQTR1 accumulation much smaller in the chemotherapy-resistant human myeloid leukemia K562 cells. The accumulation was stimulated by pluronic L61, an inhibitor of the MDR pump [25–27]. Comparison of accumulation of SkQR1 and SkQTR1 by cancer cells (K562, Fig. 4D, and Fig. S3A and B; Hela, Fig. 4E) and immortalized 3T3 fibroblasts (Fig. 4F) and by normal cells (human fibroblasts, Fig. 4G) clearly showed that the rate of this process is much slower for SkQTR1 than for SkQR1 provided cancer cells with induced MDR pumps were studied. The difference between SkQTR1 and SkQR1 strongly decreased in cancer cells without MDR pumps. Normal cells, such as fibroblasts, accumulate both compounds with one and the same rate. As found by Gate et al. [28], methylation of anthracyclines inhibits the rate of their accumulation in cancer cells due to the suppression of the activity of the MDR pump. This may explain the above-described relationships if we take into account that SkQR1 can, in fact, be regarded as methylated derivative of SkQTR1 (see above, Fig. 1B).

3. Comparison of effects of thymoquinone, SkQs and SkQTs

As shown in the preceding section, thymoquinone-like analogs of SkQ1 (i) are mitochondria-targeted antioxidants and (ii) their antioxidant activity in isolated mitochondria and living cells are, as a rule, higher than SkQ1. Historically, there is a principal difference between derivatives of plastoquinone and thymoquinone. The formers were introduced as compounds of potential therapeutic activity several years ago whereas thymoquinone-containing black cumin oil has been known as a medicine for thousand years, being mentioned in the Bible; it was described as the Melanthion of Hippocrates and Dioscorides and as the Gith of Pliny. Later, the Prophet Muhammad advised: "Hold onto use the black cumin, for it has a remedy for every illness except death" [29,30].

Thymoquinone is a pre-dominant (25–60%) component of the essential oil *Nigella sativa*, a 20–30 cm tall herb belonging to *Ranunculaceae* family [29,31,32]. It shows a very pleotropic favorable effect on various human diseases, being curative for osteosarcoma [33,34] and some other types of cancer [35–47], neuropathies [48,49], nephropathies [50,51], arthritis [51–53], inflammation [52–57], astma [58,59], sepsis [53], and decline in immune responses [29,35]. Remarkably, SkQs proved to be efficient in the majority of these diseases [14,16–18,23,60,61]. The difference in therapeutic effects thymoquinone and SkQs is quantitative. SkQs operate at much lower concentrations and their action is usually more pronounced than those of thymoquinone. All these relationships are hardly surprising. (i) Both SkQs [15,16] and thymoquinone [29,35,62,63] are antioxidants. (ii) To some degree, thymoquinone, like SkQs, is specifically targeted to mitochondria since both thymoquinone and SkQs are effectively recharged by reduction in center *i* of mitochondrial complex III [16,22]. This means that in non-mitochondrial membranes oxidized (inactive)

