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Short communication

EVALUATION OF THE POTENTIAL OF ALKYLRESORCINOLS AS SUPEROXIDE ANION SCAVENGERS AND SOX-REGULON MODULATORS USING NITROBLUE TETRAZOLIUM AND BIOLUMINESCENT CELL-BASED ASSAYS

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Abstract: The antioxidant activities of five alkylresorcinol (AR) homologs with alkyl chains of 1, 3, 5 6 and 12 carbon atoms were studied using molecular and cellular assays for superoxide anions ($O_2^{\cdot-}$). The effect of ARs as superoxide anion scavengers was assessed using the photochemical reaction of spontaneous photo-reduced flavin re-oxidation. In this system, ARs reaction with $O_2^{\cdot-}$ produced dye derivatives, as C_6- and C_{12}-AR prevented the $O_2^{\cdot-}$-induced conversion of nitroblue tetrazolium into formazan in AR-containing mixtures. The influence of ARs on *soxS* gene expression and bacterial cell viability was studied with the luminescent *Escherichia coli* K12 MG1655 *psoxS'::luxCDABE*-Amp^R strain, showing low basal light emission. This increased significantly during paraquat-induced oxidative stress as a consequence of the simultaneous transcription of *soxS*-gene and *lux*-gene fusion. ARs with alkyl chains containing 5–12 carbon atoms at concentrations of 0.1–1.0 μM weakly induced *soxS*-gene expression, whereas 1–10 mM repressed it. This respectively increased or decreased the bacterial cell resistance to $O_2^{\cdot-}$-related oxidative stress. AR derivatives lost their protective activity from reactions with superoxide anions, which required increased *soxS* gene expression for cell viability. These results show the dual nature of ARs, which possess direct antioxidant properties and the ability to indirectly regulate the activity of cellular antioxidative defense mechanisms.

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Abbreviations used: A – absorbance, AR – alkylresorcinol, C_1-AR – methylbenzenediol, C_3-AR – propylbenzenediol, C_5-AR – pentylbenzenediol, C_6-AR – hexylbenzenediol, C_{12}-AR – dodecylbenzenediol, CFU – colony-forming units, Eh – redox potential, Fz – formazan, NBT – nitroblue tetrazolium, $O_2^{\cdot-}$ – superoxide anion, OD – optical density, RLU – relative light units, ROS – reactive oxygen species, PQ – paraquat, SOD – superoxide dismutase, TEMED – tetramethylethanediamine

Keywords: Alkylresorcinols, Reactive oxygen species, Superoxide anion, *soxS* gene, Reporter *luxCDABE* gene, Bioluminescence

INTRODUCTION

Reactive oxygen species (ROS) are a consequence of free oxygen accumulating in the Earth's ecosystem after the occurrence of oxygenic photosynthesis. They are oxygen ions, free radicals and peroxides with an unpaired electron in their outer electron level, which confers high ROS reactivity with cell membranes, proteins and DNA [1]. The initial ROS is the superoxide anion ($O_2^{\cdot-}$), which result from the one-electron transfer to an oxygen molecule.

ROS toxicity induces cell defense mechanisms, including the production of low-molecular weight antioxidants and antioxidant enzymes [2, 3]. The destruction of superoxide anions is carried out by specialized metalloenzymes called superoxide dismutases (SOD), which are characterized by various metals in their active center: Mn, Fe or Cu/Zn [4], and more rarely Ni [5]. In *Escherichia coli,* SOD-Mn expression is controlled by *soxRS* genes [6–8], which encode the SoxR receptor. The SoxR receptor interacts with $O_2^{\cdot-}$ and activates the SoxS regulatory protein, which in turn induces transcription of the *sox*-regulon with various effectors of the antioxidant response. Scavengers of superoxide anions are another mechanism of cell defense associated with molecular antioxidants. These scavengers include amino acids (lysine), ascorbic acid [9], and components of extracellular exopolysaccharide [10].

Natural antioxidants include phenolic compounds with one (phenyls, phenolic alcohols, phenolic acids) or two (flavonoids) aromatic rings, and polyphenols [11, 12]. Antioxidant activity has also been shown for phenolic lipids such as alkylresorcinols (ARs), which are typically present in some bacteria, fungi and higher plants [13, 14]. The ability of these molecules to inactivate ROS is determined by hydrogen donations, and their antioxidant mechanism involves the ability to scavenge radicals by a hydrogen atom. This leads to the formation of an intermediate, 1,2,4-trihydroxy-6-alkylbenzene, which in turn easily converts to *o*- and *p*-quinones [15].

