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Multidrug Resistance P-Glycoprotein Inhibits Antiapoptotic Action of Mitochondria-Targeted Antioxidant SkQR1

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Abstract—Mitochondria-targeted antioxidants of the SkQ family that accumulate in energized mitochondria protect cells from oxidative stress by increasing the level of reduced glutathione and decreasing cell damage induced by hydrogen peroxide. The exposure of various human transformed cell lines to SkQR1, a fluorescent member of the SkQ family, showed that SkQR1 was pumped out of the chemotherapy resistant cells by P-glycoprotein, one of the main transport proteins that determines multidrug resistance typical for many neoplastic cells. It was also shown that SkQR1 pumping is neutralized by P-glycoprotein inhibitors (verapamil and pluronic L61). In experiments on K-562 cells, it was found that the subline sensitive to chemotherapy is protected by SkQR1 from apoptosis induced by hydrogen peroxide. The protection of resistant subline cells is only evident after the inhibition of P-glycoprotein.

Keywords: mitochondria, antioxidant, oxidative stress, multidrug resistance, P-glycoprotein, SkQ.

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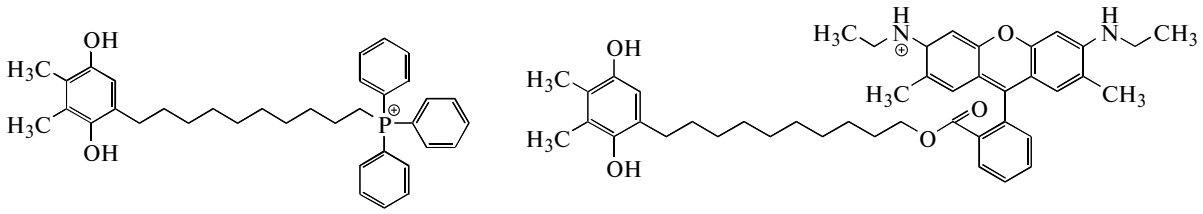
The idea that mitochondria are responsible for the production of substantial amount of reactive oxygen species has received much support in recent years (Skulachev et al., 2007). It is useful to synthesize antioxidants that specifically target mitochondria. Murphy et al. (Kelso et al, 2002; Murphy and Smith, 2007) obtained a new substance, MitoQ, that protected cells from oxidative stress via the conjugation of the natural antioxidant ubiquinone with a positively charged hydrophobic ion of decyl-triphenylphosphonium that penetrates the phospholipid bilayer of biological membranes (Liberman et al., 1969; Liberman and Skulachev, 1970, Bakeeva et al., 1970). In Skulachev's lab, other mitochondria-targeted antioxidants have been synthesized (SkQ family) that are composed of plastoquinone (Q), which is responsible for antioxidant protection; a penetrating cation (Sk) that provides compound delivery into mitochondria; and a decanoic or pentanoic linker. SkQ derivatives with the highest penetration property were selected on the planar bilayer phospholipid membranes. Synthesized antioxidants possessed more pronounced protective effect than MitoQ and had a larger concentration window between antioxidant and prooxidant properties. Members of the SkQ family are regenerative antioxidants of repeated action and are rapidly restored with complexes I and II of the mitochondria respiratory chain for the repeated antioxidant action (Antonenko et al., 2008). Here, we studied the properties of two representatives of the SkQ-family (Fig. 1), namely, plastoquinonyl-decyl-triphenylphosphonium (SkQ1) and plastoquinonyl-decyl-rhodamine 19 (SkQR1) and their possible interaction with P-glycoprotein associated with multidrug resistance (MDR).

MDR is the cell's resistance to a number of antitumor chemotherapeutic drugs of various chemical



SkQ1 SkQR1

Fig.1. Structure of SkQ family compounds: plastoquinonyl-decyl-triphenylphosphonium(SkQ1) and plastoquinonyl-decyl-rhodamine 19 (SkQR1).

structures (Van der Bliek and Borst, 1989; Stavrovskaya, 2000). The resistance to a wide range of compounds is caused by their pumping out of cells with P-glycoproteins (Pgp), which are membrane protein transporters that recognize various hydrophobic organic compounds (Higgins and Gottesman, 1992; Neifach, 2003). Transporters remove the substances from the cytoplasm into the environment, protecting cells from multiple hydrophobic poisons various in structure (Lewis, 1994). Pgp with molecular weight of 170 kDa, which is a transport ATPase of ABC syperfamily, is the best studied. Pgp is a gene product of the *mdr* family (Ng et al., 1989; Ambudukar et al., 1999). Due to the hydrophobicity and the positive charge of mitochondria-targeted antioxidants, it is highly probable that these substances may be sustained in normal tissues and withdrawn from various MDR tumors with Pgp transport protein. This is the topic of this paper (preliminary communication, see: Fetisova et al., 2010). We aimed to characterize mitochondria-targeted antioxidants, to study their release from cells with MDR, and to evaluate their ability to protect cells from the oxidative stress.

MATERIALS AND METHODS

Cells. HeLa (human cervical carcinoma), U937 (human lymphoma), and fibroblasts of human skin, as well as sensitive and resistant (with a high level of MDR) sublines of MCF-7 (human mammary carcinoma) and K562 (human erythroleukemia) cells were used in the experiments. Both resistant sublines were obtained by treating sensitive cells with doxorubicin. These cell lines are the universally accepted MDR model widely used in MDR studies (Kalinina et al., 2006). Cells were maintained on DMEM and RPMI 1640 media (PanEco, Russia) supplemented with 10% fetal calf serum (HyClone, United States), glutamine, and antibiotics (penicillin and streptomycin, 100 unite each). Cells were grown at 37°C and 5% CO_2.

Mitochondria were visualized by mitotracker green staining (Molecular Probes, United States). The accumulation of SkQ in cells was tested using SkQR1, a fluorescent derivative of SkQ with triphenylphosphonium group substituted by rhodamine 19 (Skulachev et al., 2007; Antonenko et al., 2008). The dynamics of SkQR1 accumulation and efflux was measured by a FC500 flow cytometer (Beckman Coulter, United States). The accumulation of antioxidant inside mitochondria was visualized with simultaneous SkQR1 exposure and mitotracker green staining (yellow color) using an LSM 510 confocal microscope (Carl Zeiss, Germany).

