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# Programmed Cell Death as a Target to Interrupt the Aging Program

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**Abstract**—There are two opposite points of view on aging of organisms. The traditional concept assumes that aging is a stochastic process consisting in age-dependent accumulation of random injuries in living systems. However, many pieces of evidence are recently obtained in favor of an alternative scheme suggesting that aging is genetically programmed being the final step of ontogenesis. The latter concept predicts (i) the existence of non-aging species which have lost the aging program and (ii) that the program in question can be experimentally interrupted by manipulations with corresponding genes or by small molecules operating as inhibitors of the execution of aging program. In this paper we summarize observations which are consistent with these two predictions. In both cases, interruption of the aging program is based upon inhibition of programmed cell death (apoptosis) mediated by mitochondrial reactive oxygen species (ROS). We argue that the main difference between young and old multicellular organisms consists in the *cellularity*, i.e. in number of functional cells in organs or tissues rather than in quality of these cells. The cellularity decreases due to domination of apoptosis over proliferation in aging organisms. This means that apoptosis appears to be the basis of aging program. A pharmacological approach to switch off the aging program is considered, and this approach involves mitochondria-targeted antioxidants and uncouplers. Such compounds prevent mitochondrial oxidative stress which increases with age and stimulates the age-dependent apoptosis.

*Keywords*: apoptosis, aging, mitochondria-targeted antioxidants
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## PHENOPTOSIS, A PHYSIOLOGICAL SUICIDE OF AN ORGANISM

Charles Darwin's theory of evolution is usually interpreted as a concept postulating that biological evolution is always directed to selecting the traits advantageous for an individual. However, in his second famous monograph "The Descent of Man" Darwin wrote that there could be no doubts that a community consisting of many members always ready to help one another and die for the common good would get a victory over the majority of other communities; this is natural selection [33]. In one of his letters, Alfred Russel Wallace, who formulated the hypothesis on natural selection simultaneously with Darwin, wrote that parents, once produced a sufficient offspring, become a hindrance for this offspring by competing with it for food. Natural selection removes the parents and, in many cases, provides advantages to the races that die immediately after giving birth to the offspring (cited according to [114], p. 23). Later, these ideas were developed by August Weismann in a separate monograph. He wrote that the worn out individuals were not only useless but even detrimental, occupying the place of the fitter ones, and he regarded death not as a primary necessity but as something secondary and acquired as an adaptation [114].

Weismann's theory got an unexpected support in the early 1970s after the discovery of programmed cell death (apoptosis) [58]. Indeed, if the mechanism of suicide exists at the cellular level, it seems possible that a similar phenomenon exists at the level of an organism. A positive answer to the question whether the mechanism of physiological suicide exists at least in certain species is currently out of doubts. First, let us consider the examples of such phenomena in the most primitive unicellular organisms.

Bacteria have a system referred to as quorum sensing. One of the functions of this system is cell lyzis in response to a combination of several factors, such as a high density of bacterial culture and depletion of nutrients. It is assumed that the components of lyzed cells helps the remaining bacteria to survive starvation under natural conditions. Under laboratory conditions, nutrients are always in excess and cells lose this system at a certain rate. Naturally, such mutants gain a selective advantage, and all cells in a stationary culture are eventually replaced with such mutants. This does not take place under natural conditions. Once natural selection is active, the mutants would not merely survive a temporal depletion of nutrients (see [69, 102] for reviews of other mechanisms of bacterial cell programmed death).

The mechanism of programmed death is also present in the primitive eukaryotes, for example, the

unicellular yeast *Saccharomyces cerevisiae*. It has been shown that under laboratory conditions, various stress impacts kill yeast via a specific biological mechanism—yeast mitochondria respond to stress by producing reactive oxygen species (ROS), which leads to a loss of the membrane potential and fragmentation of mitochondria thereby initiating cell death. Knockout of some genes or addition of antioxidants prevents death (for review, see [25, 39, 40, 86, 87]). Interestingly, an excess of sex pheromone induces yeast death with typical markers of apoptosis [86, 94]. It is still unclear what is the role of such a suicide (see [8, 95, 100] for discussion). Nonetheless, it is clear that the mere fact that such a mechanism does exist is hardly in line with the traditional evolutionary science.