The hydrocarbon chain length is also important, especially for preventing membrane lipid oxidation [16]. Early studies of AR antioxidant activity showed the prevention of induced Fe^{2+} and H_2O_2 peroxidation or lipid autoxidation [17]. Subsequently, these mechanisms were enhanced by ARs chelating the binding of mixed-valence transition metal ions [18], and the inhibition of pro-oxidant enzymes [19]. At the same time, the role of ARs in the control of *soxS* gene activity is unexplored despite evidence of a number effects on other stress response genes [20, 21].

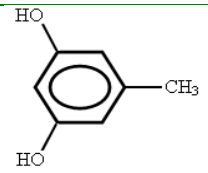
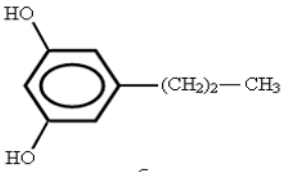
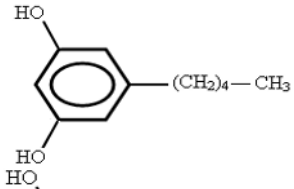
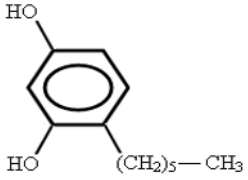
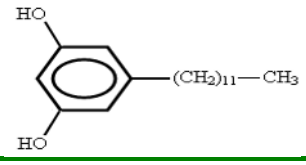
The aim of this paper is a comparative study of the antioxidant activity mechanisms of some alkylresorcinols against superoxide anions. The study included an assessment of the scavenger and regulatory effects using molecular and cellular assays.

MATERIALS AND METHODS

Alkylresorcinols

The AR analogs orcinol (5-methylbenzene-1,3-diol; C_1-AR), olivetol (5-pentylbenzene-1,3-diol; C_5-AR) and caprokol (4-hexylbenzene-1,3-diol; C_6-AR), which differ in the length and location of the hydrophobic alkyl radical, were purchased from Sigma-Aldrich. Divarin (5-propylbenzene-1,3-diol; C_3-AR) and 5-n-dodecyl resorcinol (5-dodecylbenzene-1,3-diol; C_{12}-AR) were synthesized de novo according to standard organic procedures and purified to 99% homogeneity by Enamine Ltd. The same natural phenolic organic compounds occur in certain species of bacteria [22] and lichen [23], and they can be used as topical pharmaceuticals or food additives. The chemical structures and molecular weights of these compounds are listed in Table 1. The compounds were dissolved in aqueous solutions and biologically relevant through to extremely high concentrations were used to identify their full range of scavenging and regulatory effects.

Table 1. Alkylresorcinol chemical homologs used in this study

Abbreviation	Structural formula	Mol. weight	CAS No.	Manufacturer
C_1-AR		124	504-15-4	Sigma
C_3-AR		152	500-49-2	Enamine
C_5-AR		180	500-66-3	Sigma
C_6-AR		194	136-77-6	Sigma
C_{12}-AR		278	-	Enamine

In vitro system for superoxide anion generation and detection

We used photochemical induction of superoxide anions, based on the spontaneous re-oxidation of photoreduced flavin [24]. The reaction system included 100 µl of 0.05 mM riboflavin solution and 5 µl of 1 mM N,N,N,N-tetramethylethane-1,2-diamine (TEMED) solution in 0.2 M phosphate buffer (pH 7.8). The irradiation of this system by visible light with 20 W power sources from a 10-cm distance at a temperature of 20 ± 1ºC led to the generation of superoxide anions.

The quantitative yield of $O_2^{\cdot-}$ was assessed by conversion of nitroblue tetrazolium (NBT) [25] that had been previously introduced into the system (10 µl; 1 mM) to the reduced dye product formazan, which has a maximum absorption (A) of $\lambda = 560$ nm. Analysis of the "exposure-effect" dependencies showed that there was an optimal 5-min photo-induction period to provide linearity of data. An A_{560} value of about 1.0 was given.

