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Mitochondria-targeted antioxidant SkQR1 selectively protects MDR (Pgp 170)-negative cells against oxidative stress

Elena K. Fetisova, Armine V. Avetisyan, Denis S. Izyumov, Mariya V. Korotetskaya, Boris V. Chernyak, Vladimir P. Skulachev *

A.N. Belozersky Institute of Physico-Chemical Biology and Institute of Mitoengineering, M.V. Lomonosov Moscow State University, Moscow 119991, Russia

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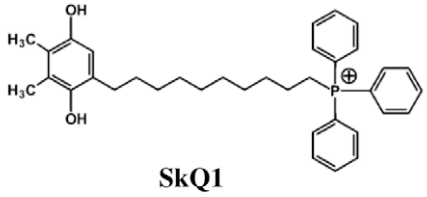
ABSTRACT

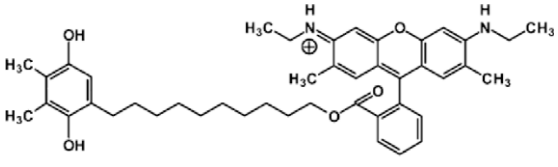
A conjugate of plastoquinone with decylrhodamine 19 (SkQR1) selectively accumulates in mitochondria of normal and tumor cells. SkQR1 protected the cellular pool of reduced glutathione under oxidative stress. Overexpression of P-glycoprotein (Pgp 170) multidrug resistance pump strongly suppresses accumulation of SkQR1. The inhibitors of Pgp 170 stimulate accumulation of SkQR1 in various cell lines indicating that SkQR1 is a substrate of Pgp 170. The protective effect of SkQR1 against oxidative stress is diminished in the cells overexpressing Pgp 170. It is suggested that mitochondria-targeted antioxidants could selectively protect normal (Pgp 170-negative) cells against the toxic effect of anti-cancer treatments related to oxidative stress.

1. Introduction

Mitochondria are one of the major sources for production of reactive oxygen species (ROS) and at the same time one of the critical targets of ROS-induced damage to the cell [1–3]. Recently a novel approach based on mitochondrial targeting of bioactive molecules has been developed (reviewed in [4]). The most popular targeting strategy consists in conjugation of an active molecule with a vehicle that accumulates in the mitochondria due to its positive charge since mitochondria is the only negatively charged compartment of the cell. Penetrating cations necessary for this purpose were developed by one of us [5] and an idea of electrophoretic accumulation of uncharged molecules conjugated with these cations as with "molecular electric locomotives" was introduced soon after this [6]. This approach was realized for the first time to deliver antioxidants thiobutyl, vitamin E and ubiquinone to mitochondria by Murphy and co-workers [7]. Recently we have developed the novel more effective mitochondria-targeted antioxidants based on cationic conjugates of plastoquinone [8–10]. Cations of triphenylphosphonium and rhodamine 19 were shown to be the most effective vehicles in artificial lipid membranes and in isolated mitochondria. The corresponding compounds named 10-(6′-plastoquinonyl) decyltriphenylphosphonium (SkQ1) and 10-(6′-plastoquinonyl) decylrhodamine 19 (SkQR1), respectively, have high antioxidant efficiency in living cells and in animal models [9,11].



SkQ1



SkQR1

Abbreviations: SkQ1, 10-(6′-plastoquinonyl) decyltriphenylphosphonium; SkQR1, 10-(6′-plastoquinonyl) decylrhodamine 19; MDR, multidrug resistance; Pgp 170, P-glycoprotein; ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); GSH, glutathione; CM-DCFH-DA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate

* Corresponding author.
E-mail address: skulach@genebee.msu.ru (V.P. Skulachev).

