Assessment of the Toxic and Protective Effects of Initiators and Inhibitors of Free Radical Reactions Using a Wild-Type Strain of *Escherichia coli* and a Strain Deficient for Superoxide Dismutase

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny,* Vol. 121, № 1, pp. 74-79, January, 1996
Original article submitted December 23, 1994

The toxic effects of H_2O_2, paraquat, and oxidized low density lipoproteins are more expressed on superoxide dismutase-deficient *E. coli* strains than on its wild-type strains, and the effect of tert-butyl-hydroperoxide is less dependent on the presence or absence of this enzyme in the bacterium, whereas that of bleomycin does not depend on it at all. The toxicity of the agents increases in the following series: H_2O_2<oxidized low density lipoproteins<tert-butyl-hydroperoxide<paraquat<<bleomycin. A culture of *E. coli* strains AB 1157 and JI 132 may be used for assessing the toxic effect of prooxidants, and an *E. coli* JI 132 culture with oxidative stress induced by prooxidants as a test system for detecting the potential antioxidants and assessing the mechanism of their action.

Key Words: *Escherichia coli culture; oxidative stress; hydrogen peroxide; paraquat; tert-butyl-hydroperoxide; low density lipoproteins; bleomycin; antioxidants; superoxide dismutase*

It is now evident that free radical reactions (FRR) appreciably contribute to the pathogenesis of many pathological states, including stress and shock of various etiology, irradiation, malignant growth, ischemia/reperfusion, etc. In view of this, the development of methods for the detection of potential initiators and inhibitors of FRR among new drugs and environmental pollutants is assuming greater and greater importance.

Cultures of *Escherichia coli* have been used for assessing the pro- and antioxidant activities of diverse compounds in recent years. Such methodology is preferable to other *in vitro* and *in vivo* methods, being simpler, more readily available, and rapid. The appearance of mutant strains of *E. coli* not containing the antioxidative enzymes superoxide dismutase (SOD), hydroxylase or glutathione and the use of a culture in the logarithmic phase of growth [9] greatly enhance the sensitivity of the method and extend the range of its applications.

We investigated the possibility of using wild-type and SOD-deficient strains of *E. coli* as a test system for detecting potential initiators and inhibitors of FRR, comparing their effects, and determining possible applications.

MATERIALS AND METHODS

Experiments were carried out with *E. coli* K-12 AB 1157 (wild-type) and JI 132 (SOD-deficient) strains. The mutant JI 132 strain does not contain Sod A and Sod B genes [5], and therefore does not produce Fe-SOD and Mn-SOD, but produces $O_2^{\bullet}$ 17 times more actively than the wild-type strain, without differing from it in other characteristics [1]. *E. coli* was grown