In the case of another primitive eukaryote—the amoeba *Dictyostelium*—the logics of a natural suicide are more understandable. Under favorable conditions, *Dictyostelium* is a unicellular organism. However, upon deterioration of such conditions, the amoebas form a multicellular agglomerate, which matures into a fruiting body. Cells differentiate within the fruiting body; a fraction of the cells transforms into spores for further sexual reproduction, and the other part forms the stalk of the fruiting body, which degenerates after spore maturation via apoptosis. How is the decision made within the fruiting body on which particular amoeba develops into a spore and which undergoes the terminal differentiation into a stalk cell? Under laboratory conditions, isogenic amoebas were divided into two groups so that one group was cultured in a poor medium and the other one (marked with a neutral genetic marker), in a rich medium. Both groups were then pooled to induce the development of a fruiting body. As a result, the "starved" cells formed the stalk and then died, having switched on the cascade of the programmed cell death, whereas the "fat" cells developed into spores [110]. Thus, the most adapted cells survived, which is favorable from the standpoint of evolution of this amoeba species.

Social amoebas can be regarded as a primitive multicellular organism. The mechanisms of "physiological suicide" have been reported for a number of "true" multicellular organisms. Such examples are rather common among insects. For example, adult mayflies have no functional mouth and die of the lack of nutrients [114]. The male praying mantis ejaculates only after the female decapitates he during sexual intercourse [36]. The larvae of *Adactylidium* tick hatch by gnawing their way through maternal body tissues, thereby killing the mother [15]. Males of some squids die immediately after mating [10]. Specialized programs of this type have been also observed in higher organisms. For example, male marsupial mice die 2 weeks after rutting because of an excess of their own pheromones. Pacific salmon die soon after spawning (for review, see [15, 59]).

A rapid programmed death of multicellular organisms is not obligatorily associated with reproduction. Death of an ill organism can be favorable for others because it prevents the spreading of infection. It has been discovered that higher animals possess a mechanism killing them in response to the appearance of Gram-negative bacteria in the blood. The lipopolysaccharides (LPS) of the bacterial cell wall are toxic because the LPS appear in the blood of the experimental animals. However, this toxicity can be drastically decreased by blocking of a specialized LPS-binding protein in the blood or by inhibiting the specialized cell receptors of LPS–protein complex (for review, see [41, 60, 101]).

A new term has been recently proposed for description of such phenomena—"rapid phenoptosis"—as well as it has been postulated that aging is nothing else than a "slow phenoptosis" [14].

## IN THESE TERMS, AGING OF AN ORGANISM IS A "SLOW PHENOPTOSIS" PLAYING A ROLE OF EVOLUTIONARY ACCELERATOR

In the case of rapid phenoptosis, potential benefits for population are often evident; however, the biological significance of its slow analog requires explanations. It has been recently postulated that a gradual programmed deterioration of certain body functions promotes an increase in the selection efficiency for other functions of the organism [102]. Take the following example. Two young hares, one smarter than the other, both have good chance to escape a fox because they both run quicker. However, with age, the smarter hare gets an advantage as their speed of running decreases due to a senile illness known as sarcopenia (a decrease in the number of cells in muscles). Now, the smarter hare, which takes to heel immediately after noticing the fox, has a better chance to escape than the other one, which pauses at the start. This means that only the smart hare continues to produce offspring, making the hare population more clever [102]. This example of the effect of aging being favorable for evolution implies that the muscle age quicker than brain; moreover, it implies that muscle strength starts to weaken when reproduction is still possible. These conditions are met, at least when we speak about humans. The first signs of muscle atrophy in humans become apparent as early as the age of 25 [70]. This process is initially very slow. Currently, a considerable decrease in muscle strength is recorded at the age of 60 for Swedish men [67] and at the age of 40 for Saudi Arabian men [18]. On the other hand, a decrease in this parameter was evident in the early 19'th century as early as in 25 years olds (examination of Belgian men, see references in [67]). These dynamics can be explained by an elevation in the living standards over the last two centuries. In any case, as T.C. Goldsmith noted, as even a minor deterioration causes a statistically significant increase in the mortality rate, it can be expected that the evolutionary effect of aging in wild animals would appear at a compara-

tively young age [43, p. 15] (see the article by Goldsmith [44] on the role of aging as a mechanism increasing evolvability).

## THE MUTATIONS EXTENDING LIFESPAN

How is it possible to experimentally test the existence of a program for slow phenoptosis? The most radical way is to find that the genes being inactivated (1) would cancel or delay aging and (2) would not have any pronounced effect on the other functions of the organism. The latter should be necessarily satisfied to avoid "false-positives" when searching for the specialized proteins involved in the aging program. For example, a severalfold (by more than one order of magnitude) increase in the lifespan of a filamentous fungus (*Podospora*) was recorded due to inactivation of the genes encoding one of the cytochrome oxidase subunits or a protein necessary for transformation of long filamentous mitochondria into small spherical structures (a marker of early apoptosis) [38, 93]. Analysis of this phenomenon has demonstrated that it is accompanied by a decrease in the mitochondrial ROS generation and an increase in cell resistance to apoptosis induced by $H_2O_2$. In this case, a conventional respiratory chain is replaced by a shortened one with a decreased efficiency of adenosine triphosphate (ATP) synthesis so that one of the sites for ROS generation disappears, whereas the other site, even when producing ROS, performs this at a slow rate [38, 93, 104]. The same change in the life pattern of a wild-type fungus is achievable simply by growing it in a liquid medium [112].