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Multidrug resistance (MDR) is a state of tumor cells developing during chemotherapy and responsible for decrease in efficiency of traditional chemotherapy of cancer. In the majority of human tumors, this phenomenon is related to overexpression of a 170–180 kDa plasma membrane glycoprotein (Pgp 170) [12,13]. This ATPase belongs to the superfamily of ATP-binding cassette transporters (ABC) and actively extrudes a wide range of structurally diverse drugs used for chemotherapy of cancer. The positively charged rather lipophilic compounds are extruded with maximal efficiency. The mitochondria-targeted antioxidants perfectly fit the requirements to substrates for Pgp 170. If so, these antioxidants could be used to protect of normal tissues against toxicity of anti-cancer drugs that are often inducing ROS overproduction in mitochondria and oxidative damage of these organelles. As to tumor cells, they will remain unprotected since SkQ will be extruded from these cells by Pgp 170.

In this work, we have shown that SkQR1 is a good substrate of Pgp 170 and selectively protects MDR-negative cells from oxidative stress.

2. Materials and methods

Reagents for cell culture were from Gibco, Mitotracker Green and 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-DCFH-DA) were from Molecular Probes. SkQ1 and SkQR1 were synthesized in our laboratory as described early [8,9]. Pluronic L61 was a kind gift of Dr. N.S. Melik-Nubarov (A.N. Belozersky Institute, Moscow State University). The other reagents were from Sigma–Aldrich (USA).

2.1. Cell culture

Normal human subcutaneous fibroblasts (Cell Collection, Institute of Medical Genetics, Russian Academy of Medical Sciences, Moscow), cervical carcinoma Hela, and breast carcinoma MCF-7 were cultivated in DMEM medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 U/ml). Human erythroleukemia K562 was cultivated in RPMI 1640 medium with the same additions. Cells were grown in humidified atmosphere containing 5% CO_2 at 37 °C. MCF-7 [14] and K562 [15] sublines with high level of MDR (the kind gift of Dr. N.S. Melik-Nubarov) were selected for resistance to doxorubicin. Overexpression of Pgp 170 was confirmed by Western analysis [14,15].

2.2. Intracellular accumulation of SkQR1

Intracellular accumulation of SkQR1 was detected using confocal microscope (Carl Zeiss with LSM 510 equipment) and measured by FACS (Beckman-Coulter FC500). Staining with Mitotracker Green (200 nM, 15 min) was used for visualization of mitochondria.

2.3. Non-protein thiols

Non-protein thiols (mainly reduced glutathione) were measured after mixing of cells suspended in PBS with solution containing 5% $HClO_4$, 2.5% Triton X-100, and 2.5 mM EDTA, in the ratio of 1:1. Denatured proteins were separated by centrifugation. Thiols were measured by reduction of 6 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) in 250 mM sodium phosphate (pH 7.5) and