MDR was suppressed by both well-known and recently synthesized inhibitors of P-glycoprotein, i.e., verapamil and pluronic L61. Verapamil is a calcium channel blocker widely used as P-glycoprotein inhibitor (Safa, 1988; Tsuruo and Yusa, 1989). Pluronic L61, a pluronic polymer (composed of ethylene- and propylene oxide blocks), is the least toxic P-glycoprotein inhibitor (Kabanov et al., 2002; Demina et al., 2005; Zhirnov et al., 2006),.

The accumulation of SkQR1 in resistant and sensitive MCF-7 and K562 cells was studied in the presence or absence of P-glycoprotein inhibitors. Cells were cultivated in medium with 50 nM SkQR1 for 1.5–3 h. In some experiments, SkQR1 was then washed out and the cells were cultivated without SkQR1 for 0.5–2 h. The inhibitors (50 μM verapamil or 30 μg pluronic L61) were added 15–20 min before the introduction of SkQR1 and were not withdrawn during the experiment. Fluorescence was measured with a FC 500 flow cytometer (Beckman Coulter, United States).

The membrane potential of the mitochondria was diminished using 10 μM FCCP (carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone) (Sigma, United States).

The accumulation of fluorescent antioxidants was studied by the HPLC method with UV and MS/MS detection. The analysis was performed by HPLC/MS with a Waters Acquity chromatographer with a diode-matrix UV-detector and tandem quadrupole MS-detector TQD (Waters). The conditions for the analysis included 11.2 μl probe volume (complete loop); 0.21 × 5.0-cm column Acquity HPLC BEH C18 (1.7 μm); a 35°C temperature column; 5–100% acetonitrile gradient for 3 min in presence of 20 mM formic acid; flow rate of 0.5 ml/min; UV detection in the range of 200–500 nm; and MS detection in the regime of positive ions. The major detector parameters included the capillary voltage +3 kV, cone voltage +55 kV, a capillary temperature of 450°C, a source temperature of 120°C, a flow rate of drying gas 800 l/h, a gas flow rate in cone 50 l/h, and scanning in a mass diapason of 100–1000 units.

Protective effect of SkQ1 (10 nM) and SkQR1 (20 nM) under conditions that induce the oxidative stress was studied after the preliminary cultivation of the cell with antioxidants for 28 h or 6 days (Saretzki et al., 2003). Oxidative stress was induced by hydrogen peroxide (100–400 μM, 20 h). The number of apoptotic cells was measured after cell incubation with Hoechst 33342 fluorescent dye (Sigma, United States) for 1 h. In each of three experiments, 500 cells were counted. The cell viability was assayed by the reduction in 3-(4,5-dimethyltiazol-2)-2,5-diphenyltetrazolium (MTT) with Mutiscan (Thermolabsystems) at a 541-nm wavelength (Pannecouque et al., 2008). In experiments on glutathione (GSH) oxidation, hydrogen peroxide was used in a concentration of 1 mM.

GSH concentration was determined using the Griffith method (Griffith, 1985) based on DTNB (5,5-ditio-bis 2-nitrobenzoic acid) reduction. K562 cells were disrupted with cold solution composed of 10% $HClO_4$, 5% Triton X-100, and 5 mM EDTA in a 1 : 1 volume ratio. Denatured protein was sedimented at 4°C. Supernatant was added to phosphate buffer (125 mM sodium phosphate, 6 mM EDTA, pH 7.5,

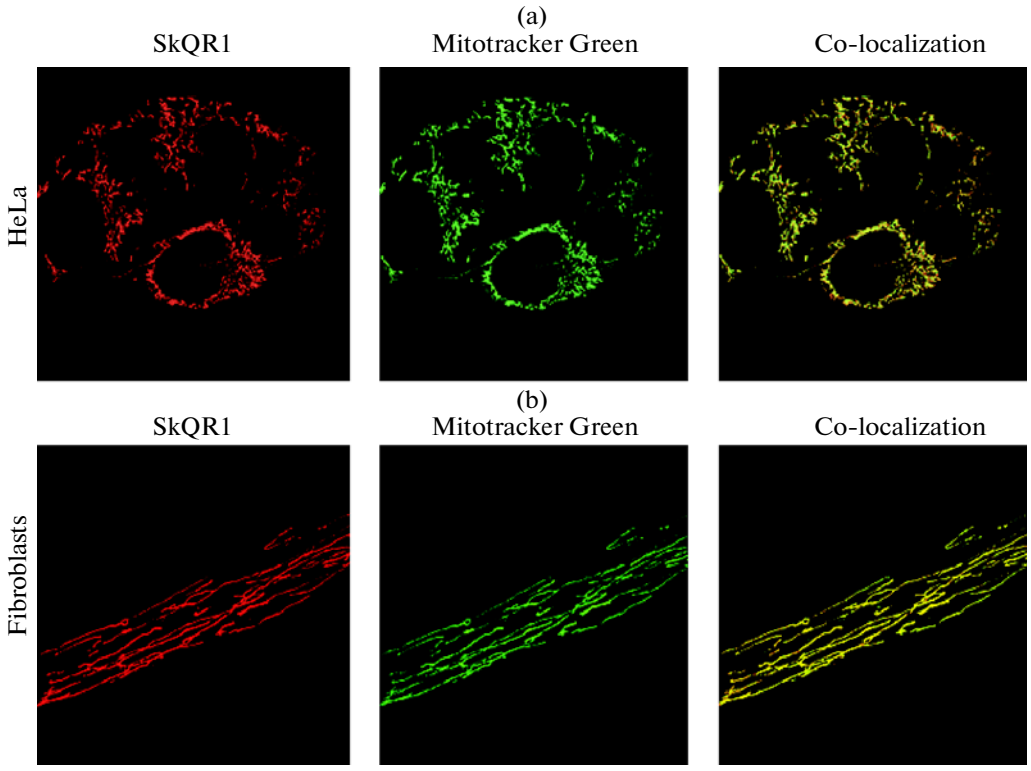


Fig. 2. Accumulation of SkQR1 in mitochondria of human cervical carcinoma HeLa cells (a) and human skin fibroblasts (b). Cells were incubated with 50 nM SkQR1 for 3 h and 200 nM mitotrecker green for 15 min and then visualized under confocal microscope. Accumulation of SkQR1 inside mitochondria (*yellow color*) is evident by colocalization of SkQR1 red fluorescence and mitotracker green fluorescence.

