ISSN 0006-2979, Biochemistry (Moscow), 2024, Vol. 89, No. 2, pp. 212-222 © Pleiades Publishing, Ltd., 2024.

Relationship of Cytotoxic and Antimicrobial Effects of Triphenylphosphonium Conjugates with Various Quinone Derivatives

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Received December 4, 2023
Revised January 30, 2024
Accepted February 21, 2024

Abstract—Quinone derivatives of triphenylphosphonium have proven themselves to be effective geroprotectors and antioxidants that prevent oxidation of cell components with participation of active free radicals – peroxide (RO_2·), alkoxy (RO·), and alkyl (R·) radicals, as well as reactive oxygen species (superoxide anion, singlet oxygen). Their most studied representatives are derivatives of plastoquinone (SkQ1) and ubiquinone (MitoQ), which in addition to antioxidant properties also have a strong antibacterial effect. In this study, we investigated antibacterial properties of other quinone derivatives based on decyltriphenylphosphonium (SkQ3, SkQT, and SkQThy). We have shown that they, just like SkQ1, inhibit growth of various Gram-positive bacteria at micromolar concentrations, while being less effective against Gram-negative bacteria, which is associated with recognition of the triphenylphosphonium derivatives by the main multidrug resistance (MDR) pump of Gram-negative bacteria, AcrAB-TolC. Antibacterial action of SkQ1 itself was found to be dependent on the number of bacterial cells. It is important to note that the cytotoxic effect of SkQ1 on mammalian cells was observed at higher concentrations than the antibacterial action, which can be explained by (i) the presence of a large number of membrane organelles, (ii) lower membrane potential, (iii) spatial separation of the processes of energy generation and transport, and (iv) differences in the composition of MDR pumps. Differences in the cytotoxic effects on different types of eukaryotic cells may be associated with the degree of membrane organelle development, energy status of the cell, and level of the MDR pump expression.

DOI: 10.1134/S0006297924020032

Keywords: antioxidants, SkQ1, MDR pumps, AcrAB-TolC, bacteria, mammalian cell cultures, cytotoxicity, antibiotic, mitochondria

Abbreviations: CFU, colony-forming unit; MDR, multidrug resistance; MIC, minimum inhibitory concentration; MTAs, mitochondria-targeted antioxidants; SkQs, "Skulachev ions" used in the study; SkQ1, 10-(6-plastoquinonyl)decyl triphenylphosphonium; SkQ3, 10-(6′-methylplastoquinonyl) decyl triphenylphosphonium; SkQThy, 10-(2-isopropyl-5-methyl-1,4-benzoquinonyl-6) decyltriphenylphosphonium; SkQT, a mixture of SkQT-para and 10-(5-toluquinonyl) decyltriphenylphosphonium; SkQT-para, 10-(6-toluquinonyl) decyltriphenylphosphonium; TPP, triphenylphosphonium.

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INTRODUCTION

Bacteria and mitochondria have much in common. Mitochondria are cellular powerhouses that generate cellular energy in the form of adenosine triphosphate and, like bacterial cells, have a negative membrane potential (–180 mV) on their inner membrane [1]. Therefore, the positively charged compounds accumulate in the mitochondrial matrix and within bacterial cells against their concentration gradient.

In 1970s, the research team of Vladimir Petrovich Skulachev [2-4] proposed the use of triphenylphosphonium (TPP) derivatives as mitochondria-targeted substances in which TPP serves as a "locomotive" for their transport into mitochondria. Two decades later, Michael Murphy et al. [5-7] applied this approach to the delivery of an antioxidant moiety into mitochondria, which prompted creation of a variety of molecules based on the triphenylphosphonium derivatives [8], including a series of "Skulachev ions", mitochondria-targeted antioxidants (MTAs) created within the framework of the "megaproject" on anti-aging penetrating ions [9].

MTAs based on quinone derivatives have become widespread both in the studies of the role of mitochondria in various physiological processes and as therapeutic agents [10, 11]. It was shown that in addition to their antioxidant action, these compounds also exhibit an uncoupling effect on mitochondria, which manifests itself as stimulated respiration and drop in the mitochondrial membrane potential. Moreover, this uncoupling effect is not necessarily toxic for the organism, since partial mitochondrial uncoupling was shown to be protective in the case of pathologies associated with oxidative stress [12-15], which may be linked to the dependence of reactive oxygen species (ROS) generation on the membrane potential of mitochondria [16]. The proposed mechanism of this uncoupling effect of SkQ1 (10-(6-plastoquinonyl)decyl triphenylphosphonium) on the mitochondrial membrane is based on its ability to interact with the endogenous fatty acids and facilitate fatty acid diffusion across the membrane by electrostatic interaction of fatty acid anions and SkQ1 cations [17]. The protonated forms of fatty acids penetrate well through the membrane, ensuring cyclic transmembrane proton transfer similar to the functioning of conventional protonophores, such as 2,4-dinitrophenol.

Despite the relative similarity between mitochondria and bacteria, MTAs such as SkQ1 were long thought to lack antibacterial properties [18], however, alkyltriphenylphosphonium cations (CnTPPs) and SkQ1 were subsequently shown to exhibit a potent antibacterial effect on Gram-positive and Gram-negative bacteria [19, 20]. It turned out that the observed lack of antibacterial action on the *Escherichia coli* bacterium is due to operation of the main multidrug resistance (MDR) pump AcrAB-TolC [20, 21], which is the only known bacterial MDR pump capable of pumping out SkQ1 [22].