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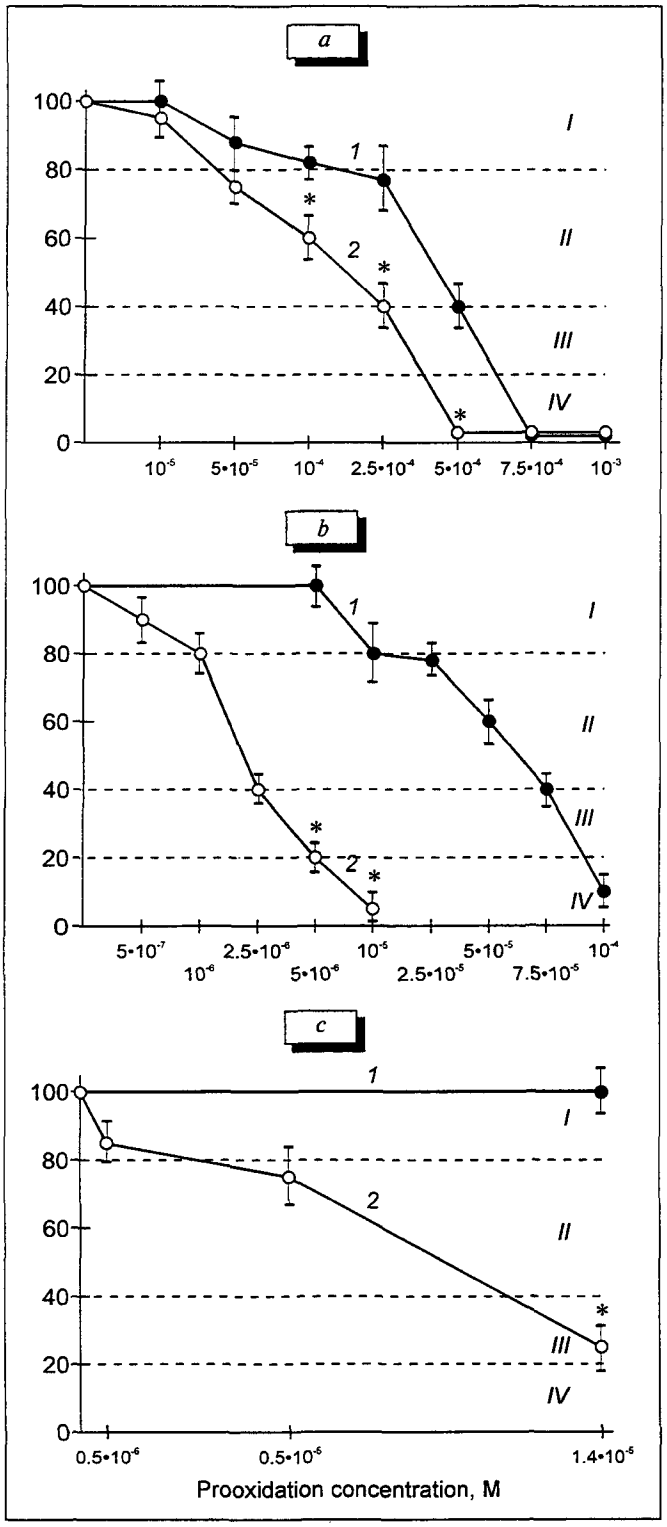

Fig. 1. Effect of H_2O_2 (*a*), PQ^{2+} (*b*), and oLDL (*c*) on the growth of wild-type (AB 1157, *1*) and SOD-deficient (JI 132, *2*) *E. coli* strains assessed after 6-hour incubation (*M±m*). Here and in Figs. 2 and 3: the ordinate gives the culture density (λ=600 nm), % of control. *$p<0.01$ between *1* and *2*. Here and in Fig. 3: are shown the intervals of toxic effect of prooxidants assessed from the delay of culture growth in experimental samples in comparison with control: *I*) no toxicity, culture density >80%; *II*) weak bacteriostatic effect, culture density >40<80%; *III*) moderate bacteriostatic effect, culture density >20<40%; *IV*) bacteriolytic (lethal) effect, culture density <20%.

for 18 h at 37°C in Luriya-Bertani's medium containing bactopeptone (10 g/liter), yeast extract (5 g/liter, Difco), and NaCl (10 g/liter); the culture was then diluted 1:9 with fresh medium, incubated for 1 h to transfer the culture to the logarithmic phase of growth, and then diluted again 1:100 with the medium [9]. The compounds of interest were incubated in conical flasks on a shaking water bath at 37°C for 6 h. The density of the growing culture was recorded every hour from absorption at λ=600 nm.

As FRR initiators we used H_2O_2, the antibiotic bleomycin (Latvian Institute of Organic Synthesis), paraquat (PQ^{2+}, Sigma), lipophilic organic tert-butylhydroperoxide (t-BuOOH, Aldrich), and native and oxidized low density lipoproteins (LDL and oLDL). LDL were isolated from human serum by preparative centrifugation [2] and dialyzed in Sigma sacks against 0.02 M phosphate buffer containing 8 g/liter NaCl, 0.2 g/liter KCl, 1.15 g/liter Na_2HPO_4, and 0.2 g/liter KH_2PO_4, pH 7.4, for 18 h at 4°C. LDL were oxidized in the absence of EDTA with 50 μM $CuSO_4$ at 37°C for 24 h. LDL oxidation was stopped by adding EDTA (1 mM) and cooling to 4°C. oLDL were assessed from the content of products reacting with 2-thiobarbituric aci; protein was estimated after Lowry.

As FRR inhibitors we used SOD (Sigma) and catalase (Boehringer Mannheim), ionol (BHT, Merck), the iron chelator o-phenanthroline (Sigma), and ethanol. Antioxidants were added to the *E. coli* incubation medium before the following prooxidants: PQ^{2+}; xanthine and xanthine oxidase (Sigma), and t-BuOOH. The experiments were repeated at least 3 to 5 times, and the results were statistically processed using Student's *t* test.