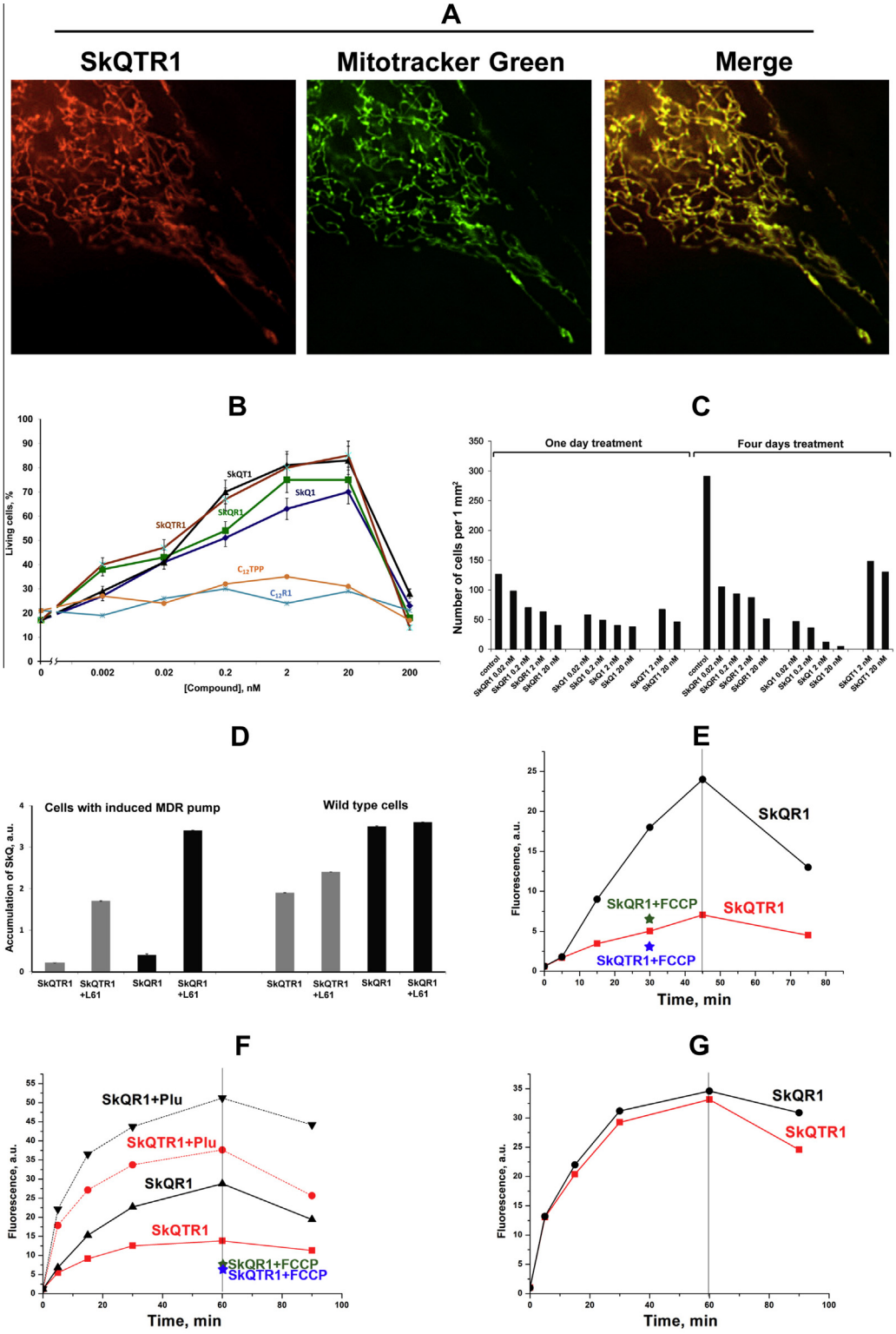

Fig. 4. Effects of SkQT1 and SkQTR1 on cell cultures. (A) SkQTR1 specifically stains mitochondria in human fibroblasts. Fibroblasts were incubated with 100 nM SkQTR1 for 45 min or 200 nM MitoTracker Green (mitochondria-specific fluorescent dye) for 15 min, and analyzed with an Axiovert microscope (Carl Zeiss) equipped with a Neofluar 100× NA 1.3 objective. (B) Effect of penetrating cations on H_2O_2-induced apoptosis. Human fibroblasts were pre-incubated for 24 h with various concentrations of SkQs and their analogs lacking quinone (C_{12}TPP or C_{12}R1). Then 0.5 mM H_2O_2 was added. The number of living cells was estimated 24 h after H_2O_2 addition. For methods, see [15]. (C) SkQs inhibit growth of the human rhabdomyosarcoma cells. Cells growth for one day or four days was measured in the absence (control) or in the presence of indicated concentration of SkQR1, SkQ1 or SkQT1. (D) K562 myeloid leukemia cells with or without induction of MDR pumps were studied. The cells were loaded with 50 nM SkQR1 or SkQTR1 for 90 min., washed and incubated for 30 min. Where indicated, pluronic L61 (plu) was added (60 μg/ml) on 10th min. before adding SkQ. Fluorescence was measured by FACS (Beckman-Coulter FC 500). (E) HeLa cells were studied. 100 nM SkQs and 10 μM FCCP were used. (F) 3T3 cells were studied. (G) Human fibroblasts were studied. In (E), (F) and (G), the samples were washed on 45th or 60th min. with a medium containing no SkQs.

forms of these quinones cannot be regenerated to their reduced (active) state. Therefore, in these membranes, quinol forms of SkQ or thymoquinone can act as antioxidant only once. (iii) Higher efficiency of SkQs comparing to thymoquinone (see, e.g., Fig. 3C) is explained by their electrophoretic accumulation of such cations as SkQs inside mitochondria [16,17] while thymoquinone cannot be accumulated being electroneutral. Moreover, SkQs are amphiphilic and, hence, have very high distribution coefficient between a membrane and a water phase [17]. This coefficient appears to be much lower for the thymoquinone. Besides, the SkQs being penetrating cations were shown to catalyze fatty acid cycling, decreasing thereby $\Delta\psi$ on mitochondrial membrane and inhibiting in this way reactive oxygen species (ROS) production by respiratory chain [20]. Thymoquinone lacking any cationic residues is incapable of performing such a function. Thus, thymoquinone is less efficient antioxidant comparing with SkQT1 and SkQRT1 although it is, like SkQs, mitochondria-targeted antioxidant being rechargeable by the mitochondrial respiratory chain (Fig. 3B). It is not surprising, therefore, that the list of diseases cured by thymoquinone resembles that of the SkQ-cured diseases. It seems promising to construct a new generation of mitochondrial medicines on the basis of SkQT1 which is more efficient than other SkQs. It is especially interesting that SkQTR1 is a much better substrate for MDR pumps of cancer cells. This pump expels SkQTR1 from cancer much faster than SkQR1. In a normal cell, the amount of MDR pumps is negligible and therefore SkQTR1 concentration in such cells should be much higher than in tumors, an effect allowing to specifically preserve tissues other than tumors at anticancer therapies [24]. Investigations into such a possibility are now in progress in our group.

Conflict of interest

M.V.S. is the general director of Mitotech LLC, a biotech company which owns rights for compounds of SkQ type. V.P.S. is a board member of Mitotech LLC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.04.043.

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