The antioxidant activity of ARs was evaluated in two variants: 1) in the system of the superoxide anion photochemical induction supplemented with ARs only as a dye product yield with a maximum absorption at $\lambda = 465$ nm (see below); and 2) in mixtures containing both ARs and NBT as prevention of product yield with a maximum absorption at $\lambda = 560$ nm.

Redox potential measurement

The redox potential of ARs and NBT at a concentration of 1 mM was measured in single-component, degasified, aqueous solutions at a temperature of 20 ± 1ºC, using ESL-43-07 and chlorine–silver reference electrodes and precision pH/ion meter Expert-001-3(0.1), according to the manufacturer's instructions.

Whole-cell lux-biosensor for detection of $O_2^{\cdot-}$-induced oxidative stress

We used the *E. coli* K12 MG1655 strain carrying the recombinant *psoxS'::lux*-Amp^R plasmid, containing a transcriptional fusion of host *soxS* promoters to a truncated *Photorhabdus luminescens* Zm 1 *luxCDABE* operon [26]. This strain has low basal light emission, which increased significantly during the oxidative stress response against superoxide anions as a consequence of the simultaneous transcription of *soxS* gene and *lux* gene fusion.

The strain was cultivated for 16–18 h at 37ºC on LB-broth (Sigma) supplemented with 20 µg/ml ampicillin. Before the experiment, the strain was diluted 1:20 in fresh nutrient medium and incubated for 3–5 h until early log-phase ($OD_{640} = 0.2$). This procedure made it possible to investigate the induction of *sox* regulon, involving the σ^{70} (RpoD) sigma factor that transcribes both genes of bacterial growth and of the oxidative stress response.

Bioluminescent cell-based assay

In an experimental series, the *E. coli psoxS'::lux*-Amp^R strain was incubated for 60 min with ARs at concentrations of 1 µM, 10 µM, 0.1 mM and 1 mM, and with the solvent (control), and then stressed with intracellular $O_2^{\cdot-}$ inductor. The $O_2^{\cdot-}$-reacted AR derivatives previously produced in the photochemical system

were analyzed in the same manner. The induction of oxidative stress in *E. coli* cells was caused by paraquat (PQ) – 1,1'-dimethyl-4,4'-bipyridinium dichloride (Sigma) at a final concentration of 4 mM.

Bioluminescence measurements were carried out using an LM-01T microplate luminometer (Immunotech), which dynamically registered the luminescence intensity of the samples, estimated in relative light units (RLU). To quantify the bioluminescence induction, we used the algorithm $F_i=(I_{60}/I_0)/N_B$, where I_{60} and I_0 are RLU values 60 and 0 min after the induction of oxidative stress; and N_B is the proportion of living cells against the control. To determine N_B, 10 µl-aliquots derived from control and experimental samples were transferred onto the LB-agar surface with ampicillin and harvested 24 h later at 37ºC so that the colony-forming units (CFUs) could be calculated.

Statistical analysis

Data are reported as ± standard errors of the mean of determinations performed in triplicate on three different samples. They were analyzed using the Student's t-test and p values of 0.05 or less were considered significant. Calculations were performed with Statistica V8 for Windows (StatSoft Inc.).

RESULTS

AR antioxidant activity in superoxide anion photochemical reactions

The mixtures of riboflavin, TEMED and AR mixtures gave dye derivatives with maximum absorptions of λ = 465 nm (Fig. 1A). That provided evidence of the ARs as final acceptors for electrons transferred from superoxide anions. Moreover, this experimental series shows the dependence of final derivative A_{465} values on the original AR fine structure. Although small differences within groups (C_1-, C_3-, C_5-) and (C_6-, C_{12}-) were reported, it was between the groups that significant differences were shown, as increased values consistently changed from C_1-AR to C_{12}-AR (Fig. 1B), which displays the role of the alkyl radical length in antioxidant activity manifestation.

Another result that was significant to the current experimental context was the weak absorption of $O_2^{\cdot-}$-reacted derivatives at λ = 560 nm ($A_{560} \leq 0.05$). These differences between the absorption spectra of formazan and AR derivatives gave the possibility to study the oxidative reaction in poly-component mixtures, where riboflavin and TEMED compounds provided photo-induced superoxide anion production, and the antioxidant activity of ARs was detected at λ = 560 nm, which prevented NBT-to-formazan conversion.