A major increase in the lifespan of a nematode *Caenorhabditis elegans* was observed in the case of a double mutation in the genes encoding insulin receptor and an enzyme converting the ubiquinone precursor demethoxyubiquinone into ubiquinone [65]. As it took some time to clarify this issue, the authors already named the second gene *Clk1* ("Clock"). This gene has been found in mice; moreover, the mutation at this gene also somewhat extended their lifespan and decreased the sensitivity of their fibroblasts to the apoptosis induction by reactive oxygen species (ROS) [72].

The list of candidates for players in the aging program also includes p66shc protein. It has been shown that an average lifespan of mice lacking p66shc gene is 30% longer as compared with the control. Note that neither the initial study nor further experiments succeeded in finding any pathologies in such animals [24, 77, 79, 82]. p66shc protein forms a complex with cytochrome *c*, presumably, making the cytochrome capable of self-oxidizing. If this is correct, cytochrome *c*, once bound p66shc, reduces $O_2$ to superoxide which in turn triggers apoptosis.

A manuscript is now being prepared in the laboratory of A.G. Ryazanov, which describes another example of such protein - EF2 (elongation factor 2) kinase. The EF2 phosphorylation by this kinase blocks protein synthesis, thereby inducing apoptosis in cell culture. The assumed reason is the difference in the lifespans of pro- and antiapoptotic proteins. Importantly, a number of antiapoptotic proteins are both synthesized and degraded in cells at higher rates than the proapoptotic proteins. Consequently, a decrease in the total intensity of protein biosynthesis can change the balance between the anti- and proapoptotic proteins towards the latter [34, 47, 50, 53]. It has been demonstrated that a knockout of this kinase in mice increases the lifespan by 30% and also elevates the resistance to ionizing radiation and hydrogen peroxide (A.G. Ryazanov, personal communication).

## AGING: THE ROLE OF APOPTOSIS AND MITOCHONDRIAL ROS

If certain genes are responsible for aging, then the animals that have lost such genes and, consequently, the aging program, should exist in nature. Such animals, in fact, do exist.

It is known that birds on average live three times longer than the terrestrial mammals of a comparable size. In addition, the level of a bird's metabolism is approximately 2.5-fold higher compared to the one of terrestrial warm-blooded animals of comparable size. There are some avian species with mortality rate being independent of age, they also display no aging of certain components of the endocrine system and lack any age-related decrease in fertility (for review, see [48]).

Perhaps, a non-aging mammal that is most studied from a biochemical standpoint is the naked mole-rat. This rodent of a size of a mouse is famous for its average lifespan being 28 years instead of 1.5-4 years typical for mice; moreover, it dies of certain age-unrelated reasons rather than of aging (its mortality rate remains at a low and constant level during the entire life [28]). It is known that the arterial cells of naked mole-rats are considerably more resistant to injuries compared to murine cells. In particular, the cells of naked mole-rats are resistant to the $H_2O_2$-induced apoptosis similar to fibroblasts of mice lacking the p66shc protein or EF2 kinase [64]. In this connection, recent data on elevated sensitivity of naked mole-rat fibroblasts (as compared to murine fibroblasts) in culture to hydrogen peroxide [91] may look rather unexpected. Presumably, this is due to the fact that mouse cells are, to a greater degree, damaged in culture and, consequently, catalase is released into culture medium. A fact matching this interpretation is that it has been shown that the sensitivity of mouse fibroblasts to another prooxidant, paraquat, whose action is independent of catalase, was higher than that of naked mole-rat fibroblasts [91].

Thus, a positive correlation is evident between the cell resistance to ROS-induced apoptosis and the lifespan of the organisms. Is there any functional connection between these two effects? It is considered that

the ROS-induced damage in proteins, nucleic acids and lipids accumulates in cells with age. Many advocates of the theory of aging being a result of damage accumulation use this fact as a supporting argument.

Indeed, it is known that ROS are among the main pro-aging factors. A decrease in ROS level by antioxidants contributes to elongation of the lifespan of the cells in culture and assists in treating several senile diseases (for review, see [37, 75, 103, 105, 107]). Moreover, a negative correlation is observed between the average lifespans of mammals and birds and the rate of $H_2O_2$ generation by the heart muscle mitochondria in the reverse electron transfer within the respiratory chain [22, 23, 63, 66]. Note that the non-aging naked mole-rat is the only exception to this rule [66].