Bactericidal effect of SkQ1 makes it a promising compound for use in clinical practice, in particular, to combat infections caused by Gram-positive bacteria, such as *Staphylococcus aureus*, *Streptococcus mutans*, or *Mycobacterium tuberculosis* [22-24].

Other triphenylphosphonium-based substances, such as fluorescein ester MitoFluo (10-[2-(3-hydroxy-6-oxoxanthen-9-yl)benzoyl] oxydecyl-triphenylphosphonium) [25], or chloramphenicol-triphenylphosphonium conjugate CAM-C10-TPP (N-{[(1R,2R)-dihydroxy-1-(4-nitrophenyl)propan-2-yl]amino}-11-oxoundecyl triphenylphosphonium) also exhibited antibacterial effects [26]. At the same time, chimeric molecules retained the properties of their components. For example, the CAM-C10-TPP molecule inherited the ability of chloramphenicol to inhibit protein synthesis on ribosomes and the ability of alkyltriphenylphosphonium to reduce membrane potential on the bacterial membranes. The conjugates based on quinones, which are similar to SkQ1 [20], can exhibit both antioxidant and antibacterial properties, which makes them promising research objects.

The aim of the present study was to compare the effects of several quinone derivatives on eukaryotic and prokaryotic cells. In particular, almost all derivatives were shown to exhibit antibacterial activity against Gram-positive bacteria, while Gram-negative *E. coli* bacteria were resistant, which is the result of operation of the AcrAB-TolC MDR pump. With regard to their toxic effect on mammalian cells, such quinone derivatives as SkQ3 (10-(6′-methylplastoquinonyl) decyl triphenylphosphonium), SkQT (a mixture of SkQT-para (10-(6-toluquinonyl) decyltriphenylphosphonium), 10-(5-toluquinonyl) decyltriphenylphosphonium), and SkQThy (10-(2-isopropyl-5-methyl-1,4-benzoquinonyl-6) decyltriphenylphosphonium) differ slightly from SkQ1, while their toxic effect depends on the cell type, which may be due to different levels of MDR pump expression.

MATERIALS AND METHODS

Materials. Penetrating cations (Fig. 1) were kindly provided by the Institute of Mitoengineering, Lomonosov Moscow State University, and their synthesis was carried out according to the previously published methods [27, 28]. All other reagents, unless otherwise noted, were from Sigma-Aldrich, USA.

Bacterial strains. Standard laboratory strains of *Bacillus subtilis* subs*. subtilis* Cohn 1872, strain BR151 (*trpC2 lys-3 metB10*), and *Escherichia coli* Castellani and Chalmers 1919, strain MG1655 (F-lambda-*ilvG-rfb-50 rph-1*), were used in our study. *Staphyllococcus*

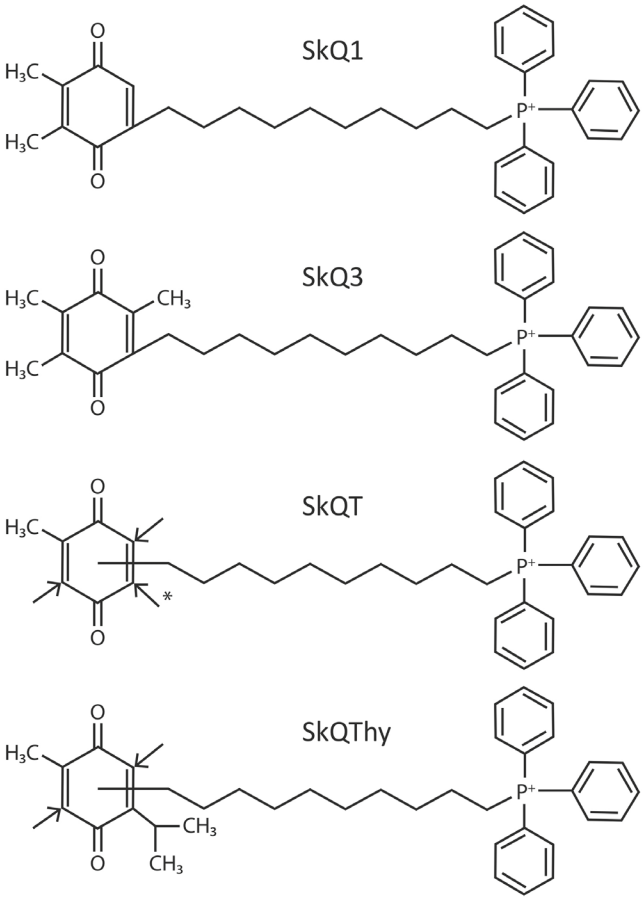

Fig. 1. Structural formulas of the "Skulachev ions" (SkQs), the cations used in the present study. The structures with arrows indicate a mixture of isomers, the arrows demonstrate position of decyltriphenylphosphonium. Asterisk (*) marks position of decyltriphenylphosphonium for the SkQT-para compound.

aureus Rosenbach 1884 (no. 144) and *Mycobacterium smegmatis* Lehmann and Neumann 1899 (no. 377) bacteria were obtained from the collection of microorganisms of Lomonosov Moscow State University.