In the experiments where both ARs and NBT were competing for superoxide anion interaction, C_6-AR in concentrations $\geq$ 10 µM and C_{12}-AR in concentrations $\geq$ 1 µM showed detectable antioxidant activity. It was characterized by inhibition of the NBT-to-formazan conversion by 10% or more (Fig. 1C). At the maximum effect, C_6-AR and C_{12}-AR respectively decreased the A_{560} values to 87 ± 8% and 89 ± 6% ($p < 0.05$) in comparison with the control.

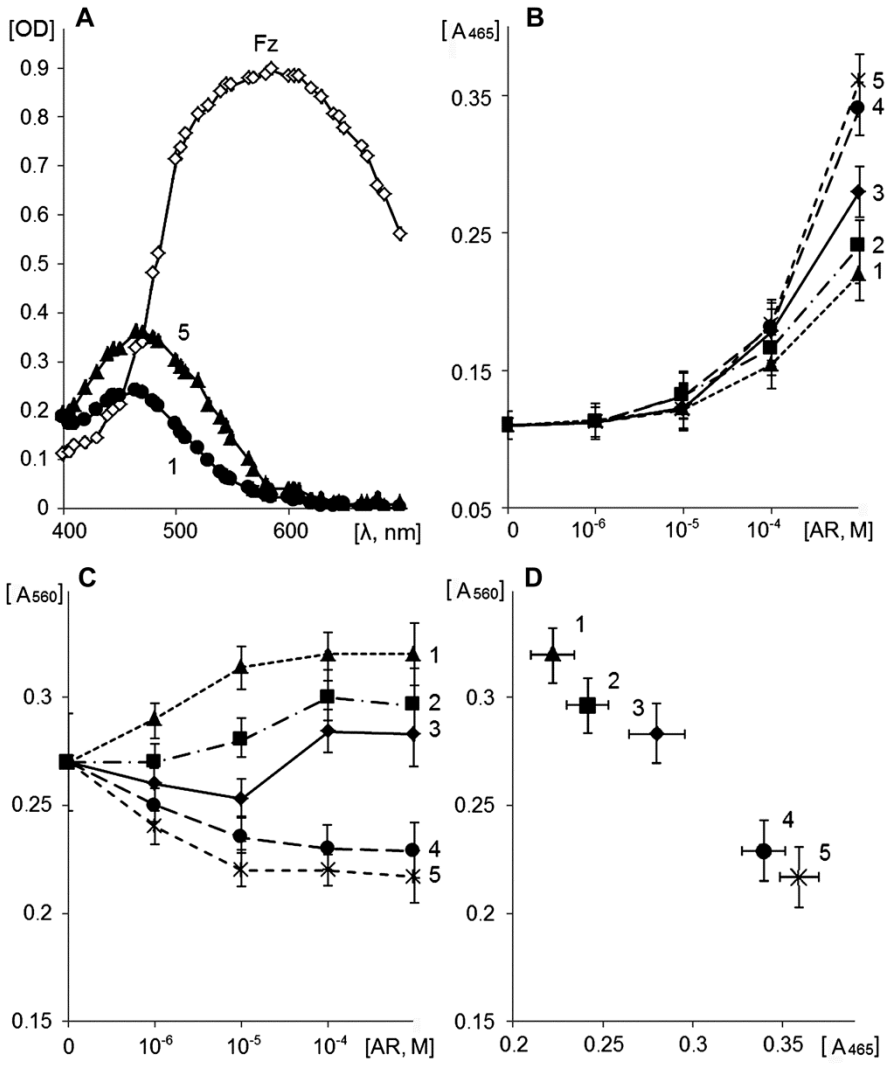


Fig. 1. Antioxidant activity of alkylresorcinols in superoxide anion photochemical reactions. A – Absorption spectra of formazan (Fz) as C_1- (1) and C_{12}-AR (5) $O_2^{\cdot-}$ reacted derivatives. B – A_{465} values of C_1- (1), C_3- (2), C_5- (3), C_6- (4) and C_{12}-AR (5) $O_2^{\cdot-}$ reacted derivatives. C – A_{560} values in riboflavin+TEMED+AR+NBT mixtures. D – the interdependence between A_{465} values in the system of superoxide anion generation in the presence of ARs, and the A_{560} values in the system with the simultaneous presence of NBT and ARs at a concentration of 1 mM. Legend: abscissa – wavelength, nm (A); concentration of ARs, M (B, C); A_{465} values (D); ordinate – absorbancy, relative unit (A); A_{465} values (B); A_{560}, values (C, D).