6 mM DTNB). GSH nonenzymatically reduces DTNB to TNB (2-nitro-5-tiobenzoic acid) which amount was determined by spectrophotometer at 412 nm. Calibration was performed with standard GSH solution.

RESULTS AND DISCUSSION

SkQR1 accumulation was studied in various cultured cells. Rhodamine 19, an SkQR1 component with red fluorescence, allowed us to follow the distribution of the substance in cellular organelles. The simultaneous application of SkQR1 (red) and mitochondrial dye mitotracker green (green) to HeLa and normal fibroblast cultures showed that similar organelles were stained yellow as a result of the merged red and green colors. This indicates that SkQR1 and mitotracker green dye (Fig. 2) have similar cellular localizations, i.e., SkQR1 accumulates inside mitochondria. This is probably because the mitochondrial matrix is the only negatively charged intracellular compartment. The exposure of the cell to FCCP, which uncoupled the oxidative phosphorylation and diminished the membrane potential, prevented the accumulation of the antioxidant inside the mitochondria (Fig. 3), i.e., SkQR1 selectively accumulated only inside of energized mitochondria.

Accumulation of SkQR1 in HeLa cells and its efflux from cells. A preliminary UPLC analysis of cell extracts showed that no substances with 500-nm absorbance except for SkQR1 accumulated in the cells. Hence, it was possible to conclude that possible products of SkQR1 degradation had no absorbance at 528 nm and, therefore, were incapable for fluores-

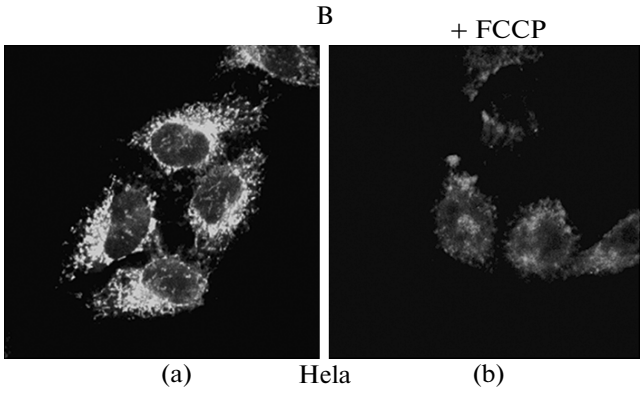


Fig. 3. Accumulation of SkQR1 in HeLa cells in presence of uncoupling agent of oxidative phosphorylation FCCP. (a) accumulation of SkQR1 in cells cultivated with 20 nM SkQR1 for 5 h; (b) 10 μM FCCP was added 1 h before SkQR1 exposure.

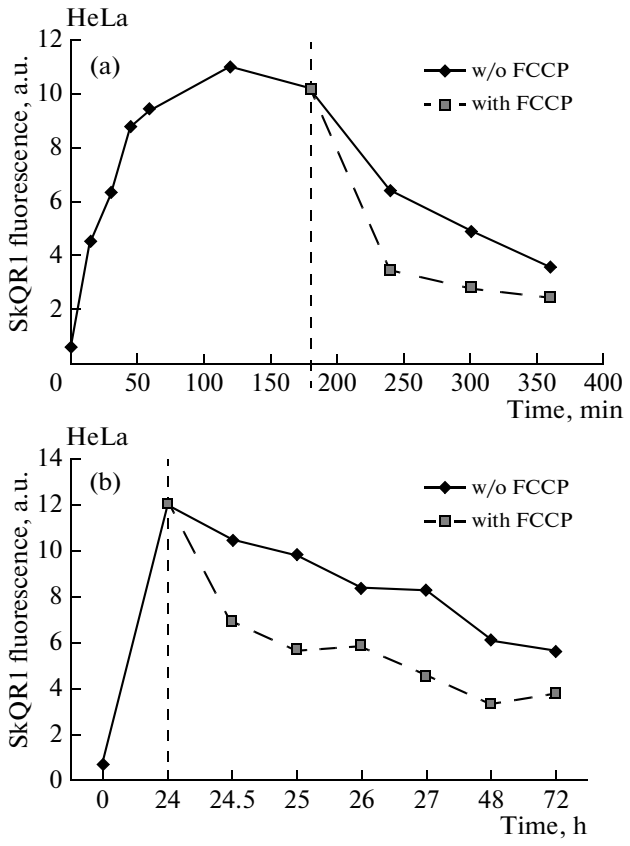


Fig. 4. Dynamics of accumulation of SkQR1 in HeLa cells. (a) Cells were cultivated with 50 nM SkQR1for 3 h, then the medium was exchanged for SkQR1-free medium; (b) kinetics of SkQR1 release from HeLa cells in 24 h of cultivation with SkQR1. Some samples were supplemented with 10 μM FCCP after washing.