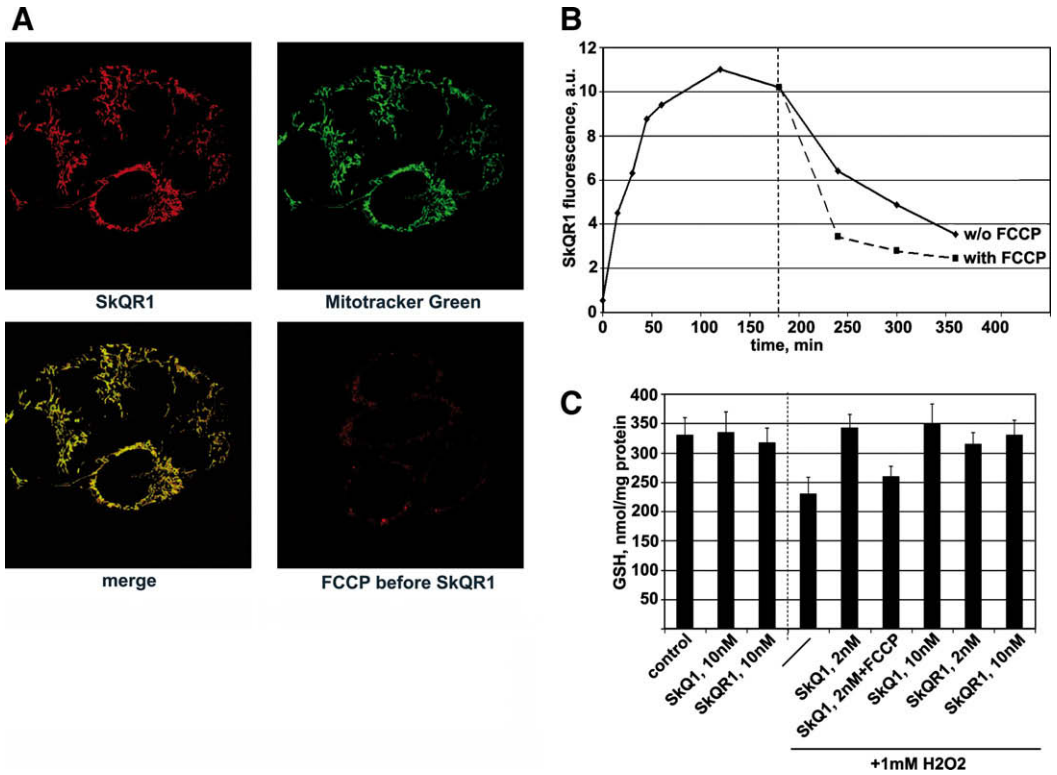

Fig. 1. Accumulation of SkQR1 in mitochondria of HeLa cells. (A) Confocal images of the cells incubated with 50 nM SkQR1 for 3 h and stained with Mitotracker Green (200 nM, 15 min). Where indicated, 10 μM FCCP was added 15 min before SkQR1 (Mitotracker Green was omitted). (B) Accumulation of SkQR1 (50 nM) measured by FACS. After 3 h incubation, SkQR1 was washed out (dotted line) and its release was measured. In the separate assay, 10 μM FCCP was added after washing. The mean values of fluorescence are shown. (C) Reduced glutathione was measured after 2 h of incubation with 1 mM H_2O_2. SkQ1 and SkQR1 were added 2 h before H_2O_2. Where indicated, 5 μM FCCP was added simultaneously with SkQ1.

6 mM EDTA at 412 nm. Protein was measured with the Lowry reagent.

2.4. Cell viability

Cell viability was analyzed by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) using microplate reader as described [16]. Apoptotic cells were stained with Hoechst 33342 (1 μg/ml) for 30 min and apoptotic nuclei were counted using Axiovert microscope (Carl Zeiss).

2.5. Statistical analysis

Each assay was performed at least three times. The data were expressed as mean ± SD and Student's *t*-test was used to determine the significance of differences in multiple comparisons. $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. SkQR1 is selectively accumulated in mitochondria of normal and tumor cell

HeLa cells were incubated with SkQR1 (50 nM) for 3 h and then stained with Mitotracker Green, specific mitochondrial dye (Fig. 1A). Confocal images demonstrated complete colocalization of two dyes, indicating that SkQR1 was selectively accumulated in mitochondria. The detailed analysis of the images revealed that (i) all mitochondria in the cell accumulated SkQR1 and (ii) no other organelles were stained with SkQR1. The protonophorous uncoupler FCCP, that caused mitochondrial membrane depolarization prevented SkQR1 accumulation (Fig. 1A) in full agreement with an assumption that this antioxidant is electrophoretically accumulated in mitochondria. Similar images demonstrating selective accumulation of SkQR1 in mitochondria were obtained for the other cell lines used here, namely normal human subcutaneous fibroblasts, HepG2, MCF-7, K562 (not shown).