On the other hand, ARs with 5 and less carbon atoms in the chain did not show the same activity. C_1-AR even increased formazan production. At the maximum effect (in concentration of 1 mM), production exceeded the control by 19 ± 1.4% ($p < 0.05$). Thus, the evaluated differences between C_1-, C_3-, C_5-AR and C_6-, C_{12}-AR show the second group as strong antioxidants that act like superoxide anion scavengers.

Interestingly, the comparative analysis of the superoxide anion generation system with ARs only and with both AR and NBT (Fig. 1D) showed an inverse correlation dependence between the A_{465} and A_{560} values ($r = –0.987$; $p < 0.01$). This result led to two interdependent conclusions: (i) the high reactivity of individual AR homologs to $O_2^{\cdot-}$ increased the NBT protection in the polycomponent mixtures; (ii) the affinity of ARs to $O_2^{\cdot-}$ increases with increasing alkyl radical length in these molecules. In the current experimental system, these effects can be explained by differences in the oxidation-reduction (redox) values, which increased from Eh = 41.3 mV (C_1-AR) to Eh = 82.0 mV (C_6-AR) and Eh = 86.0 mV (C_{12}-AR). They were significantly higher than those for NBT (Eh = 12.4 mV).

AR activity in the whole-cellular $O_2^{\cdot-}$-related stress model

In the control experiment, the *E. coli psoxS'::luxCDABE*-Amp^R recombinant strain showed an intensive bioluminescent response to superoxide anion-related oxidative stress as a result of the synchronous induction of the natural *sox* regulon and reporter *luxCDABE* genes transcribed under the *soxS* promoter. It was modeled by PQ at a concentration of 4 mM, causing the transfer of electrons from respiratory chains on molecular oxygen with the formation of intracellular $O_2^{\cdot-}$, which led to a more than 100-fold increase in bioluminescence (F_i = 109.9 ± 9.7).

Preliminary incubation of *E.coli psoxS'::luxCDABE*-Amp^R with alkylresorcinols changed the number of colony-forming bacterial cells and the level of their luminescence, which was nonlinear at the concentration used and dependent on the fine structure of AR molecules. The C_6-AR and C_{12}-AR homologs at concentrations of 1 and 10 μM did not affect cell viability and weakly induced the *soxS* gene with F_i values from 1.2 ± 0.2 to 1.3 ± 0.1, respectively. Increasing C_5-, C_6- and C_{12}-AR concentrations to 0.1 mM led to a combined decrease both in the number of living bacterial cells and bioluminescence, which approached zero values at maximum concentrations of 1 mM. On the other hand, C_1- and C_3-AR homologs at low concentrations did not induce bioluminescence, and at high ones decreased light production by 30–35% against the control without a detectable effect on bacterial cell viability. The obtained data was important for following the assessment of *E. coli psoxS'::luxCDABE*-Amp^R viability and bioluminescence under oxidative stress conditions modeled after AR pre-treatment (Fig. 2), and correct quantification of induction factor F_i values (Table 2).

The luminescence measurement of AR-pretreated bacterial cells stressed with PQ showed trends similar to the original AR effects. The most common AR activity at concentrations of 1–10 μM was slightly increased *E. coli*

psoxS'::luxCDABE-Amp^R bioluminescence. This may have resulted from additive *soxS* gene stimulation by $O_2^{\cdot-}$ and ARs that had progressed in the C_1-AR → C_{12}-AR range (Fig. 2A). A study of the bacterial viability after pretreatment with 1–10 μM concentrations and following PQ-induced oxidative stress showed some increase in the CFUs (Fig. 2C), as determined by both direct AR antioxidant activity and stimulated *soxS* gene expression.

Table 2. The influence of ARS on the bioluminescence induction index F_i, showing *soxS* gene expression in *E.coli psoxS'::luxCDABE*-Amp^R cells under various conditions.