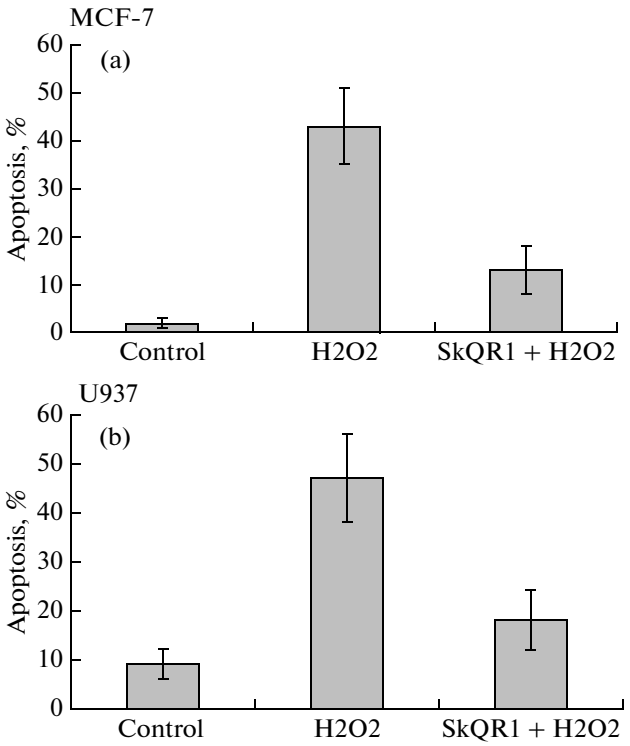


Fig. 5. Antioxidant SkQR1 cell protection from oxidative stress induced by hydrogen peroxide. (a) Human mammary carcinoma MCF-7 cells were cultivated with 20 nM SkQR1 for 28 h; (b) human lymphoma U937 cells were cultivated with 10 nM SkQR1 for 6 days. 400 μM hydrogen peroxide was added to both cell types for 20 h after SkQR1 removal. Cultivation with antioxidant reduced the number of apoptotic cells induced by hydrogen peroxide.

cence similar to SkQR1. HeLa cells were cultivated with 50 nM SkQR1 for 3 h. Then, SkQR1 was removed and cells were cultivated without the antioxidant, which results in the slow release of SkQR1 from the cells. Figure 4a shows that SkQR1 accumulated for about 1 h; then, the curve reaches a plateau. The kinetics of the accumulation did not depend on the antioxidant concentration (Fig. 4 only shows the data for 50 nM SkQR1). The FCCP respiration uncoupling agent stimulated SkQR1 efflux from the cells (Fig. 4b).

Cell protection from oxidative stress induced by hydrogen peroxide. To prevent apoptosis caused by hydrogen peroxide MCF-7 and U937 cells were cultivated with SkQR1 for 28 h or 6 days. Figure 5 demonstrates that the cultivation of the cell with antioxidant reduces the number of apoptotic cells produced by treatment with hydrogen peroxide.

Antioxidant effect on GSH level at the oxidative stress. GSH, a major intracellular antioxidant, was evaluated in HeLa and K562 (human erythroleukemia) cells. Treatment of the cell with hydrogen peroxide diminished the GSH level, which indicates the development of oxidative stress (Schulz, et al., 2000). SkQ and SkQR1 in very low concentrations (0.5 nM) prevented GSH oxidation. FCCP blocked the protective action of SkQ (Fig. 6).

Effect of P-glycoprotein (Pgp) inhibitors on accumulation of mitochondria-targeted antioxidants. The study of the intracellular accumulation of SkQR1 showed that HeLa cells accumulated SkQR1 two to three times less than human normal skin fibroblasts (Fig. 7). The difference depended on neither the size of the cell nor the number of mitochondria. Direct measurements showed that both types of cells are similar in size and the number of mitochondria (Fig. 7). Since MDR is a common property of various tumor cells, it has been suggested that the difference in SkQR1 accumulation is a result of being pumped out from HeLa cells with Pgp. Indeed, the exposure of the cell to inhibitors of the most common Pgp170 isoform (verapamil or pluronic L61) resulted in SkQR1 accumulation in HeLa cells to the level detected in the fibroblasts (Figs. 7, 8). Most likely, Pgp 170 is expressed in HeLa cells as was observed in many other tumor cells. The small increase in SkQR1