RESULTS

Incubation of an *E. coli* culture under aerobic conditions without adding any compounds (control) showed (Table 1) that the growth of strain JI 132 appreciably lagged behind the growth of AB 1157. The delay was most pronounced after 2-4 h of incubation (4-5-fold differences in culture densities) and was evidently due to the toxic effect of $O_2^{\bullet}$ forming in a SOD-deficient strain under aerobic conditions of incubation. The delay in the growth of a SOD-deficient *E. coli* strain under conditions of aerobic incubation was demonstrated previously [1,5], whereas under anaerobic conditions the growth of both strains was reported to be the same [5].

The effects of FRR initiators on the growth of *E. coli* AB 1157 and JI 132 are presented in Fig. 1. We see (Fig. 1, *a*) that H_2O_2 was nontoxic in the lowest concentrations (10^{-5} M) but highly toxic (lethal) for both strains in the highest concentrations (5-7.5×10^{-4} M). H_2O_2 in concentrations of 10^{-4} and 2.5×10^{-4} M exerted

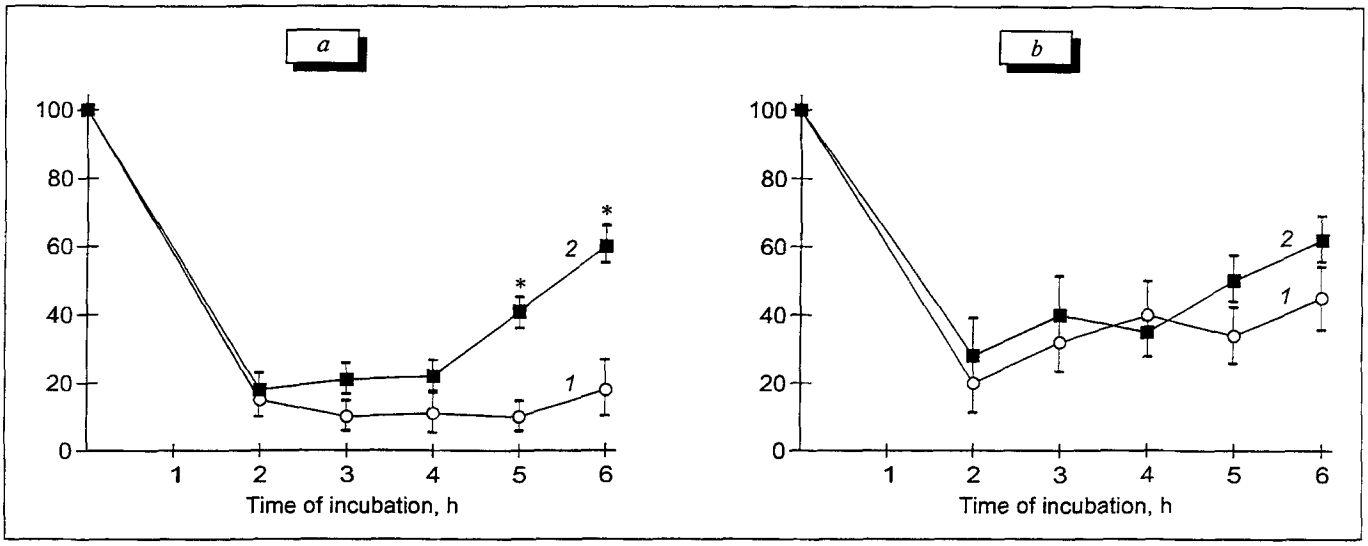

Fig. 2. Time course of growth of *E. coli* strains AB 1157 (*a*) and JI 132 (*b*) not adapted (*1*) and adapted (*2*) to H_2O_2 (*M*±*m*). *$p<0.01$ between *2* and *1*. Adaptation was attained by incubation of *E. coli* with H_2O_2 for 6 h, after which the culture was diluted with fresh medium (1:100) and incubated for 16 h. Second incubation with H_2O_2 was carried out as initially. The concentration of H_2O_2 was 5×10^{-4} M for AB 1157 and 2.5×10^{-4} M for JI 132 for the primary and secondary incubations. Cultures of both *E. coli* strains incubated once and twice without H_2O_2 were controls.

a 2-2.5 times stronger toxic effect on JI 132 in comparison with AB 1157 ($p<0.01$), while H_2O_2 in a concentration of 5×10^{-4} M was moderately toxic for AB 1157 and lethal for JI 132.