The test compound	Concentration, M	Induction regulon *soxS* factor (F_i) in the presence of AR	Induction regulon *soxS* factor (F_i) in the presence of oxidized AR
C_1-AR	10^{-6}	109.8 ± 9.3	116.8 ± 9.1
	10^{-5}	101.5 ± 10.2	107.6 ± 8.9
	10^{-4}	59.0 ± 4.5*	82.7 ± 7.6
	10^{-3}	44.9 ± 5.4**	68.7 ± 6.7*
C_3-AR	10^{-6}	114.6 ± 10.5	115.6 ± 9.8
	10^{-5}	105.7 ± 9.9	110.6 ± 10.1
	10^{-4}	72.3 ± 6.7*	85.8 ± 8.5
	10^{-3}	42.7 ± 6.5**	71.9 ± 8.0*
C_5-AR	10^{-6}	116.6 ± 9.8	128.6 ± 11.2
	10^{-5}	112.5 ± 11.3	128.0 ± 10.0
	10^{-4}	89.9 ± 9.6	103.9 ± 9.4
	10^{-3}	9.0 ± 0.7**	13.5 ± 0.9**
C_6-AR	10^{-6}	119.9 ± 11.8	143.9 ± 11.0
	10^{-5}	115.3 ± 10.6	145.0 ± 9.6*
	10^{-4}	104.5 ± 9.0	126.5 ± 9.9
	10^{-3}	1.0 ± 0.2**	1.1 ± 0.1**
C_{12}-AR	10^{-6}	114.3 ± 13.6	146.2 ± 10.1*
	10^{-5}	116.6 ± 9.9	151.6 ± 10.3*
	10^{-4}	107.5 ± 10.7	122.9 ± 9.9
	10^{-3}	1.9 ± 0.2**	2.0 ± 0.3**
Control		109.9 ± 9.7	

*$p < 0.05$; **$p < 0.01$.

The increased AR concentrations suppressed the bioluminescence, which was interdependent on the residual colony-forming cell numbers that were ambiguous. Therefore, C_5-, C_6- and C_{12}-AR at concentrations of 0.1 mM decreased the luminescence values two-fold, and at concentrations of 1 mM led to zero level light emission (Fig. 2A). Another effect of pretreating cells at concentrations of 0.1–1 mM was the reduced cell viability under subsequent oxidative stress, which progressed in the range of C_5-AR → C_6-AR → C_{12}-AR (Fig. 2C). Based on this data, F_i quantification showed the repression of *sox* gene transcription by ARs with alkyl chains consisting of 5–12 carbon atoms at high concentrations (F_i values from 1.0 ± 0.2 to 9.0 ± 0.7 against F_i = 109.96 ± 9.7 in control; $p < 0.01$), which explained the increased sensitivity of the bacterial

cells to $O_2^{\cdot-}$-mediated oxidative stress. On the other hand, C_1-AR and C_3-AR at concentrations of 0.1–1 mM repressed *soxS*-controlled bioluminescence moderately, but paradoxically increased bacterial cell resistance. Therefore, at a maximum concentration of 1 mM, the ARs with alkyl chains of 1–3 carbon atoms kept alive at 16.1–40.9% more bacterial cells against the control values (without preliminary AR treatment but with PQ-induced oxidative stress).