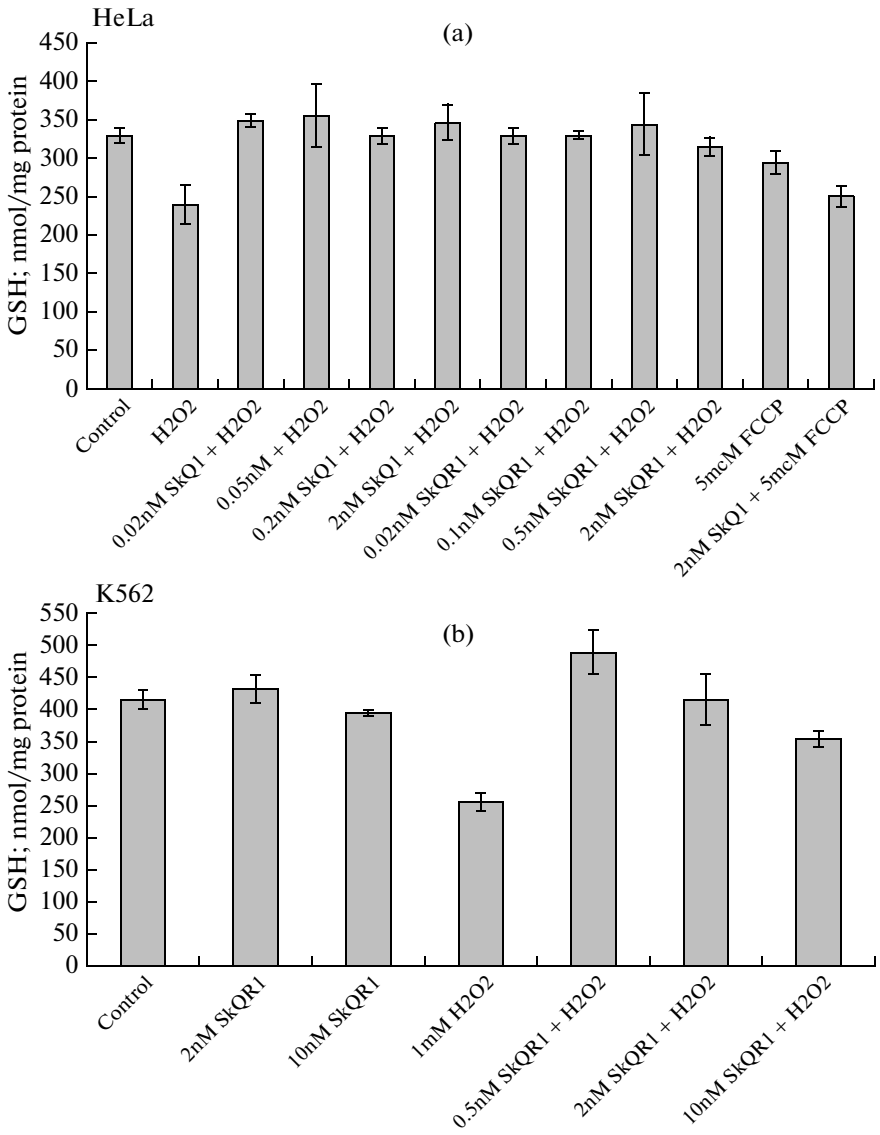


Fig. 6. Reduced glutathione (GSH) content in cells exposed to SkQ. (a) HeLa cells incubated with SkQ1 and SkQR1, FCCP blocks SkQ protection; (b) K562 cells cultivated with SkQR1. SkQ1 and SkQR1 were added for 4 h, then cells were treated with 1 mM hydrogen peroxide for 2 h and the GSH level was estimated. FCCP 5 μM was added 30 min before SkQ exposure.

accumulation was found in cultured human skin fibroblasts treated with inhibitors (Figs. 7, 8). The expression of specific MDR-related transport proteins in human skin fibroblasts is a well-known phenomenon and is associated with the implication of these cells in skin inflammatory reactions (Dreuw et al., 2005). Indeed, the inhibition of these proteins leads to not only an increase in the cell's susceptibility to antitumor drugs, but also a simultaneous decrease in organism inflammatory reactions. This was apparently connected to leukotriene and prostaglandin synthesis by some MDR transporters (Dreuw et al., 2005).

To confirm that SkQR1 is released from cells with Pgp, we have studied the accumulation of SkQR1 in the MCF-7 human mammary carcinoma and K562 human erythroleukemia, which have a high level of MDR. Each cell line was presented by two sublines, i.e., sensitive and resistant. MCF-7 and K 562 resistant sublines were previously tested for Pgp expression. It was found (Demina, 2007) that the basic mechanism of MCF-7 and K562 resistance was mediated by Pgp-170. Pgp inhibitors verapamil and pluronic L61 were applied for MDR suppression. We added 20 nM SkQR1 to sensitive and resistant sublines in the presence or absence of Pgp inhibitors. Treated cells were examined under a confocal microscope.

SkQR1 accumulation (20 nM, 3 h) was much higher in sensitive MCF-7 and K562 cells than in

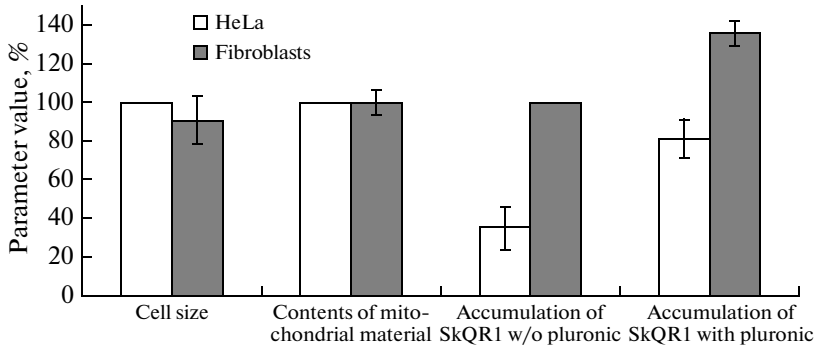


Fig. 7. Accumulation of SkQR1 in HeLa cells and human normal skin fibroblasts.

Relative cell size was calculated by light scattering with flow cytometry. Mitochondria content was determined by total mitotracker green fluorescence per a cell. accumulation of SkQR1 was measured in the presence of 5 μg/ml pluronic L61 an inhibitor of multiple drug resistance (MDR). Data of single experiment with calculation of various parameters are presented. Cell size, content of mitochondrial material, and accumulation of SkQR1 in human fibroblasts (without pluronic L61) were taken as 100%. Standard deviation was zero.