Deletion strains ECK3026 (Δ*tolC*), ECK0456 (Δ*acrB*), ECK2465 (Δ*acrD*), ECK3253 (Δ*acrF*), ECK2071 (Δ*mdtB*), ECK3498 (Δ*mdtF*), ECK0870 (Δ*macB*), ECK2680 (Δ*emrB*) and ECK2363 (Δ*emrY*) were kindly provided by Dr. H. Niki (National Institute of Genetics, Japan) [29].

Cultivation of microorganisms. Bacteria were grown in LB medium overnight at 30 or 37°C on a shaker at 200 rpm until optical density of 1.5 at 600 nm was achieved. Optical density at 600 nm was measured with an Ultrospec 1100 pro spectrophotometer (Amersham Biosciences, UK).

Measurements of minimum inhibitory concentrations. Minimum inhibitory concentrations were measured by double dilution method according to the protocol [30] recommended by the Clinical and Laboratory Standards Institute (CLSI) in a liquid Mueller–Hinton medium.

TolC screening. Screening of a panel of deletion mutants of TolC-containing transporters was carried out according to the previously published works [20, 21, 31] in LB medium in 96-well plates (Citotest, China). Preselected concentrations of SkQs (5, 30, and 50 μM) were added to each mutant, which was allowed to grow for 21 h at 37 °C. After that, optical density was measured at 620 nm by using a Multiskan FC plate spectrophotometer (Thermo Fisher Scientific, USA).

Analysis of the dependence of antibacterial action on the number of cells. Various volumes of *B. subtilis* culture were added to fresh LB medium containing 1 μM SkQ1 and incubated for 3 h at 37°C and 220 rpm. Optical density was measured at 600 nm by using an Ultrospec 1100 pro spectrophotometer.

Analysis of the effect of volume of the incubation SkQ1-containing medium on survival. An overnight culture of *S. aureus* bacteria was diluted to ~15,000 CFU (colony-forming units)/ml and incubated for 3 h at 37°C and 220 rpm in various volumes of saline solution of glucose (0.9% NaCl, 5 mM D-glucose) with 1 μM SkQ1, after which the bacteria were plated on LB agar to determine CFU.

Analysis of the protective effect of dead cells. Killed *S. aureus* cells were obtained by heating the bacteria for 90 min at 65°C; loss of bacterial viability was confirmed by plating on LB agar. A mixture of various proportions of live and dead *S. aureus* cells was incubated for 3 h at 37°C and 220 rpm with 0.25 μM SkQ1 in saline with glucose, after which CFU for the survived bacteria was determined.

Molecular modeling and docking. Molecular docking was performed by using the QuickVina2 program [32] as part of the ODDT software modules [33] for Python. Lack of information about the binding site and size of the protein make it impossible to effectively scan the entire volume at once in a single experiment. To solve this problem, the entire volume was divided into 10 intersecting cells measuring 40 × 40 × 40 Å with a center shifted by 15 Å with each step. Docking was performed with redundant space scanning with exhaustiveness = 128 for two TolC states: closed (PDB ID: 1ek9) and open (PDB ID: 2×mn). Independent calculations were made on all three axes to fully cover the volume of TolC. The docking results were visualized in the PyMol program [34].

Experiments with human RKO and MRC5-SV40 cell lines. Human colon carcinoma cell line RKO (ATCC CRL-2577) and MRC5-SV40 fibroblasts (EcACC Cat. no. 84100401) were cultured in a modified DMEM supplemented with 10% fetal bovine serum (FBS), streptomycin (100 units/ml) and penicillin (100 units/ml). Cell viability was analyzed using a CellTiter-Blue reagent (Promega, USA). The cells were seeded into 96-well plates at 10,000 cells per well and cultured for 24 h at 37°C. The cells were treated with SkQ1 for 17 h,

then a Cell Titer-Blue reagent (20 μl per well) was added, after which the cells were incubated for 1 h before fluorescence was measured (ex = 560 nm; em = 590 nm) by using a Fluoroskan Ascent Microplate Fluorimeter (Thermo Fisher Scientific).

Experiments with cultures of renal tubular epithelial cells. Primary cultures of renal tubular cells were obtained from the kidneys of 5-7-day-old male Wistar rats. The protocols for working with animals were approved by the ethical committee of animal research of the Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University (Protocol 3/19 of 18 March 2019). The kidneys were sterilely isolated, cut into small pieces and incubated with a 0.125% solution of type II collagenase (Gibco, Thermo Fisher Scientific) in DMEM/F12 without bicarbonate at 37°C for 15 min.

After incubation, the kidney pieces in the collagenase solution were dispersed by pipetting for several minutes, and the resulting suspension was centrifuged for 5 min at 400*g* to sediment the tubule fraction. The resulting pellet was resuspended in a complete culture medium consisting of DMEM/F-12 (1/1) containing 10% FBS, 2% amino acids, 1% vitamins, and 1% L-glutamine, and seeded in a 96-well plate. Cells were cultured in an incubator with 5% CO_2. After 24 h of cell seeding, the culture medium was replaced to remove cell debris. After 24 h, SkQT, SkQ3 and SkQ1 were added at concentrations of 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 μM and the renal tubular epithelial cells were incubated with these substances for 24 h in a complete nutrient medium. Control cells were incubated in the same medium without addition of SkQT, SkQ3, or SkQ1. Cell viability was assessed by the standard MTT test, for which the culture medium was replaced with a DMEM/F-12 without bicarbonate containing 5 mg/ml of MTT reagent and incubated for 1 h. Then the medium was removed and 50 μl of DMSO were added to each well. Formazan absorbance was measured at 540 nm with a Zenyth 3100 plate spectrofluorimeter (Anthos Labtec, Austria).