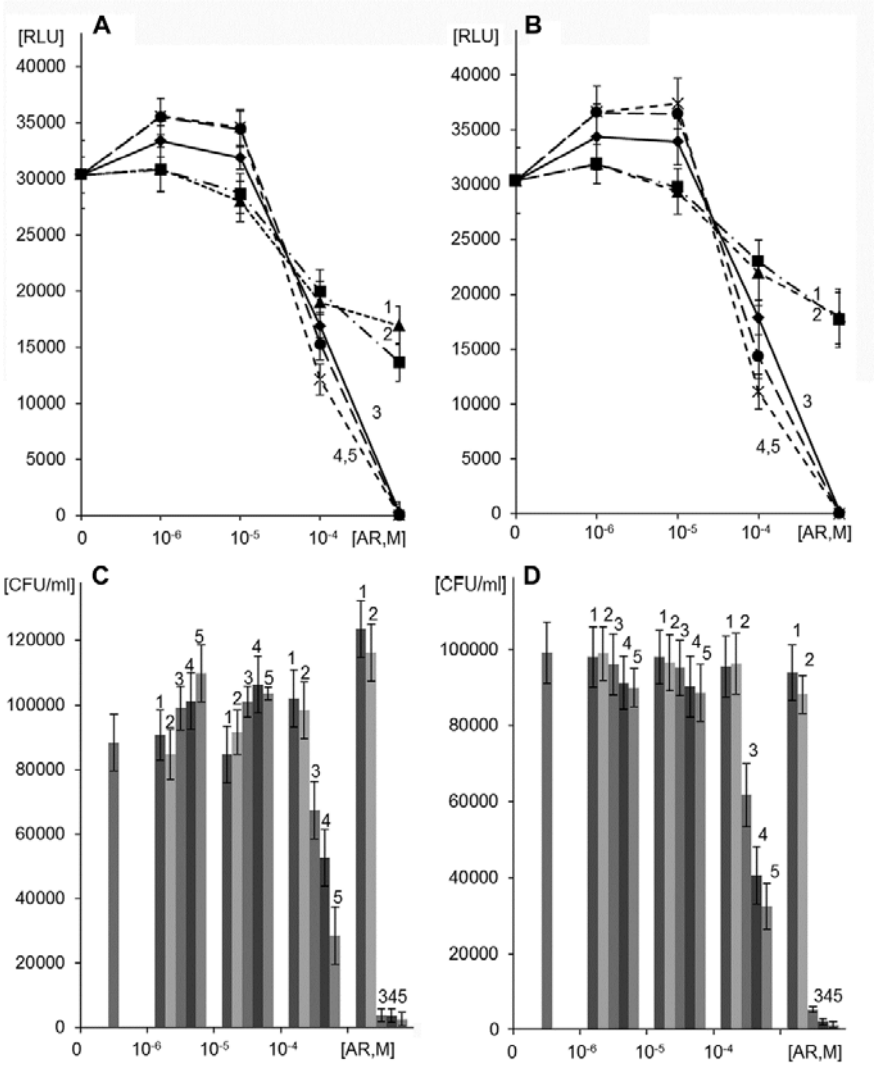


Fig. 2. The influence of $O_2^{\cdot-}$-related oxidative stress on the bioluminescence (A, B) and growth ability (C, D) of the *E.coli psoxS'::luxCDABE*-Amp^R strain, pre-incubated with intact ARs (A, C) and with $O_2^{\cdot-}$-reacted AR derivatives (B, D). Legend: abscissa – ARs concentration, M; ordinate – the intensity of bioluminescence, RLU (A, B); the number of viable cells, CFU/ml (C, D).

The results show that combined AR activity developed as superoxide anion scavenging and as *soxS* gene modulation. However, the ratios of these effects (induction/inhibition) were non-linear in a dose-dependent manner and varied according to the ARs homologs with different alkyl chain length.

The activity of $O_2^{\cdot-}$-reacted AR derivatives in the cell-based oxidative stress model

Prior to the experiment, the AR individual homologs at concentrations of 1 mM were placed into the riboflavin+TEMED system and irradiated with visible light to produce dye derivatives with a maximum absorption at λ = 465 nm (as described above). This reaction led to the depletion of the scavenging potential of ARs but the retention of *soxS* gene expression regulation.

Pretreatment of *E. coli psoxS'::luxCDABE*-Amp^R with $O_2^{\cdot-}$-reacted AR derivatives, as opposed to intact ARs, changed the measured parameters after PQ-induced oxidative stress. This result included the loss of the protective effect of C_5-, C_6- and C_{12}-AR derivatives at low concentrations (1–10 μM) for cell viability, and the disappearance of the protective effect of C_1-AR and C_3-AR derivatives at high concentrations (0.1–1.0 mM). Simultaneously, more contrast dose-dependent modulation of bioluminescence was observed (Fig. 2B). Since PQ-inducible oxidative stress in bacterial cells treated with AR derivatives led to a significant decrease of CFUs in comparison with intact ARs (Fig. 2D), the quantifications based on these data showed higher values of induction factor F_i (Table 2). Therefore, the AR derivatives with alkyl chains of 5–12 carbon atoms at concentrations of 1–10 μM increased the F_i from 145.0 ± 9.6 to 151.6 ± 10.3 compared to an F_i of 109.9 ± 9.7 in the control ($p < 0.05$), which was 1.2–1.3-fold higher than similar values for the intact ARs. On the other hand, AR derivatives with an alkyl chain of 1–3 carbon atoms at concentrations of at least 0.1 μM resulted in F_i values that were 1.2–1.4 times less than those of intact ARs. Thus, in the absence of AR scavenging effects, cell viability under oxidative stress demanded a more expressive *soxS*-regulated antioxidant response.