Dynamics of SkQR1 accumulation in HeLa cells was studied using flow cytometry. It was found that accumulation of SkQR1 takes approx. 2 h and reached a plateau (Fig. 1B). The amount of accumulated dye was proportional to the SkQR1 concentration but kinetics of the process proved to be concentration independent within the range 20–1000 nM (data not shown). The latter effect might be due to that SkQR1 formed a complex with serum albumin (which was present in high excess in the culture medium) and could play a role of "SkQR1 buffer". Release of SkQR1 from the cells after washing was practically completed in 3 h. It was strongly accelerated by FCCP, indicating that release of the dye from mitochondria was rate-limiting for the whole process. Interestingly, prolonged (24 h) incubation with SkQR1 resulted in much slower release and some staining of mitochondria was detected even after 48 h of washing (not shown).

To analyze antioxidant effect of SkQR1, we have studied oxidation of gluthatione induced by hydrogen peroxide in HeLa cells. Glutathione (GSH) is a major intracellular redox buffer [16] and its depletion caused by H_2O_2 results in an oxidative stress. Incubation of the cells with 2 nM SkQR1 for 4 h almost completely prevented depletion of GSH (Fig. 1C). The parental antioxidant of SkQ family, SkQ1 demonstrated the same efficiency in this model. FCCP ceased the protective effect of SkQ1 confirming the critical role of their accumulation in mitochondria. These data are in perfect agreement with high antioxidant activity of SkQ1 and SkQR1 demonstrated earlier in isolated mitochondria and in living cells where oxidative stress was detected using a ROS-sensitive fluorescent dye (CM-DCFH-DA). Measurements of GSH confirmed the conclusion that oxidative stress induced by exogenous H_2O_2 is mediated by secondary ROS production in mitochondria [17].

3.2. SkQR1 is a substrate of the multidrug efflux protein Pgp 170 (MDR1)

SkQR1 accumulation in HeLa cells was increased by the inhibitors of multidrug efflux verapamil and pluronic L61 (Fig. 2A). These inhibitors also slowed down the release of SKQR1 from the cells upon washing. A similar effect was observed with hepatoma HepG2 cells (not shown). Normal human subcutaneous fibroblasts accumulated almost three times more SkQR1 per cell than HeLa (Fig. 2B). This difference was not related to different cell size or mitochondrial content since flow cytometry measurements of light scattering and Mitotracker Green staining did not reveal any difference between the two cell types (not shown). Verapamil and pluronic L61 only slightly stimulated accumulation of SkQR1 in human fibroblasts (Fig. 2B) indicating a low activity of multidrug efflux pumps in these cells. High activity of multidrug efflux pumps is typical for various tumor cells in culture and in situ [18,19]. They predominantly determine the phenomenon of multidrug resistance [12].

In our experiments, accumulation of SkQR1 was stimulated by verapamil and pluronic L61 which were known as specific inhibitors of the main multidrug efflux protein Pgp 170 [20–22]. We suggested that SkQR1 is a substrate of Pgp 170. To test the role of Pgp 170 in efflux of SkQR1, we have used the MCF-7 and K562 cell sublines with elevated expression of this protein. These sublines were