RESULTS

Antibacterial action of SkQs. It was previously shown that addition of the micromolar concentrations of SkQ1 or C_{12}TPP to bacteria results in inhibition of their growth and bactericidal effect [20, 24]. Table 1 shows measured minimum inhibitory concentrations (MICs) for three Gram-positive (*B. subtilis*, *S. aureus*, *M. smegmatis*) and one Gram-negative bacterium (*E. coli*). For all the SkQs we studied, MIC values comparable to those of SkQ1 were obtained for Gram-positive bacteria as well as for Gram-negative *E. coli*. Just as in the case of SkQ1, all the SkQs we studied demonstrated antibacterial action only against the deletion mutants deficient in the *tolC* and *acrB* genes, while against other deletion mutants deficient in the *acrD*, *acrF*, *mdtB*, *mdtF*, *macB*, *emrB*, and *emrY* genes, antibacterial activity was comparable to that exhibited against the wild-type *E. coli*. The MIC values for all deletion mutants (except Δ*tolC* and Δ*acrB*) were similar for all SkQs, indicating that all other SkQs, just like SkQ1, are pumped out only by the AcrAB-TolC MDR pump.

SkQ1 docking in TolC. Our data demonstrate important role of the AcrAB-TolC MDR pump in bacterial resistance to SkQ1 [20-22]. We started the work on modeling the process of SkQ1 interaction with this pump. In the first step, molecular docking calculations were performed for the TolC pump component. The results (Fig. 2) allow us to conclude that no obvious binding pockets for SkQ1 are present on the inner surface of TolC in both of its states (closed and open). However, an interesting clustering of sites appears at the entrance of the pump in the closed state, where the phosphonium group interacts with the abundant GLU and ASP residues, the aromatic rings stack with three tyrosine residues, and ASP371 forms hydrogen bonds with the quinone. Unfortunately, scoring functions for ranking the docking results do not allow estimating affinity of such binding even approximately. It can be concluded that the most specific binding site of SkQ1 in TolC is the entrance of the pump in the closed state.

Table 1. Minimum inhibitory concentrations (μM)

SkQ	*B. subtilis*	*S. aureus*	*M. smegmatis*	*E. coli*	Δ*tolC*	Δa*crB*	other*
SkQ1	1	1	1	35	1	1	35
SkQ3	1	1	1	35	1	1	35
SkQT	2	2	2	35	2	2	35
SkQT-para	1	1	1	35	1	1	35
SkQThy	1	1	1	35	1	1	35

Note. Asterisk (*) denotes deletion mutants deficient in other TolC-containing pump proteins (AcrD, AcrF, MdtB, MdtF, MacB, EmrB and EmrY).

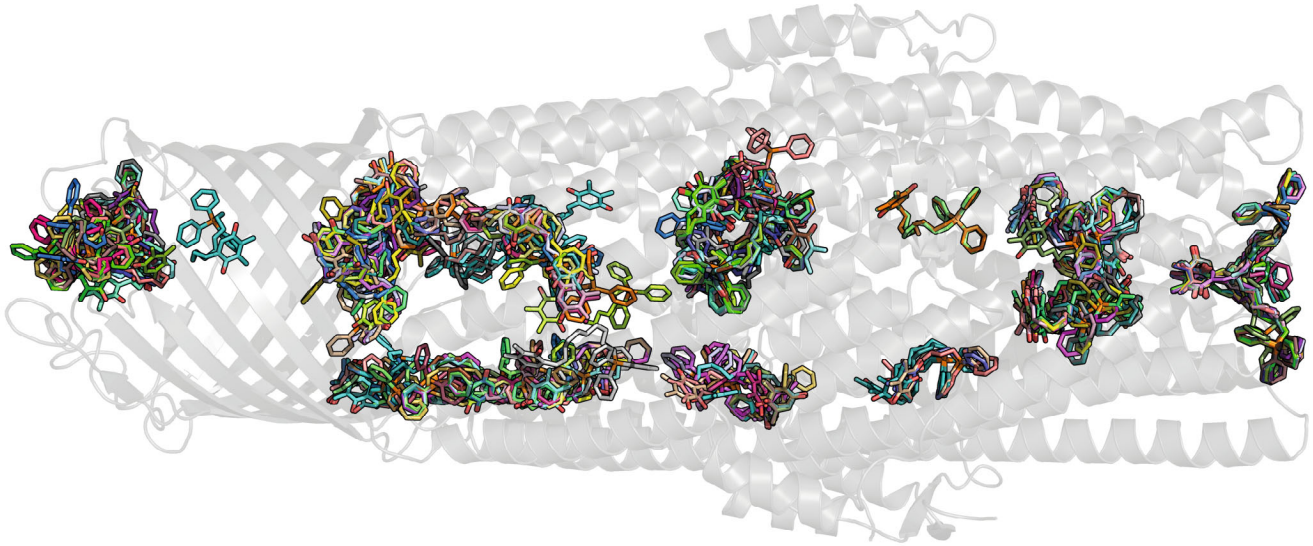

Fig. 2. Results of modeling SkQ1 docking to the internal cavity of TolC in its closed state. Structure of TolC is shown in gray, demonstrating secondary structure elements. SkQ1 positions are shown in different colors to display covalent bonds.