Let us compare the levels of antioxidant defenses and the degree of oxidation of biopolymers in mice and their long-living relatives, naked mole-rats. It seems evident that a long-living organism should have a more powerful antioxidant defense and a lower level of oxidation damage than a short-living species. However, the data point to the opposite. Superoxide dismutase and catalase activities are rather similar in these two species, whereas the third key antioxidant enzyme, glutathione peroxidase, displays a 70-fold lower activity in naked mole-rats as compared with mice. In addition, both the rate of ROS generation by the heart muscle mitochondria [66] and the degree of oxidation of biopolymers are considerably higher in the naked mole-rats than in mice [19]. The key for understanding this paradoxical situation can be found in the already-mentioned observation on naked mole-rat cell resistance to the ROS-induced apoptosis.[1]

It looks as if the cells of multicellular organisms are "made with an excess degree of safety". They can, in principle, live and function at considerably higher levels of oxidation damage as compared with levels that induce apoptosis or other types of programmed cell death. A low level of the threshold ROS oxidation causing programmed cell death is necessary to shorten the cellular lifespans and, consequently, to decrease their number and eventually cause aging of the organism [14, 15, 17, 73, 104].

## HYPOTHETICAL SCHEME OF AN ORGANISM'S AGING PROGRAM

If aging is a programmed age-dependent weakening of a large number of physiological functions of an organism, perhaps the simplest way to achieve this goal is to decrease the "cellularity", that is, the number of cells constituting the corresponding organs responsible for these functions. In this case, the body can be compared to a factory with its staff being constantly reduced and still keeping the same plan for production with a hope of improving the production process.

The literature provides several examples of the observations which demonstrate that the level of damages in biopolymers in the cells of certain tissues of the old mice often does not differ in a statistically significant manner from that of the young mice (for review, see [29, 46, 49]). Therefore, it is impossible to reliably determine the age of an animal only from a tissue sample: as a rule the individual differences between the animals of the same age are more pronounced than the differences between age cohorts. The most evident age-related changes are provided by the number of functioning cells rather then their quality. Indeed, evident traits of aging, such as osteoporosis, sarcopenia (muscle degeneration), and a decrease in immunity are the primary consequences of a decrease in the number of osteoblasts, myoblasts, thymus cells, and cells of other tissues determining the immunity (for review, see [16]).

The above-described concept is illustrated by the following scheme:

Ontogenetic clock
↓
Increase in the ROS level in mitochondria
↓
Predominance of apoptosis over cell proliferation
↓
Decrease in the number of cells in organs
↓
Weakened function of organs

According to this scheme, the finishing touch in the melody of the ontogenetic clock is the signal to aging commencement. If a tissue responsible for circadian organization of life (circadian rhythm) plays the role of such clock, the ontogenetic clock should be searched for in the suprachiasmatic nucleus of the mammalian hypothalamus or avian pineal gland [102, 104]. As in the case of circadian rhythm, mammalian pineal gland might serve as the organ multiplying the signal coming from the suprachiasmatic nucleus. Some hormone in the pineal gland (e.g., melatonin) might be a mediator between the pineal gland and the other body tissues. A decrease in melatonin level with age is well-established as well as an anti-aging effect of this substance [2, 3, 20, 55, 84] (see [102] for review on other candidate mediators for aging signal transmission from the "clock" to body tissues).

[1] The most likely reason for the loss of aging program by the naked mole-rat as a species trait is its way of life. Naked mole-rats live in colonies of up to 250 individuals in underground labyrinths the size of two soccer fields; moreover, only the queen and one to three her sexual partners are involved in reproduction. The "chambers" of the queen are in the center of the labyrinth, and the approach avenues to them are securely defended by "the army of soldiers". The queen has almost no enemies. It is important that the Damaraland mole-rat, found in sub-Saharan Africa, a close relative of the naked mole-rat but living in small groups, does not differ from mice in its lifespan [66].