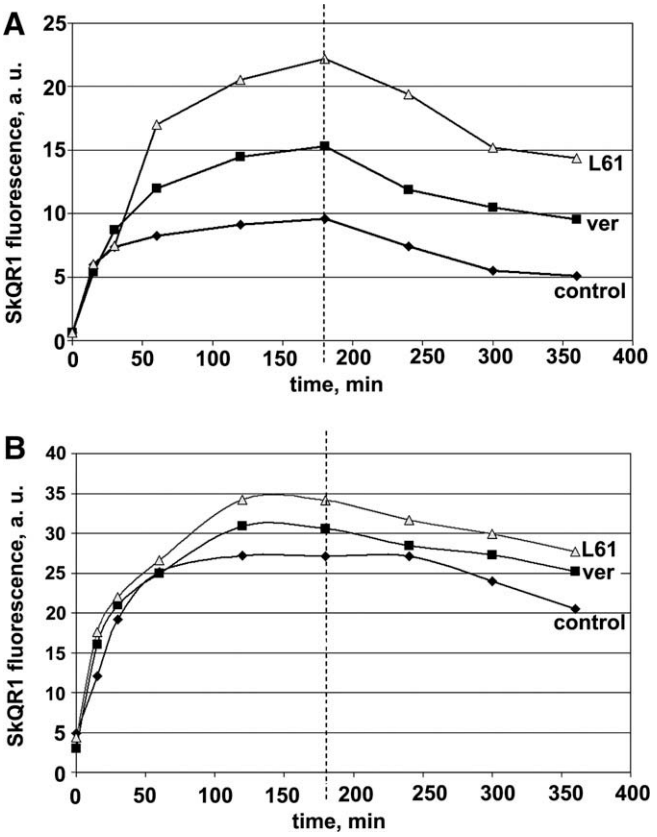

Fig. 2. Effects of inhibitors of multidrug resistance on accumulation of SkQR1. Accumulation of SkQR1 (50 nM) measured by FACS in HeLa cells (A) and fibroblasts (B). Verapamil (50 μM) and pluronic L61 (30 μg/ml), inhibitors of multidrug resistance, were added 1 h before SkQR1. After 3 h of incubation, SkQR1 was washed out (dotted line) and, where indicated, the inhibitors were added.