Antibacterial activity depends on the amount of cells. Although SkQ1 can be considered as an antibacterial agent or as an antibiotic, its properties still differ from those of the traditional antibiotics. Unlike for other antibiotics, activity of SkQ1 depends on the number of cells, which is explained by its lipophilicity and protonophore-like properties, allowing it to reduce bacterial membrane potential. Fig. 3a shows the 6-h growth curves for the samples containing different amounts of *B. subtilis* cells cultured overnight in the medium with the same concentration of SkQ1. The obtained results indicate that antibacterial action of the lipophilic cation SkQ1 decreases with the increase in the number of cells and, consequently, the amount of cellular membranes/lipids. Thus, despite the fact that the protonophore-like effect of SkQ1 is mediated by free fatty acids, addition of exogenous fatty acids may result not in the increase in the protonophoric effect, but in the protective effect due to competition of fatty acid micelles with bacterial cells for SkQ1 [35]. Such protective mechanism was previously described for *S. aureus*, which was resistant to daptomycin due to decrease in the activity of this antibiotic through the release of membrane phospholipids into the external environment with subsequent incorporation of the antibiotic in phospholipid micelles [36]. In the case of addition of exogenous fatty acids, we observed a protective effect (Table 2): MIC increased 2-4-fold, which further confirms dependence of the antibacterial action on the amount of membranes/lipids or micelles. It should be noted that addition of exogenous fatty acids did not have a negative effect on the growth of bacterial cells without the addition of SkQ1.

Protective effect of dead cell population against the action of SkQ1. Addition of the heat-killed bacteria to the live *S. aureus* cells prevented the SkQ1-mediated death of live *S. aureus* bacteria, and the more killed bacteria were present, the stronger the protective effect was (Fig. 3b). Interestingly, with a 1000-fold excess of dead bacteria, growth rate of live cells increased approximately 2-fold relative to the control, which can be explained not only by the SkQ1 sorption

Table 2. Increase in the minimum inhibitory concentration of SkQ1 upon addition of exogenous fatty acids (μM)

Acids	Formula	Fatty acid, μM	SkQ1 MIC, μM
Myristic acid	$C_{14}H_{28}O_2$	0.5	2-4
Palmitic acid	$C_{16}H_{32}O_2$	0.5	2-4
Stearic acid	$C_{18}H_{36}O_2$	0.5	2-4
Linoleic acid	$C_{18}H_{32}O_2$	0.5	2-4
Without any additives	–	0	1

Note. Addition of fatty acids did not affect growth rate of the *B. subtilis* population.

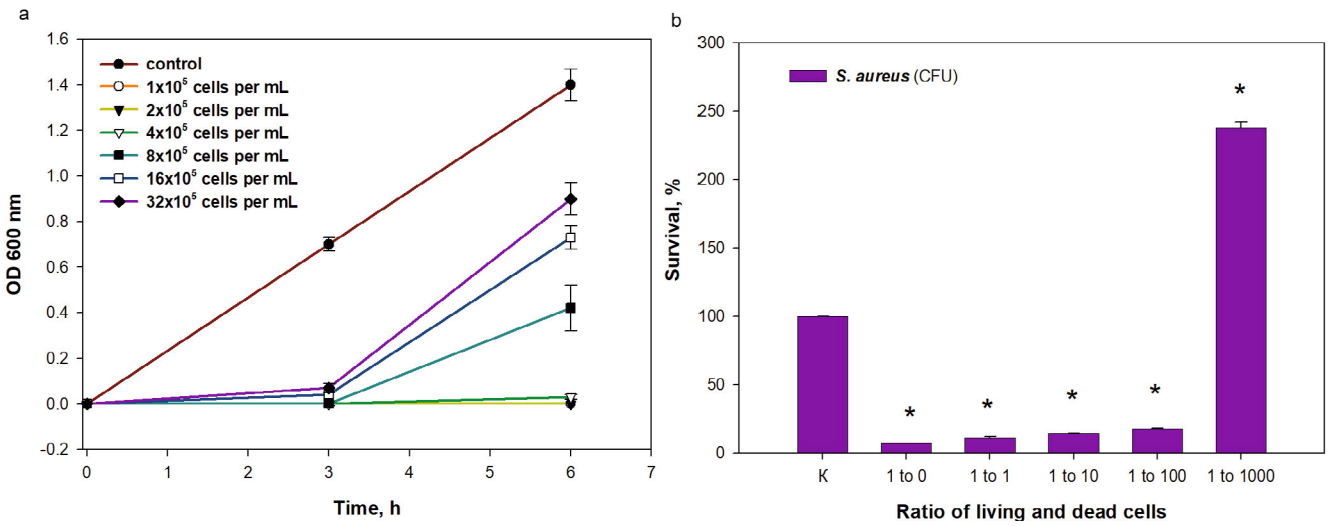

Fig. 3. Antibacterial action depends on the number of cells. a) Dependence of antibacterial action of 0.5 μM SkQ1 on the number of *B. subtilis* cells. b) Effect of dead *S. aureus* cells on bacterial survival under the action of SkQ1. Bacteria at concentration of ~60,000 CFU per ml were incubated for 3 h at 37°C in a saline solution with glucose (0.9% NaCl, 5 mM D-glucose, 1 μM SkQ1). Control cells (c) were incubated without SkQ1. Relative results of bacterial survival (CFU) when incubated with 0.5 μM SkQ1 in the presence of aliquots of dead bacteria (1 to 1; 1 to 10; 1 to 100; 1 to 1000) are presented. Mean ± SEM values are given, $n = 4$. * $p < 0.01$ when compared with the "C" sample by unpaired Student's *t*-test.

on the dead cells and prevention of its toxic effect, but also by the presence of metabolites from the dead cells in the incubation medium. The dead cells are apparently used by the live bacteria for growth and reproduction (necrotrophic growth) [37].