The problem of aging signal transduction within a cell of peripheral tissues, i.e from the cell surface, directly interacting with the juvenile or aging hormone, to mitochondria, is even vaguer. For example, the main player might be the juvenile hormone itself, if it enters the cell and reaches mitochondria. In particular, it has been shown that melatonin concentrates in mitochondria [2, 74]; however, it is unclear how melatonin influences the level of mitochondrial ROS. It is unlikely that this is due to its ability to act as an antioxidant. Indeed, the amount of melatonin is evidently insufficient to obtain a detectable decrease in ROS. It is more likely to expect the presence of a certain melatonin receptor in mitochondria that transmits the signal inducing a decrease in the ROS level. Such a decrease may be a result of deceleration in mitochondrial ROS generation or an increase in their neutralization. The former assumption is favored by the discovered negative correlation between the lifespan and the rate of the ROS generation during reverse electron transfer through complex I. This correlation was not detected in the case of a direct electron transfer though the same region of the respiratory chain. Lambert et al. [66] recently observed this when studying a large number of warm-blooded species; independent experiments in the laboratories of Sohal [63], Barja [22, 23], and Austad [27], who examined smaller samples of species, provided the confirmation. Of paramount importance is the fact that the only exclusion from this rule was the naked mole-rat, once again the only non-aging species among all the animals studied: as was already mentioned, the rate of ROS generation in the naked mole-rat is higher as compared with mice, yet it lives almost ten times longer.

A simplest hypothesis is that the level of mitochondrial ROS increases due to their accelerated generation during reverse electron transfer. According to our data, this process depends in a threshold manner on the value of mitochondrial membrane potential [61]. In particular, a 15% decrease in $\Delta\psi$ results in a tenfold decrease in ROS generation; moreover, this effect is independent of how the $\Delta\psi$ decrease was achieved, be it addition of ADP (i.e., activation of oxidative phosphorylation), of a small dose of protonophore uncoupler, or inhibition of respiration. The effect of a threshold dependence of ROS generation was later confirmed in other laboratories [108, 113]. These observations suggest that $\Delta\psi$ elevates in mitochondria with aging, which increases ROS generation during reverse electron transfer. In turn, an elevation in $\Delta\psi$ can result from a decrease in the proton leakage through the mitochondrial membrane. This process is controlled by uncoupling proteins and mitochondrial anion carriers, which enhance the electrophoresis of fatty acid anions, i. e their transfer from mitochondria (for review, see [99]). Outside mitochondria, fatty acid anions are protonated and returned back to the mitochondrial matrix carrying protons (uncoupling fatty acid circuit [97]). Its activity can be regulated by either proteins carrying fatty acid anions or concentration of these acids in the cytosol. Correspondingly, $\Delta\psi$ increase during aging can be determined by either a decrease in the intracellular level of free fatty acids or a decrease in carrier protein activities (e.g. assuming that melatonin enhances the transfer of fatty acid anions by these proteins).

Our laboratory has demonstrated how an increase in mitochondrial $\Delta\psi$ is involved in rapid phenoptosis of yeasts in response to amiodarone or excess pheromone [86]. The following chain of events was observed: inducer of phenoptosis $\longrightarrow$ $\Delta\psi$ increase $\longrightarrow$ ROS $\longrightarrow$ $\Delta\psi$ loss $\longrightarrow$ cell death.

Interestingly, yeast grown in fermentable medium typically displayed low $\Delta\psi$ levels and a low respiration that was not inhibited by oligomycin and not stimulated by small doses of an uncoupler. Addition of amiodarone or pheromone increased $\Delta\psi$, stimulated respiration and induced the respiration sensitivity to low doses of an uncoupler. Phenoptosis could be blocked by uncoupling, which drastically elevated yeast survival in response to amiodarone or pheromone [86].

Subsequent stages of senile phenoptosis, shown in our scheme, need no comments. It is well known that an increase in the mitochondrial ROS level leads to the opening of a nonspecific pore in the inner membrane of these organelles, which, in turn, leads to swelling of the mitochondrial matrix, disruption of the outer mitochondrial membrane, and release of pro-apoptotic proteins (normally hidden in the intermembrane space of mitochondria) into the cytosol. This is how apoptosis is activated [16, 81, 83, 98]. The number of cells that enter apoptosis becomes so large that they cannot be restored by new cells, and the number of cells in organs decreases as well as the ability of these organs to fulfill their functions, that is, the organism ages.

## ANTIOXIDANTS OF SKQ TYPE TARGETED TO MITOCHONDRIA AS A TOOL FOR EXTENDING YOUTH

As it follows from the scheme shown above, the aging program can be interrupted by preventing an age-related increase in ROS. It is important that here we speak only about excess ROS formed in mitochondria during organism's aging rather than all ROS types. The point is that ROS are not only involved in implementing the aging program but also perform a number of useful functions, such as activation of proliferation genes; protection from bacteria; destruction of virus-infected, malignizing, and "homeless" (those that accidentally left their tissue and entered another one) cells and so on (for review, see [26, 54, 102, 109]).

The problem of decreasing an excess ROS level in mitochondria without influencing other ROS types can be solved in two ways: using mitochondria-targeted antioxidants or using protonophore uncouplers. The former would eliminate the ROS already gener-

ated during reverse electron transfer, and the latter would prevent this electron transfer and with it, the mitochondrial ROS generation.