The decisive role of SOD in the different sensitivity of wild-type and SOD-deficient strains of *E. coli* to H_2O_2 also manifested itself in the ability of only strain AB 1157 to adapt to H_2O_2. This was detected by repeated incubation of the culture with a similar (Fig. 2, *a*) or ascending (data not presented) concentration of H_2O_2 in the medium. *E. coli* JI 132 did not possess the ability to adapt (Fig. 2, *b*). The ability of wild-type *E. coli* to adapt to H_2O_2 was demonstrated previously [6], as was its ability to adapt to PQ^{2+} [8], the adaptation being associated with an increase of SOD activity in the bacterium.

PQ^{2+} added to the incubation medium in concentrations higher than 2.5×10^{-6} M showed different toxicity toward wild-type and mutant *E. coli* strains (Fig. 1, *b*). It exerted a bacteriolytic (lethal) effect on JI 132 when added in a concentration of 10^{-5} M, but on AB 1157 only in a concentration of 10^{-4} M.

The toxicity of oLDL depended on the presence of SOD in *E. coli* (Fig. 1, *c*). They were nontoxic in a concentration of 0.5×10^{-6} M, weakly toxic in a concentration of 0.5×10^{-5} M, and moderately toxic in a concentration of 1.4×10^{-5} M, whereas the maximal concentration of oLDL used (1.4×10^{-5} M) was not toxic for AB 1157.

In contrast to H_2O_2, PQ^{2+}, and oLDL, t-BuOOH exerted just slightly different toxic effects on the different *E. coli* strains (Fig. 3, *a*); specifically, for JI 132 the dose of 2×10^{-5} M was lethal, whereas for AB 1157 the same effect required a double dose – 4×10^{-5} M.

Bleomycin, too, showed the same patterns of toxicity for both wild-type and SOD-deficient strains of *E. coli* (Fig. 3, *b*). It was nontoxic for both AB 1157 and JI 132 in concentrations of $0.1\text{-}0.2\times10^{-6}$, weakly or moderately toxic in concentrations of $0.4\text{-}0.8\times10^{-6}$ M, and bacteriolytic in concentrations of $1.6\text{-}6.5\times10^{-6}$ M. These results were somewhat unexpected, because the mechanism of toxic action of bleomycin is believed to be free-radical. It may be that the toxic effect of bleomycin is realized locally, only at the level

TABLE 1. Time Course of Wild-Type (AB 1157) and SOD-Deficient (JI 132) *E. coli* Strains under Aerobic Conditions of Incubation (Control Experiments, *M*±*m*)

E. coli strain	Number of experiments	Culture density (λ=600 nm)					
		duration of incubation, h					
		0	2	3	4	5	6
AB 1157	20	7±0	186±15	269±50	1513±72	2159±85	2589±121
JI 132	20	6±0	45±3*	123±9*	169±16*	960±29*	1361±44*

Note. *$p<0.01$ - reliable differences in the time course of growth of strains AB 1157 and JI 132.