Volume of the incubation medium affects bacterial survival under the action of SkQ1. When *S. aureus* was incubated with 1 μM SkQ1, it was found that increase in the volume of the incubation medium leads to the significant decrease in bacterial survival (Fig. 4), which apparently indicates the ability of bacteria to accumulate SkQ1 from the incubation medium.

Effect of SkQs on eukaryotic cells. Although according to the theory [27] mitochondria of eukaryotic cells should accumulate an order of magnitude more SkQs than prokaryotic cells, experiments on various cell cultures show that cytotoxic effect is observed at higher concentrations than for prokaryotic cells.

SkQT, SkQ3, and SkQ1 had a pronounced cytotoxic effect on the cells of the primary culture of rat renal tubules only at concentration of 32 μM (Fig. 5a), which is more than 2 orders of magnitude higher than the MIC for Gram-positive bacteria and is comparable to the MIC for *E. coli*.

Primary renal tubular cell culture treated with SkQT at concentrations of 0.125-16 μM did not show decrease in viability compared to the control group. Moreover, at concentration of 1 μM, which is the MIC for Gram-positive bacteria, SkQT increased the cell survival. When incubated with SkQ3, cell viability exhibited a tendency to increase up to concentration of 2 μM. However, at concentration of 4 μM or more, SkQ3 was already causing a slight decrease in viability. SkQ1 at concentration of 0.125-4 μM did not reduce cell viability in the primary renal tubules cell culture, and concentrations of 0.5 and 1 μM improved cell survival. Increasing concentration to above 8 μM led to the decrease in cell viability. Thus, toxicity for the primary cell culture was comparable with what we have previously observed for immortalized HeLa cancer cells [20].

A completely different situation was observed for the human colon carcinoma RKO cell line and MRC5-SV40 fibroblasts (Fig. 5b). Human lung fibroblast

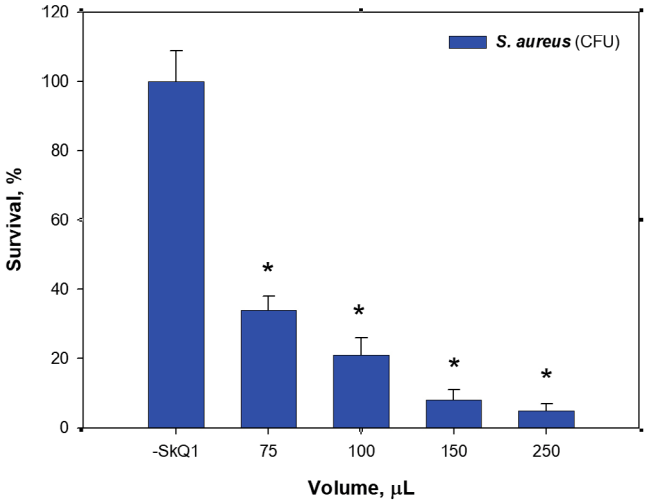

Fig. 4. Survival of *S. aureus* in various volumes of incubation medium with 1 μM SkQ1. Bacteria at concentration of ~15,000 CFU per ml were incubated for 3 h at 37°C in various volumes of the medium (0.9% NaCl, 5 mM D-glucose, 1 μM SkQ1). Results of bacterial survival (CFU) are presented. Mean ± SEM values are provided, $n = 4$. * $p < 0.01$ when compared with the control (–SkQ1) by unpaired Student's *t*-test.

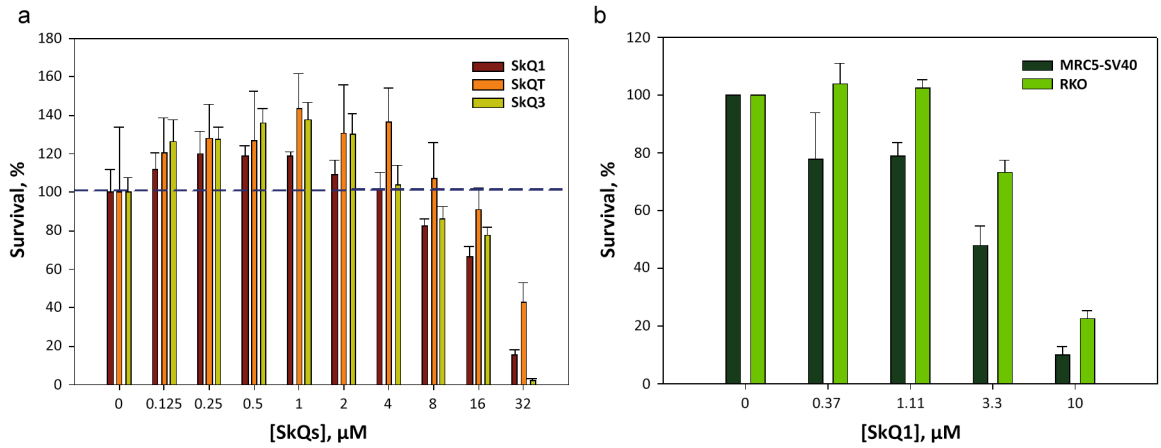

Fig. 5. Evaluation of mammalian cell viability upon addition of SkQs. a) Cell viability of the primary culture of rat renal tubule cells after addition of SkQs. The cells were incubated for 24 h. Cell viability was determined by using MTT test. b) Viability of human colon carcinoma RKO cells and MRC5-SV40 fibroblasts after addition of SkQ1. The cells were incubated for 17 h. Cell viability was determined by using CellTiter-Blue Reagent (Promega, USA).