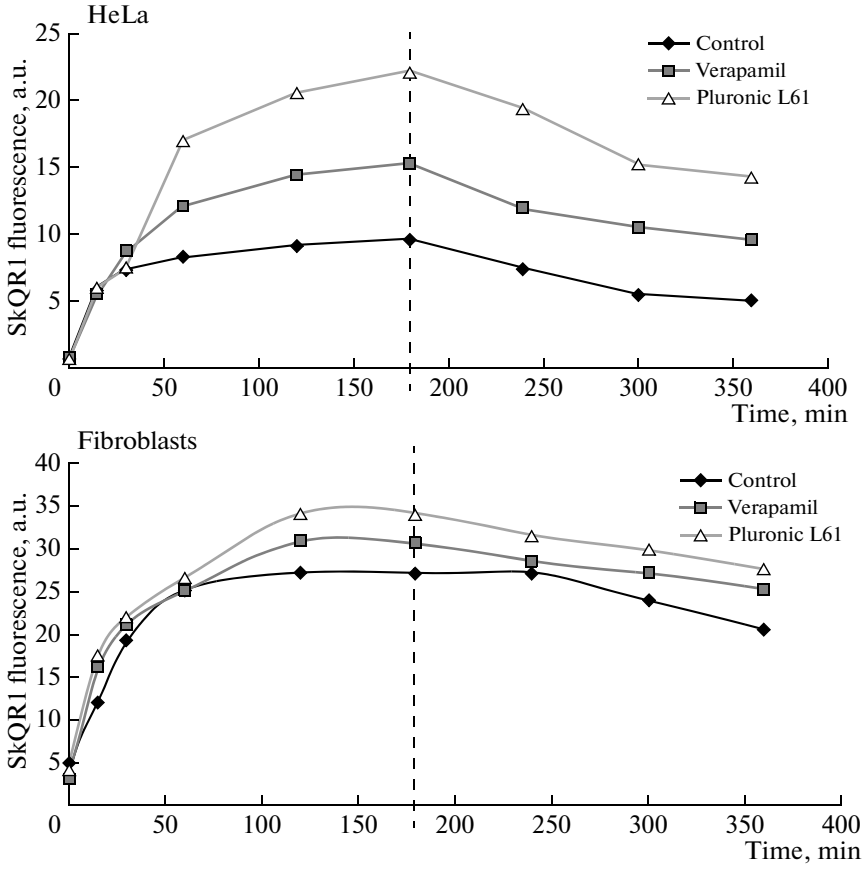


Fig. 8. Accumulation of SkQR1 in HeLa cells and normal human skin fibroblasts treated with MDR inhibitors. Cells were incubated with 50 nM SkQR1 for 30 min. 50 μM verapamil 5 μg/ml pluronic L61 were added 1 h before SkQR1 and repeatedly after SkQR1 removal. Accumulation of SkQR1 was evaluated by fluorescence intensity measured with flow cytometry.

resistant cells. In resistant cells, SkQR1 was nearly undetectable. The addition of Pgp inhibitors resulted in the accumulation of SkQR1 in resistant cells (Figs. 9, 10), i.e., it was indirectly shown that Pgp pumped SkQR1 out of resistant cells with MDR. Further experiments were done to measure the accumulation of SkQR1 in these cells using flow cytometry. In the first type of experiments, cells were cultivated with

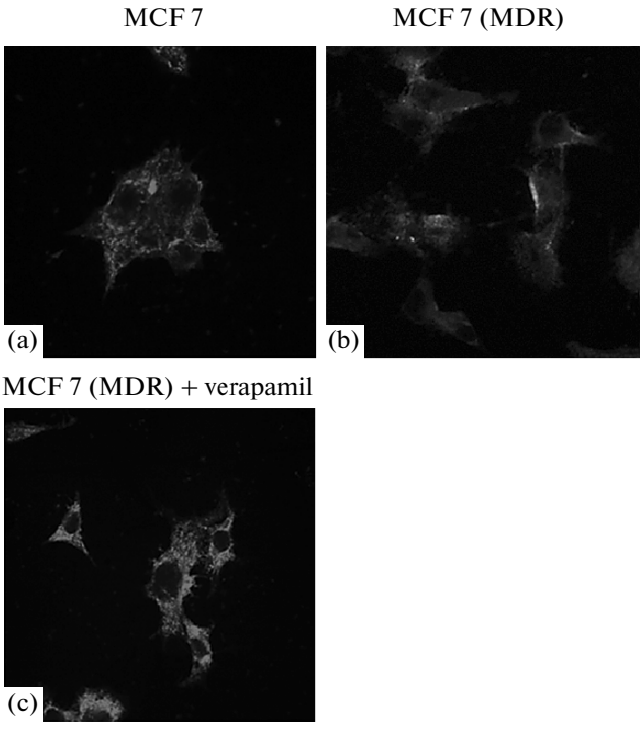


Fig. 9. Accumulation of SkQR1 in cells of human mammary carcinoma MCF-7 sublines. Confocal microscopy. Cells were incubated with 20 nM SkQR1 for 3 h: (a) sensitive subline; (b) resistant subline. Accumulation of SkQR1 is only slightly visible; (c) resistant subline in presence of 50 μM verapamil, accumulation of SkQR1 is evident.

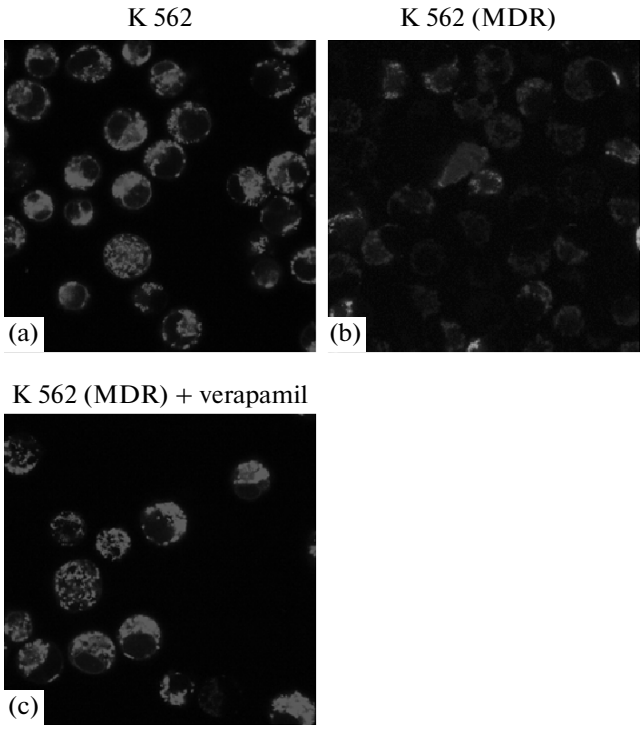


Fig. 10. Accumulation of SkQR1 in cells of human erythroleukemia K562 subline. Confocal microscopy. Cells were incubated with 20 nM SkQR1 for 3 h. Other designations are same as in Fig. 9.

50 nM SkQR1 for 3 h in the presence of (50 μM) or without verapamil; then, the amount of SkQR1 was determined. To uncouple SkQR1 influx and efflux the second group of experiments was performed. Cells were cultivated for 1 h with SkQR1 and verapamil. Then, SkQR1 was removed. Verapamil persists in culture for 30–60 min to avoid being pumped out of the accumulated antioxidant; then, the content of SkQR1 was measured. In both cases, resistant and sensitive cells differed in their accumulation of SkQR1. Figure 11 shows that the accumulation of SkQR1 in resistant K562 and MCF-7 cells was very weak. The accumulation of SkQR1 in sensitive cells was significantly higher than in resistant cells and did not depend on the presence of Pgp inhibitor. Pgp inhibitors increased the accumulation of SkQR1 in resistant cells to the level observed in sensitive cells.