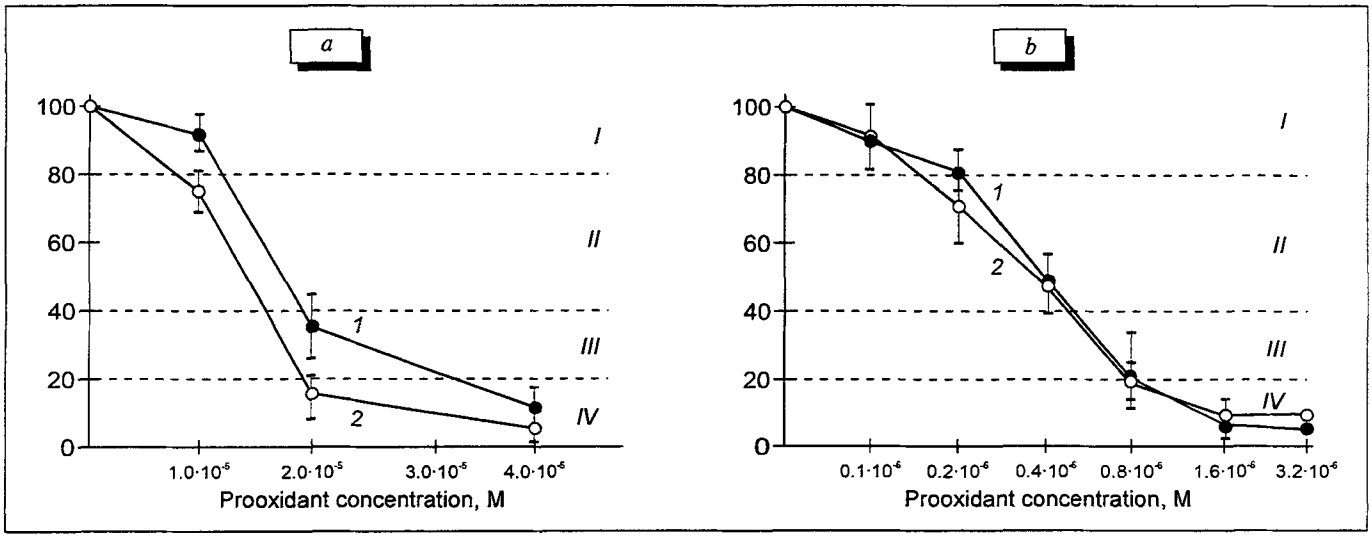

Fig. 3. Effect of t-BuOOH (*a*) and bleomycin (*b*) on the growth of cultures of wild-type (AB 1157, *1*) and SOD-deficient (JI 132, *2*) *E. coli* strains assessed after 6-hour incubation ($M \pm m$).

of DNA, causing its fragmentation and death of the cell (apoptosis) [3], or that the mechanism of its effect is different and is not realized through reactive oxygen species [12].

Comparison of the effects of the various prooxidants showed (Table 2) that their toxicities toward both *E. coli* strains increased in the following series: H_2O_2<oLDL<t-BuOOH<PQ^{2+}<<bleomycin. If we take the concentration of the most toxic drug (bleomycin) as 1, the concentrations of the drugs causing a bacteriolytic effect will approximately correspond to the following order: 300:X:10:5:1 for JI 132 and 450:X:25:50:1 for AB 1157, where X is the lethal concentration of oLDL for *E. coli,* which was not determined.

The high sensitivity of *E. coli* cultures to prooxidants becomes self-evident if we remember that PQ^{2+} in concentrations of 10^{-3}-10^{-5} M [4,15], t-BuOOH in concentrations of 0.25-1.0×10^{-3} M [7,11], and H_2O_2 in concentrations of 10^{-3}-10^{-5} [14,16] exert moderately toxic effects on mammalian tissues (cultures of endothelial cells, fibroblasts, lung slices, etc.). These concentrations are equal to or surpass the concentrations moderately toxic for *E. coli* AB 1157 and far surpass the concentrations for *E. coli* JI 132 (Table 2).

Study of the protective effect of the antioxidants carried out on 3 models of induced oxidative stress and on the most sensitive *E. coli* strain JI 132 showed (Table 3, only antioxidants with positive effects are shown) that with PQ^{2+}, catalase, and ethanol enhanced the growth of *E. coli,* whereas the effects of the other agents (ionol, o-phenanthroline, SOD) were negligible. With xanthine and xanthine oxidase, the protective effect manifested itself after the addition of catalase and SOD. The effect of ionol was weak, and that of o-phenanthroline and ethanol null. On the contrary, both ionol and o-phenanthroline exerted a protective effect in oxidative stress induced by t-BuOOH, in contrast to SOD and ethanol.