How could an antioxidant or an uncoupler be targeted to mitochondria? Here, we have to return to the work performed by E.A. Liberman and one of the authors as long ago as the late 1960s, that is, to the discovery of membrane-penetrating ions [71], later named "Skulachev ions" by D. Green ($Sk^+$ and $Sk^-$) [45]. The charge of these ions, for example, methyltriphenylphosphonium, is strongly delocalized, preventing formation of a water shell around the ion and drastically increasing its ability to pass through the hydrophobic barrier of biological membranes [13, 71]. In this set of articles, we also proposed that the penetration cations could be used as "molecular locomotives" for targeted delivery to mitochondria of the compounds lacking a positive charge or charged but unable to pass through the membranes [12]. We used this idea to explain the role of the cation group of carnitine in the transport of fatty acid residues into mitochondria [7, 12, 13, 68]; later, Murphy and Smith applied this idea to construct mitochondria-targeted antioxidants [30, 51, 52, 56, 57, 82, 106]. A substance referred to as MitoQ was used in the majority of Murphy's works where ubiquinone was selected as an antioxidant and decyltriphenylphosphonium cation as $Sk^+$ [51, 52, 56, 57, 78, 92].

We confirmed the ability of *MitoQ* at micromolar concentrations to accumulate in the mitochondria and protect them from oxidative stress. However, even a smallest overdose of this substance changed the sign of the effect: *MitoQ* became a powerful prooxidant that catalyzed the mitochondrial generation of $H_2O_2$ at a record rate (of the same order as the mitochondrial respiration rate in the absence of ADP [5, 16]). Three other laboratories, including Murphy's team, observed the same effects [35, 51, 80].

We then turned to plastoquinone, an electron carrier acting instead of ubiquinone in the photosynthetic electron transport chains of plant and cyanobacterial chloroplasts. Presumably, better antioxidant characteristics of plastoquinone as compared with ubiquinone, described in chemical experiments with model systems [62, 89], were the reason for evolutionary replacement of ubiquinone, involved in the mitochondrial respiratory chain, by plastoquinone in the photosynthetic chain of chloroplasts of the same plant cell. Actually, oxygen generating chloroplast is always exposed to a stronger oxidative stress as compared with mitochondria, which consume this oxygen. Unlike ubiquinone, plastoquinone carries methyl groups instead of methoxyl groups and the methyl group of ubiquinone is replaced by hydrogen. It has been shown that such substitutions drastically increase an antioxidant activity of the resulting compound in biological systems. The MitoQ concentrations inducing anti- and prooxidant effects differ less than 2-fold (300 and 500 nM), whereas this difference increased 32-fold (25 and 800 nM) for the plastoquinone derivative of decyltriphenylphosphonium, *SkQ1*.

Once this result was obtained, it became clear that we had a uniquely efficient antioxidant specifically targeted to mitochondria and displaying no side prooxidant effects.

Note here that our works on SkQ in 2003–2005 were funded by the Paritet Foundation (later, Vol'noe Delo), sponsored by O.V. Deripaska) and in 2005–2008, also by Deripaska, under an investment project. During these years, about $18 million was spent on research, which allowed us to perform a wide range of experiments from synthesizing new substances to in vivo testing using various types of organisms. The experimental data of these studies are summarized in six articles published in *Biokhimiya* [1, 4–6, 9, 14; available at http://www.protein.bio.msu.ru/biokhimiya], *Journal of Membrane Biology* [21, 88], *FEBS Letters* [85], and *Biochimica et Biophysica Acta* [103]. The results of the project with the participation of the Institute of Physico-Chemical Biology, several faculties of Moscow State University, and over 30 research institutions from Russia, Sweden, United States, and Ukraine can be summarized as follows. Cationic derivatives of plastoquinone (SkQ) were synthesized with decyl- or amyltriphenylphosphonium (*SkQ1* and *SkQ5*, respectively), decylrhodamine 19 (*SkQR1*), decyltributylammonium (SkQ4), and analogous derivatives of methylplastoquinone, ubiquinone (*MitoQ*), demethoxyubiquinone, and several others (Korshunova, G.A. et al., [5]) used as $Sk^+$. The penetration ability of these substances was assessed using a planar phospholipid membrane; this ability decreased in the following order: *SkQR1* > *SkQ1*, *SkQ3*, *MitoQ* > *SkQ5* > *SkQ4* (Severina, I.I., Antonenko, Yu.N., et al., [5]). Antioxidant properties on bilayer phospholipid membrane, micelles of linoleic acid, and mitochondria, which were tested in aqueous solutions, decreased as follows: *SkQR1*, *SkQ1* > *SkQ3* > *MitoQ*. Pro-oxidant properties of the compounds studied changed in the opposite order, being maximal in *MitoQ* (Vysokikh. M.Yu., Ruuge, E.K., Antonenko, Yu.N., Roginsky, V.A., et al., [5]). Based on these data, *SkQ1* and its fluorescent derivative *SkQR1* were selected for further experiments.