Compared to cells that contained verapamil in cultivation media, the cell's accumulation of SkQR1 without verapamil was three and nine times lower in resistant MCF-7 cells and seven and ten times lower in resistant K562 cells in the first and second experiments, respectively.

To evaluate the effect of MDR on the accumulation of SkQR1, we compared its effect to rhodamine 6G, which is a common agent for MDR testing. Rhodamine 6G was added to K562 sensitive and resistant cells in the same concentration as SkQR1 (50 nM) for 3 h with and without verapamil. It was found that the accumulation of rhodamine 6G in resistant cells increased 30 times after the addition of verapamil (Fig. 12a). Similar results were obtained for HeLa cells exposed to TMRM (50 nM), another rhodamine containing agent (Fig. 12b). In all cases, the oxidative phosphorylation uncoupling agent FCCP prevented the accumulation of rhodamine-containing compounds due to the reduction of the membrane potential.

In conclusion, studies on HeLa and resistant K562 and MCF-7 cells with high levels of MDR proved that compounds of the SkQ family can be excluded from resistant cells with Pgp 170 multidrug transporter.

The exposure of resistant cells to SkQR1 did not protect the cells from the oxidative stress induced by hydrogen peroxide because the antioxidant was incapable of accumulating in mitochondria. Unlike resistant cells, low doses (20 nM) of SkQR1 in sensitive cells reduced peroxide toxicity and increased the cell's viability (Fig. 13) assessed by the MTT test. MDR may be inhibited by low-toxic Pgp inhib-

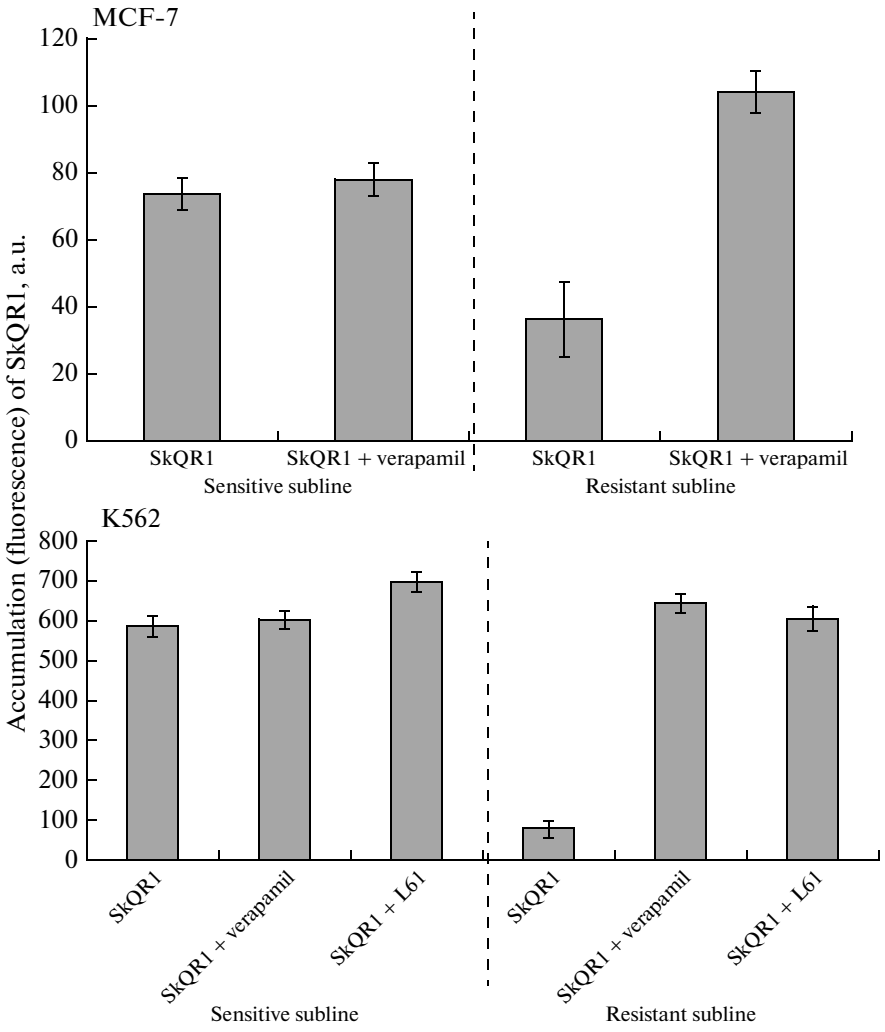


Fig. 11. Accumulation of SkQR1 in MCF-7 and K562 cells. Flow cytometry. P-glucoprotein inhibitors verapamil (50 μM) and pluronic L61 (5 μg/ml) were added 1 h before SkQR1. Accumulation of SkQR1 was measured in 3.5 h. Inhibitors stimulated accumulation of SkQR1 in resistant cells.

itor pluronic L61 after long-term cultivation with SkQR1. L61 prevented SkQR1 from being pumped out from these cells and, therefore, protected resistant cells from the toxic effect of hydrogen peroxide. Cell viability determined by the MTT test was increased (Fig. 13).