The data indicate that the protective effect of an antioxidant manifested itself mainly when the points of application of this effect and the prooxidant effect coincided. For example, in experiments with FRR initiation in the cell by intracellular (PQ^{2+}) [13] or extracellular (xanthine and xanthine oxidase) production of $O_2^{\bullet}$

TABLE 2. Comparison of Toxic Effects of Different Prooxidants on Cultures of Wild-Type (AB 1157) and SOD-Deficient (JI 132) Strains of *E. coli*

Prooxidant	Toxicity, concentration (M)							
	AB 1157				JI 132			
	none	weak	moderate	high	none	weak	moderate	high
Bleomycin	0.2×10^{-6}	0.8×10^{-6}	1.6×10^{-6}	3.2×10^{-6}	0.2×10^{-6}	0.8×10^{-6}	1.6×10^{-6}	3.2×10^{-6}
PQ^{2+}	10^{-5}	5×10^{-5}	7.5×10^{-5}	10^{-4}	10^{-6}	2.5×10^{-6}	5×10^{-6}	10^{-5}
t-BuOOH	10^{-5}	-	2.5×10^{-5}	4×10^{-5}	-	10^{-5}	-	2×10^{-5}
oLDL	1.4×10^{-5}	-	-	-	0.5×10^{-6}	0.5×10^{-5}	1.4×10^{-5}	-
H_2O_2	10^{-4}	2.5×10^{-4}	5×10^{-4}	7.5×10^{-4}	10^{-5}	10^{-4}	2.5×10^{-4}	5×10^{-4}

Note. See Fig. 1 for the assessment of the degree of toxic effect.

TABLE 3. Protective Effect of Some Antioxidants on Models of Prooxidant-Induced Oxidative Stress in *E. coli* Strain JI 132

Prooxidant	Antioxidant	Culture density (λ=600 nm) duration of incubation, h				
		2	3	4	5	6
PQ^{2+}*	Control	66	100	137	136	140
	Catalase	73	138	168	168	190
	Ethanol	68	94	113	159	170
Xanthine/xanthine oxidase	Control	67	180	435	1130	1416
	Catalase	192	475	1120	1480	1980
	SOD	105	265	650	1345	1828
t-BuOOH*	Control	59	73	170	285	544
	Ionol	41	98	189	397	720
	o-Phenanthroline	40	101	211	361	748

Note. *: a drastic inhibition of growth and a weak protective effect of antioxidants are caused by high prooxidant doses. Concentrations of pro- and antioxidants: PQ^{2+} - 2.5×10^{-5} M; xanthine - 10^{-3} M; xanthine oxidase - 4 ml/ml of medium; t-BuOOH - 2.5×10^{-5} M; catalase - 300 U/ml; SOD - 300 U/ml; ionol - 10^{-6} M; o-phenanthroline - 5×10^{-5} M; ethanol - 0.1%.

and H_2O_2 followed by the formation of •OH in Fenton and Haber-Weiss reactions, the antioxidative enzymes inactivating reactive oxygen species possessed protective effects. The toxic action of t-BuOOH is known to be realized by the formation of tBuO• and tBuOO• radicals in the presence of Fe (II) and Cu(I) [10]. These radicals trigger lipid peroxidation in respiratory membranes, glutathione peroxidase being the principal enzyme of respiratory chain detoxication [11]. Hence, the protective effect of ionol and o-phenanthroline, but not of antioxidant enzymes, becomes easy to understand.

The pronounced differences between the toxic effects of H_2O_2 and PQ^{2+} on *E. coli* wild-type and SOD-deficient strains (Fig. 1, *a*, *b*) are in good correlation with the protective effect of antioxidative enzymes on the models of paraquat- and xanthine/xanthine oxidase-induced stress, just as the slight differences in the toxic action of t-BuOOH on these strains of *E. coli* (Fig. 3, *a*) correlate with the absence of a protective effect of SOD and catalase on the model of t-BuOOH-induced oxidative stress.

Hence, a study of potential prooxidants in *E. coli* cultures elucidated their dose-dependent effect, enabled us to compare cytotoxicities, and revealed adaptogenic properties; simultaneous experiments with wild-type and SOD-deficient *E. coli* strains helped establish the point of application of the toxic effect of an agent of interest. Trials of potential antioxidants on various models of oxidative stress of *E. coli* bring to light not only their antioxidant characteristics, but also the mechanism of their action.

The authors are grateful to Prof. I. Fridovich and Dr. S. Liochev (Duke University, USA) for allowing us to study the methods of handling *E. coli* cultures and for *E. coli* strains AB 1157 and JI 132, and to E. I. Voshchinnikov from the Institute of Biomedical Chemistry, Russian Academy of Medical Sciences, for bleomycin.

The study was sponsored by the Foundation for Basic Research, Russian Academy of Sciences, 1994.

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