In the in vitro experiments with heart mitochondria, it has been demonstrated that the respiratory chain can reduce *SkQ1*, that is, *SkQ1* can be used as a rechargeable antioxidant. The center *i* of complex III, located near the inner surface of the mitochondrial inner membrane, appeared to be the site of reduction (Vysokikh, M.Yu. et al., [5]).

Using *SkQR1*, it has been found that the SkQ substances added to a cell culture selectively accumulate in mitochondria (Chernyak, B.V. et al.). This result was predicted based on the fact that the mitochondrial matrix is the only negatively charged structure in the cell. The studied substances arrested the $H_2O_2$-induced apoptosis of HeLa cells and human fibro-

blasts (activity order: *SkQR1* > *SkQ1* > *MitoQ*). ROS-induced necrosis was also sensitive to SkQ (Chernyak, B.V. et al., [5]). *SkQ1* increased the median lifespan of the fungus *Podospora anserine*, crustacerian *Ceriodaphnia affinis*, *Drosophila*, and mice up to twofold in the last species (Anisimov, V.N., Pasyukova, E.G., Vysokikh, M.Yu., Filenko, O.F., et al., [4]). In all cases, SkQ1 was especially efficient in decreasing the mortality rate at early stages of aging (the first 20% of death cases) and had a weak effect on the maximal lifespan (the last 10% of death cases). Thus, the mortality rates were rectangulized. In the experiments with mice kept in a nonsterile facility, *SkQ1* added to drinking water (0.5–5 nmol/kg per day) drastically decreased mortality from infectious diseases yet had an insignificant effect on cancer mortality at old age (Anisimov, V.N., et al.). Experiments with mice and rats have shown that *SkQ1* delayed the development of several aging signs, such as the involution of thymus cells and spleen follicular cells, decrease in the lymphocyte-to-neutrophil ratio in the blood, osteoporosis, cataract, retinopathy, glaucoma, hair loss, grey hairs, cessation of estrous cycles in females, disappearance of libido in males, hypothermia, and catalepsy (Kolosova, N.G., Anisimov, V.N., Drize, N.I., Ryazanov, A.G., Cannon, B., et al. [4, 9, 96, 103]). Experiments with fibroblasts isolated from mice which did or did not receive SkQ during their entire lives demonstrated that *SkQ1* prevents the age-related onset of beta-galactosidase activity and phosphorylation of histone H2AX. Using the same model, it was possible to observe an age-related increase in spontaneous apoptosis and its stimulation by added hydrogen peroxide; both effects were completely removed by *SkQ1* (Spivak, I.M., Mikhel'son, V.M., et al. [4]). It is important that *SkQ1* did not completely arrest the apoptosis but only prevented its threefold increase in old mice. This result agrees well with our scheme of aging (see above).

In several cases, we succeeded in observing that SkQ hindered the development of progeria. This was demonstrated in the experiments with OXYS rats, which suffered from constant oxidative stress, when *SkQ1* not only prevented (and even reverted) early cataract and retinopathy but also interfered with accumulation of oxidized lipids and proteins in tissues (Kolosova, N.G., Zinovkin, R.A., et al. [9]). In the same model, *SkQ1* prevented the age-related decrease in the number of thymus cells and spleen follicles, playing key roles in the immune response (experiments of L.A. Obukhova et al. [103]). In the case of irradiation-induced progeria, *SkQ1* prevented appearance of grey hairs (black mice were used in the experiments; A.G. Ryazanov, personal communication).

The results of experiments with mutant mice where progeria was induced by replacing the aspartate at position 257 by alanine in the corrector domain of mitochondrial DNA polymerase γ were extremely interesting. The enzyme with such a mutation synthesized mitochondrial DNA but was unable to control and repair the errors. Consequently, the number of mutations in mitochondrial DNA drastically increased with age, which was accompanied by manifestation of numerous aging signs and pronounced decrease in the lifespan [111]. Administering *SkQ1* with drinking water increased the lifespan and—even more importantly—extended the youth period: mice that received SkQ died without a set of senile diseases which were observed in dying mutants that did not receive *SkQ1* [e.g., osteoporosis (kyphosis)], baldness of cheeks and body, catalepsy, decreased body temperature, disappearance of estrous cycles, and others [96]). The effect of *SkQ1* on mortality from infectious diseases was not assessed in these experiments because the mice were kept under sterile conditions (Cannon, B. et al.).