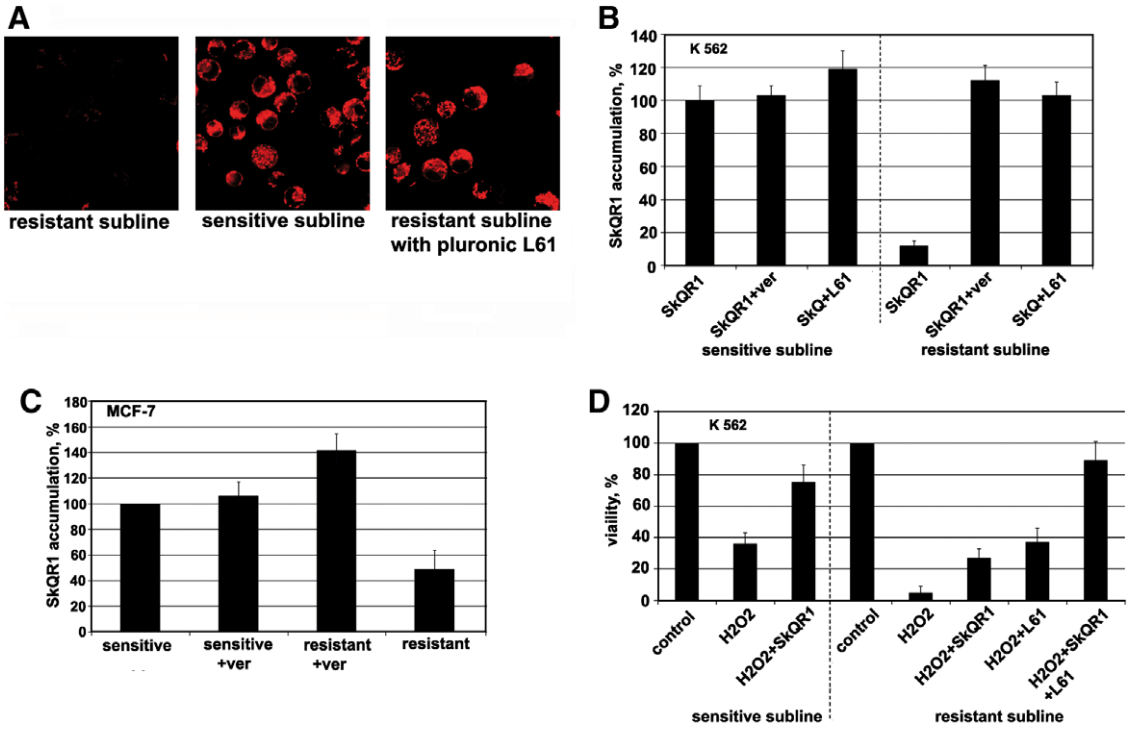

Fig. 3. Effects of SkQR1 in K562 and MCF-7 cell lines and sublines with overexpression of Pgp 170. (A) Accumulation of SkQR1 in resistant and sensitive sublines of K562 cells. Cells were incubated with 50 nM SkQR1 for 3 h. Pluronic L61 (30 μg/ml) was added 1 h before SkQR1. Confocal microscopy. (B and C) Accumulation of SkQR1 (50 nM, 3 h) in the resistant and sensitive sublines of K562 and MCF-7. Verapamil (50 μM) and pluronic L61 (30 μg/ml) were added 1 h before SkQR1. Measurements were made by flow cytometry. (D) Protective effect of SkQR1 in sensitive and resistant K562 sublines. Cells were pretreated with SkQR1 (10 nM) for 6 days before H_2O_2 addition. The sensitive and resistant K562 sublines were incubated for 20 h with 100 μM H_2O_2 or 300 μM H_2O_2, respectively, to reach the similar toxic effect. Where indicated, pluronic L61 (5 μg/ml) was added for 6 days together with SkQR1. Cell viability was measured by MTT-test.

selected for resistance against doxorubicin and tested for MDR phenotype and for overexpression of Pgp 170 was confirmed by Western analysis [14,15]. Accumulation SkQR1 in the resistant sublines of the both cell types was significantly lower than in the sensitive counterparts (Fig. 3B and C). Verapamil and pluronic L61 increased an SkQR1 accumulation in the resistant sublines, being ineffective in the sensitive ones. As a result, accumulation of SkQR1 in the presence of the inhibitors did not differ in these sublines. Confocal images demonstrated that the MDR inhibitors did not modify mitochondrial localization of SkQR1 (Fig. 3). These data confirmed the leading role of Pgp 170 in the efflux of SkQR1.

As it was shown earlier, SkQR1 (as well as SkQ1) protected various cells from apoptosis induced by oxidative stress [4,9]. We have studied the protective action of SkQR1 in sensitive and resistant sublines of K562. Hydrogen peroxide was used to induce oxidative stress and it was found that Pgp 170 overexpressing subline is more resistant to this treatment. The inhibitors of MDR did not sensitize these sells (even the opposite effect was observed with pluronic L61), indicating that resistance to H_2O_2 was acquired during selection procedure independently from Pgp 170 overexpression. This observation was not unexpected since doxorubicin (used for selection) has pronounced prooxidant effect which can induce the increase in various antioxidant defense systems [15]. We titrated the cytotoxic effect of H_2O_2 and treated the resistant K562 cells with higher dose of H_2O_2 to reach the same level of cell death. It was found that a 6 day pretreatment with SkQR1 strongly protected the sensitive K562 subline against oxidative stress, while Pgp 170 overexpressing subline remained sensitive to H_2O_2 (Fig. 3D). When Pgp 170 was inhibited by pluronic L61 during pretreatment with SkQR1, the protective action of antioxidant was restored. These data clearly show that the inability of SkQR1 to protect the resistant subline from oxidative stress is related to activity of Pgp 170. It can be concluded that the protective action of SkQR1 was limited by it efflux catalyzed by Pgp 170.

Multidrug resistance mediated by Pgp 170 is typical for various tumors highly resistant to conventional chemotherapy [23,24]. Elevation of therapeutic doses is limited by non-selective cytotoxicity of the drugs. Cytotoxic action of some anti-cancer agents is based upon ROS formation and induction of oxidative stress. Doxorubicin (adriamycin), for example, induces ROS formation interacting with the respiratory chain and cardiotoxicity of this drug is mediated by ROS [25]. Nephrotoxicity of cisplatin also depends on excessive ROS production [26]. It seems probable that mitochondria-targeted antioxidants could be effective protectors against these drugs. Since tumor cells overexpress Pgp 170, they will not be protected or protected to much lesser extent than normal cells. The selective protection of normal (MDR negative) cells by mitochondria-targeted antioxidants could be a promising strategy for improvement of antitumor therapy.

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