MRC5-SV40 cells were also not affected by the SkQ1 cytotoxicity at concentration of 1 μM, but a significant decrease in their viability was observed at 10 μM of SkQ1. For the RKO carcinoma cells, 1 μM concentration of SkQ1 was already causing noticeable cytotoxicity. At the concentration of 10 μM, survival was only 10%. Thus, toxicity was significantly higher than what we had previously observed for the HeLa cells [20], which apparently indicates a difference in metabolism and gene expression in these immortalized cells.

DISCUSSION

Despite the significant progress in the study of MTAs, the mechanisms of their action on prokaryotic and eukaryotic cells remain not fully understood. Just like SkQ1, a well-studied MTA, other quinone derivatives, such as SkQT [38, 39], SkQThy [40, 41], and SkQ3 [9, 42] also exhibited pronounced antioxidant properties, however, antibacterial activity has not been demonstrated experimentally for any of them.

All living cellular organisms without exception contain MDR pumps localized on their cell membrane. Bacteria have six classes of these pumps divided into two large groups: ATP-dependent pumps and H^+/Na^+ gradient-dependent pumps [43], so it was very likely that one of them could recognize SkQs and start pumping them out, thereby increasing bacterial resistance. All the more surprising is the observed fact that all of the SkQs we have studied are pumped out by the single AcrAB-TolC pump.

Thus, if only the AcrAB-TolC pump is capable of pumping out SkQs, then many Gram-positive bacteria would be sensitive to SkQs since they simply cannot possess such pumps (with the possible exception of Negativicutes). In the case of Gram-negative bacteria, in which existence of such pumps is possible, resistance would depend on the presence of the AcrAB-TolC pump or a similar one, structure of which is close to that of the *E. coli* AcrAB-TolC pump [22]. In the case of eukaryotes, such as *Saccharomyces cerevisiae* yeast, the SkQ1 pumping occurs due to operation of at least several ATP-dependent pumps, including Pdr5 [44].

Moreover, the main MDR pumps in eukaryotes are ATP-dependent, since the process of energy generation is separated from the process of substance transport and occurs within mitochondria and not on the plasma membrane. Thus, decrease in the membrane potential due to the SkQ1 protonophore-like cycle leads to the shutdown of the H^+/Na^+ gradient-dependent pumps (such as AcrAB-TolC) in prokaryotes, but does not result in a shutdown of the ATP-dependent pumps in eukaryotes.

Unlike in the case of prokaryotes where the mechanism of cytotoxic action of SkQ1 and protection against it is sufficiently clear, no such clarity exists for eukaryotes. Indeed, eukaryotic mitochondria should theoretically pump out an order of magnitude more SkQs than bacterial cells. The difference in electric potentials between the extracellular environment and the mitochondrial matrix in eukaryotes is approximately –240 mV (~ –60 mV on the plasma membrane and –180 mV on the inner mitochondrial membrane), whereas potential on the bacterial membrane is only –180 mV. Since the eukaryotic plasma membrane has a lower electric potential than the bacterial cell membrane, accumulation of SkQs in the cytoplasm of eukaryotic cells should be less efficient than in bacterial cells. It should be noted that operation of the AcrAB-TolC pump in the Gram-negative bacteria only reduces the rate of SkQs accumulation on the inner membrane of bacteria, giving the Gram-negative bacteria a chance to increase their biomass and, thereby,

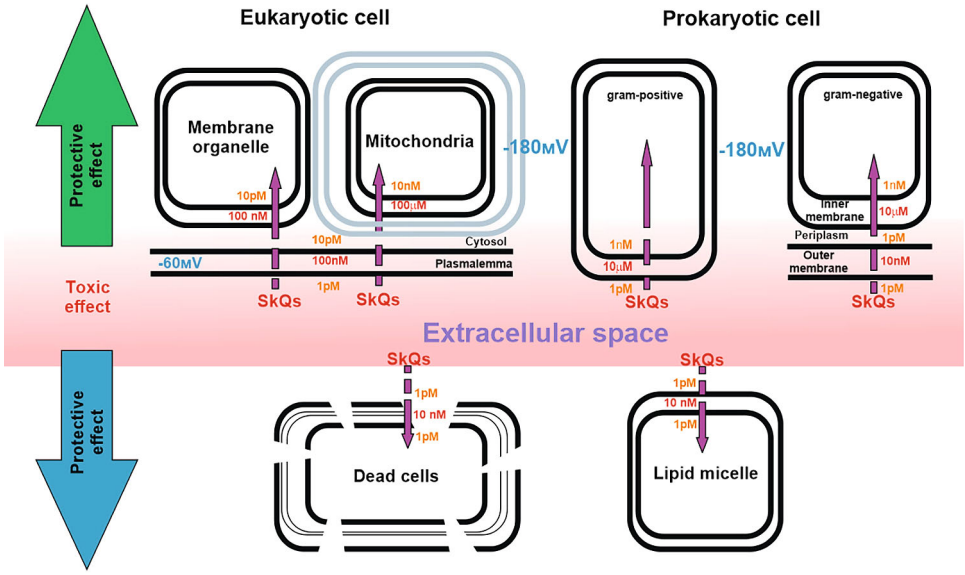

Fig. 6. Scheme illustrating accumulation of SkQs in eukaryotic and prokaryotic cells and protective effect of the membrane-enclosed intracellular organelles, lipid/membrane micelles, dead cells (due to SkQs deposition) and live cells (due to decrease in the SkQs/membrane ratio) ([27], with modifications).

reduce the ratio of SkQs to membrane fractions due to the cell division. Results of our experiments studying dependence of the antibacterial action of SkQ1 on the number of cells and on the presence of dead cells confirm this conclusion.