It is well known that chemotherapeutical antitumor drugs are frequently extruded from malignant cells with multidrug transporters (Van der Bliek and Borst, 1989). This is a serious obstacle for cancer treatment. Nevertheless, there is a number of therapeutic agents that are not related to MDR; i.e., irradiation, physicochemical exposure, and some chemical substances (cisplastin, etc.). Their action is based on the production of reactive oxygen species and the induction of the oxidative stress. Theoretically, anticancer drugs should destroy only malignant cells and do not negatively affect normal tissues. However, the majority of cytostatics do not possess specific activity and destroy not only malignant, but also normal cells. The development of new drugs and the intensification of cytostatics therapy assists the efficiency of treatment, but simultaneously increases toxicity. Multiple but not highly successive efforts are aimed at finding agents with reduced toxic effects on normal tissues that do not affect the efficiency of chemotherapy. In the interests of patients, it seems prospective to use the MDR-properties of tumor cells, which is usually considered to be a serious obstacle in tumor therapy. In antitumor therapy, mitochondria-targeted antioxidants will be extruded from malignant cells, which does not prevent the efficiency of antitumor action. Antioxidants retained in normal tissues that lack MDR reduced the damaged therapy effects and protect nonmalignant cells.

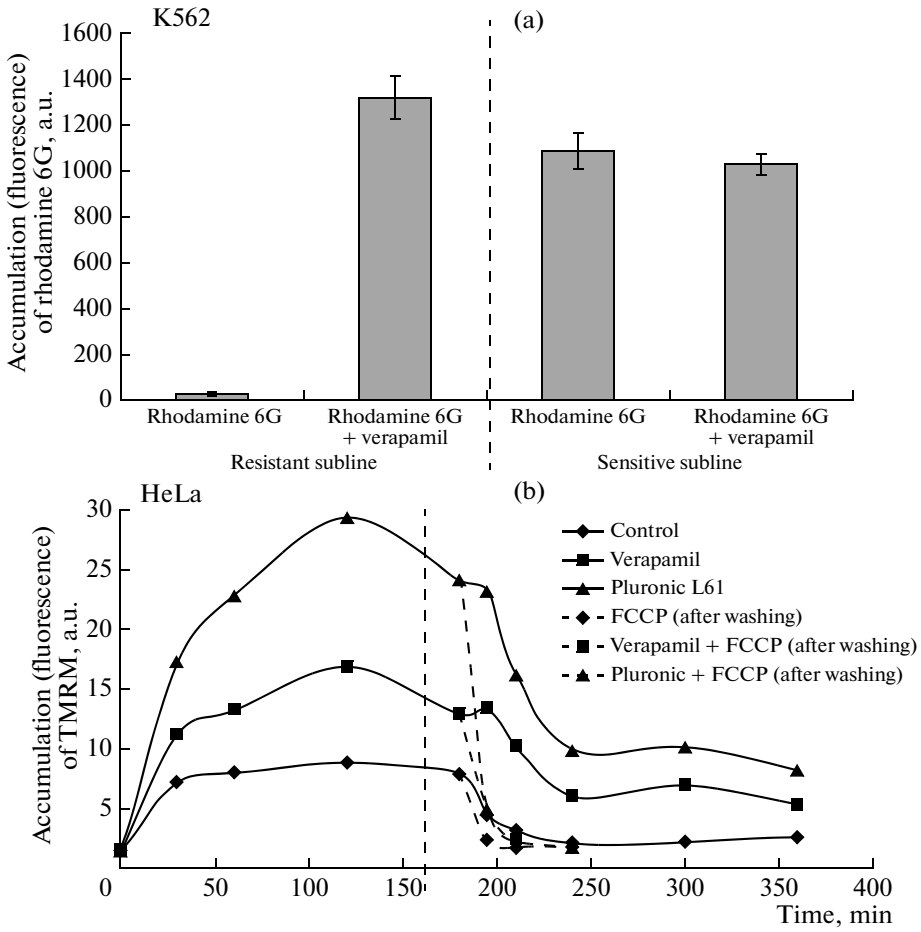


Fig. 12. Rhodamine accumulation in K562 (a) and Hela (b) cells. Flow cytometry. (a) Rhodamine 6G (50 nM) accumulation in various K562 sublines was measured for 4 h, verapamil concentration was 50 μM; (b) kinetics of TMRM (50 nM) accumulation in HeLa cells. Cells were incubated with 50 nM SkQR1 for 3 h. Verapamil and pluronic L61 (5 μg/ml) were added 1 h before SkQR1 treatment and repeatedly after SkQR1 removal; 10 μM FCCP was added after TMRM removal (were is indicated).

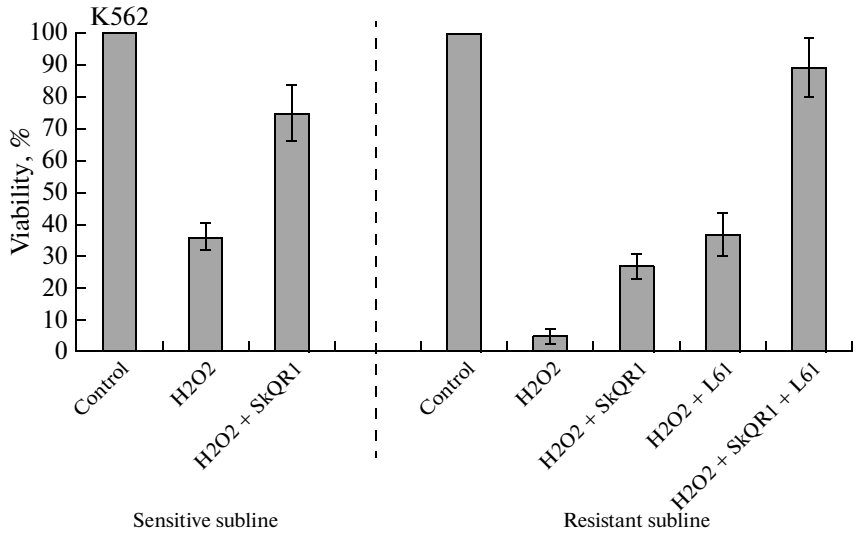


Fig. 13. SkQR1 protection of various K562 subline cells from hydrogen-peroxide toxicity. Cells were cultivated with 20 nM SkQR1 for 6 days. Then, SkQR1 was withdrawn and 0.1 or 0.3 mM hydrogen peroxide was added to sensitive and resistant cells for 20 h. Cell viability was assessed with MTT test. 5 μM pluronic L61 was present permanently (where indicated).

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