Further experiments have demonstrated that administration of *SkQ1* or SkQR1 to animals drastically decrease the unfavorable effect of subsequent ischemia and reperfusion on the heart rhythm and decrease the zones of myocardial infarction and brain stroke. Both compounds prevented death of the rats with one kidney excised and the other subjected to 20-min ischemia (teams of V.I. Kapel'ko and D.B. Zorov, [6]). The experiments with $p53^{-/-}$ mice demonstrated that *SkQ1* or an unaddressed antioxidant, N-acetylcysteine (NAC), hindered development of lymphomas, decreased the ROS level in the spleen, and extended the lifespan; noteworthy, a sufficient dose of *SkQ1* was 5 nmol/kg per day. The dose of NAC necessary to induce the same effect was 6 mmol/kg per day, which is *1200000-fold higher* as compared with *SkQ1* (team of B.P. Kopnin [1]). Even smaller amounts of *SkQ1* were sufficient to control the heart arrhythmia in rats and increase the mouse lifespan (0.05 and 0.5 nmol which corresponds to 0.025 and 0.25 ng per 1 kg animal weight per day) [4, 6]. The efficiency of such small amounts of SkQ is explainable by several facts:

(1) *SkQ1* is electrophoretically accumulated in the cell cytosol in an approximately tenfold excess as compared with its concentration outside the cell due to the voltage difference on the outer membrane ($\Delta\psi$ of about 60 mV);

(2) Even more considerable accumulation (thousandfold) takes place in the mitochondria ($\Delta\psi$ of about 180 mV); and

(3) The partition coefficient for *SkQ1* in system octanol/water is 13000 [5]; correspondingly, *SkQ1* concentration in the inner mitochondrial leaflet should be $10 \times 10^3 \times 1.3 \times 10^4 = 1.3 \times 10^8$-fold higher than in the extracellular space [5].

The mechanism of SkQ antioxidant action can be dual. First, $SkQH_2$ accumulating in the inner mitochondrial membrane is able to quench the oxide radicals of polyunsaturated fatty acids of phospholipids ($LO_2^{\cdot}$), localized to this membrane (first and fore-

most, cardiolipin [5]). Thus, $LO_2^{\cdot}$ . transforms into a fatty acid peroxide (LOOH), thereby interrupting a chain reaction causing oxidative damage of mitochondrial lipids [5, 76, 90]:

$$LO_2^{\cdot} + SkQH_2 \longrightarrow LOOH + SkQH^{\cdot}. \qquad (1)$$

The produced $SkQH^{\cdot}$ is reduced by cytochrome $b_h$ of complex III in the respiratory chain near the inner membrane surface with regeneration of the initial $SkQH_2$:

$$SkQH_2^{\cdot} + \text{cytochrome } b_h^{2+} + H^+ \longrightarrow SkQH_2 + \text{cytochrome } b_h^{3+}. \qquad (2)$$

The fate of the fatty acid residue (LOOH)is unclear. Most likely, it is cleaved from cardiolipin and transferred from the inner to outer membrane leaflet as a fatty acid peroxide anion with subsequent cleavage with involvement of cytochrome c or specialized glutathione peroxidase, able to use lipid peroxides as a substrate (for review, see [42]). In this process, either minor uncoupling proteins (UCP2, UCP3, UCP4, or UCP5) [42] or again UCP can play the role of carriers. Our team (I.I. Severina, Yu.N. Antonenko, M.Yu. Vysokikh et al.) has recently demonstrated [103] that SkQ and its analog lacking plastoquinone ($C_{12}$TPP) can act as carriers of fatty acid anions in artificial and mitochondrial membranes. This additional *SkQ1* activity allows it to be regarded as a mitochondria-targeted uncoupler. It is noteworthy that unlike classical protonophores, such as dinitrophenol, SkQ must act as a mild uncoupler. Discharging $\Delta\psi$, SkQ prevents its accumulation in mitochondria; therefore, a more pronounced protonophore effect is expected only at high $\Delta\psi$ values. Such a mild uncoupling is the optimal mechanism that prevents mitochondrial ROS generation: a small decrease in $\Delta\psi$ drastically hinders the ROS generation yet does not interfere with ATP synthesis [61]. (See refs. [11] and [31] for data on life extension of Drosophila and mice and prevention of age-related oxidation of biopolymers with small doses of the uncoupler dinitrophenol.)

Summing up, we can conclude that SkQ is the most active of all the known geroprotectors decreasing the mortality rate at early and medium stages of aging and preventing development of a large set of senile pathologies. In other words, we believe that SkQ is able to extend youth by interfering with the age-dependent decrease in the number of functioning cells in organs and tissues.

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