Another mechanism protecting eukaryotic cells from the toxic effects of SkQs is the presence of membrane organelles (intracellular vacuoles, Golgi apparatus, endoplasmic reticulum, lysosomes, endosomes, etc.), in which lipophilic SkQs molecules could be temporarily deposited, which also reduces the rate of SkQs accumulation at the inner mitochondrial membrane. Results of our experiments on the effect of addition of exogenous fatty acids on the antibacterial action of SkQ1 confirm this conclusion. Figure 6 shows a scheme of the distribution of the penetrating SkQ cation outside and inside eukaryotic (left) and prokaryotic (right) cells. To be specific, for this diagram, we assume that SkQ concentration outside of the cells is 1 pM, with the external space serving as an infinite source of this compound. Then, as a result of its potential-dependent accumulation in the cytoplasm of eukaryotic cells, SkQ concentration will be 10 pM, and ~100 nM within the plasma membrane (due to the high membrane-water distribution coefficient). In the case of prokaryotic cells, SkQ concentration in the cytoplasm should be 1000 pM = 1 nM, and ~10 nM SkQ should accumulate in the mitochondrial matrix of eukaryotic cells. When the outside source of SkQ is limited, which should be the case of a real-life situation, concentration of SkQ inside the mitochondria should be below 10 nM due to the presence of other membrane organelles in the cells, which should effectively accumulate hydrophobic SkQ. Similarly, dead cells and lipid micelles outside of the cell will effectively accumulate hydrophobic SkQ and reduce its concentration within the live cells in a real-life situation.

The $\Delta\psi$ values on the plasma membrane and the inner mitochondrial membrane are taken to be –60 and –180 mV, respectively. The membrane/water distribution coefficient for SkQ is assumed to be 10,000 : 1 [27].

CONCLUSION

The obtained results allow us to conclude that antibacterial activity of SkQs depends on the amount of lipid components of the membranes or micelles (Fig. 6). This allows us to formulate several major reasons that determine the increased resistance of eukaryotic cells, namely: (i) presence of a large number of membrane organelles (endoplasmic reticulum, Golgi apparatus, etc.) which deposit a certain amount of SkQs within; (ii) difference in the cell membrane potential in prokaryotes (~180 mV) and eukaryotes (~60 mV), which determines slower penetration of SkQs into eukaryotic cell; (iii) spatial separation of the processes of energy generation (mitochondria) and substance transport (cell membrane) in eukaryotes, in contrast to combination of these processes on the cell membrane of prokaryotes; (iv) difference in the composition of MDR pumps on the membranes of eukaryotes (mainly ATP-dependent pumps) and prokaryotes (mainly H^+/Na^+ gradient-dependent pumps). Together, all four of these major factors determine the increased resistance of eukaryotic cells compared to their theoreti-

cally expected sensitivity. Difference in the sensitivity of different eukaryotic cell types is apparently determined by: (i) the degree of membrane organelle development (endosome system, Golgi apparatus, etc.), (ii) energy status of the cell [45], and (iii) level of the MDR pump expression in them.

Acknowledgments. The authors wish to express their gratitude to N. V. Sumbatyan for assistance in the study and fruitful discussion of its results. The authors are grateful to A. I. Sorochkina for help in translating the article.

Contributions. P.A.N., Yu.N.A., M.V.S., R.A.Z., and E.Yu.P. study concept and management; P.A.N., L.A.Z., A.A.B., K.G.L., and A.V.G. conducting experiments; P.A.N., Yu.N.A., M.V.S., R.A.Z., E.Yu.P., M.V.K., A.V.G., and K.G.L. discussion of the results; P.A.N., M.V.S., A.V.G., E.A.K., R.A.Z., E.Yu.P., M.V.K., and Yu.N.A. writing the manuscript; P.A.N., E.A.K., E.Yu.P., and M.V.K. editing the manuscript.

Funding. The present study was carried out with financial support of the Russian Science Foundation (grant no. 22-15-00099, P. A. Nazarov). The experiments on human cell lines were carried out with financial support of the Russian Science Foundation (grant 23-14-00061, K. G. Lyamzaev). Molecular docking was performed with support of the Moscow State University Interdisciplinary Scientific and Educational School "Brain, Cognitive Systems, Artificial Intelligence" (A. V. Golovin).

Ethics declarations. All the applicable international, national and/or institutional guidelines for the care and use of animals were followed. The protocols for working with animals were approved by the ethical committee of animal research of the Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University (Protocol 3/19 of March 18, 2019).

Conflict of interests. M. V. Skulachev is the director of the Mitotech company which develops and commercializes drugs based on SkQ-type MTAs. The authors of this work declare that they have no conflicts of interest.

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