DISCUSSION

The antioxidant activity of alkylresorcinols has been the subject of numerous studies [16–19, 23, 27–32]. The ARs are involved in the suppression of oxidation reactions induced by mixed-valence transition metal ions, ROS scavenging, and inhibition of pro-oxidant enzymes, while the influence of ARs on the expression of antioxidant response genes has not yet been studied. This prompted an integral study of AR antioxidant activity, including direct (scavenging) and indirect (regulatory) effects. These effects were evaluated in this study using molecular and cell-based assays of superoxide anion generation and detection.

The first principal result was the confirmation of AR participation in the non-enzymatic antioxidant defense system, proof of which included: (i) the production of AR derivatives with a maximum absorption of λ = 465 nm through

reaction with superoxide anions; (ii) the prevention of superoxide anion-induced conversion of nitroblue tetrazolium into formazan in AR-containing mixtures; and (iii) the well-coordinated growth of both activities through increases in the alkyl radical length in AR molecules, correlating with an increase in redox potential values.

Thus, the obtained data confirm the role of the alkyl chain length in providing AR antioxidant effect. However, in addition to the previously described effect in lipid membranes [28, 29], it now can be achieved in aqueous solution. We believe that the fundamental nature of the $O_2^{\cdot-}$ reactivity for different AR homologs is identical [13], and only quantitative differences are influenced by the alkyl chain length. In part, the electron-density distribution in the reacting resorcinol ring affects the hydrogen-donation possibilities, which may be determined by the influence of the alkyl chain [30].

The second principal result was the influence of the ARs on bacterial cell viability under paraquat-induced oxidative stress. This effect manifested differently in various AR homologs and developed in a non-linear concentration-dependent manner with ambiguous association of *soxS* gene induction/inhibition. Low concentrations of ARs with alkyl chains of 5–12 carbon atoms weakly activated *soxS* gene transcription, which suggests that the ARs act as potential inducers of a wide range of stress reactions [21], pre-adapting bacterial cells to subsequent environmental stresses. In our experiments, the combination of the stress-inducing activity with scavenging activity of C_5-, C_6- and C_{12}-AR led to increased resistance of bacterial cells to $O_2^{\cdot-}$-related oxidative stress.

On the other hand, high concentrations of these ARs homologs caused the repression of *soxS* gene expression and simultaneously decreased the resistance to oxidative stress. Against this background, ARs with alkyl chain of 1–3 carbon atoms at low concentrations had no effect on *soxS* gene regulation, whereas at high concentrations they repressed it moderately, but paradoxically increased bacterial cell viability. In our opinion, this phenomenon can be explained by scavenger effects of these ARs homologs against the "late" ROS – hydrogen peroxide and hydroxyl-radical [31, 32], which interfere with the enzymatic antioxidant mechanisms and lead to bacterial cell survival without *soxS* gene induction. In this context, the presence of ARs in natural systems as isomers and homolog mixtures [13, 14] became clear due to the additive activity of various in structure molecules against various reactive oxygen species.

A special opportunity to compare AR activity mechanisms under $O_2^{\cdot-}$-related oxidative stress was provided by their derivatives, which lose their scavenging potential when produced in a superoxide anion generation system. The main action of $O_2^{\cdot-}$-reacted AR derivatives was the strengthening of *soxS* gene induction, which mobilized the antioxidant enzymatic response but was not comparable with the protective effect of intact ARs.

In summary, these results indicate a dual nature for ARs, which possess direct antioxidant properties and the ability to indirectly regulate the activity of cellular antioxidative defense mechanisms. We have also provided evidence of the

regulatory activity of ARs supporting their $O_2^{\cdot-}$ scavenging activity, whereas regulatory effects on the active-stress protection mechanisms are limited to some concentration ranges of individual AR homologs or to situations in which the scavenging potential is depleted.

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