Combination of Methods for *in Vitro* Study of Antioxidant Properties of Chemical Compounds

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We proposed a combination of methods to study antioxidant properties of compounds, including evaluation of the capacity of the test preparations to inhibit peroxidation of unsaturated lipids (in model systems with oxidation of ethyl oleate; aqueous emulsion medium) and low-density lipoproteins (in the presence of transition metal ions), generation of superoxide anion radical (system of lucigenin, xanthine oxidase, and xanthine) and $NO^{\cdot}/ONOO^{-}$ (system of SIN-1 and lucigenin), and induction of respiratory burst in blood granulocytes (luminol-induced and lucigenin-induced reaction after zymosan stimulation). *In vitro* study showed that the antioxidant properties of synthetic water-soluble phenols depend strongly on masking of the phenol OH group and nature of the ionogenic fragment in the *p*-propyl substituent.

Key Words: *antioxidants; combination of methods; water-soluble phenols*

Generation of reactive oxygen species (ROS) with prooxidant properties is an essential component of normal aerobic life. This process is balanced by deactivation of prooxidants by endogenous and exogenous antioxidants. Prooxidant/antioxidant imbalance leads to the development of oxidative stress, which accompanies more than 100 pathological processes and diseases [5]. The appropriateness of total antioxidant therapy is under discussion. However, the necessity of antioxidant treatment is beyond doubt. The possibility of using natural substances for reducing the severity of oxidative stress is now extensively studied. In parallel, the search for new compounds with antiradical activity is in progress.

Studying the potential antioxidant activity (AOA) of chemical compounds and pharmacological agents should take into account that this parameter depends on the medium and conditions of functioning. For example, mitochondrial ubiquinone can play a role of both antioxidant and prooxidant [7]. Transition metal ions can inhibit (oxidized state) or activate (reduced state) lipid peroxidation in erythrocyte membranes [1]. Under certain conditions, ROS ($NO^{\cdot}$ and O_2^{-}) have an inhibitory effect on free radical oxidation [6]. Studying the AOA of test compound should include complete evaluation of its properties under experimental conditions that modulate ROS generation *in vitro*.

We proposed a combination of methods for studying the capacity of chemical compounds and pharmacological agents to inhibit peroxidation of unsaturated lipids, superoxide anion radical, $NO^{\cdot}/ONOO^{-}$, and respiratory burst in granulocytes. The method was used to perform a quantitative structure—activity relationship (QSAR) study for antioxidant properties of synthetic water-soluble compounds from bromine-substituted alkyl phenols.

MATERIALS AND METHODS

S-Alkyl thiosulfonates and S-alkyl sulfonates were obtained during the interaction of halogen alkanes

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with thiosulfate and sodium sulfite, respectively, in an aqueous alcohol solution (Fig. 1). Potassium phenosan (3-(3',5'-di-tert-butyl-4'-hydroxyphenyl)-propionate) served as the reference preparation.

Oxidation of ethyl oleate in an aqueous emulsion medium was performed in the presence of catalytic additives (Cu II) at 60°C. The effectiveness of the test preparation was evaluated from the induction period (τ). This parameter was estimated graphically as an intersection point of 2 tangents to the kinetic curve. The slope ratio of these tangents was 0.5 and 0.75 of the slope ratio of the uninhibited reaction curve.

Low-density lipoproteins (LDL) were isolated from blood plasma of donors by sequential ultracentrifugation in a KBr density gradient [3]. The effectiveness of the test preparation was evaluated from accumulation of thiobarbituric acid-reactive substances (fluorescence assay) [9] during oxidation of LDL in the presence of 5 mM $CuSO_4$ or 25 mM $FeSO_4$ at 37°C for 30 min.

The ability of preparations to inhibit O_2^- was estimated from chemiluminescence (CL) quenching in a system of lucigenin (10 mM), xanthine oxidase (5×10^{-3} U/ml), and xanthine (50 mM) at 37°C [4]. The test compound was added 7 min after the start of reaction.

Quenching of peroxynitrite ($ONOO^-$) by phenol compounds was studied in a system of SIN-1 (20 mM) and lucigenin (100 mM) at 37°C [8]. The test compounds were added simultaneously with SIN-1 and lucigenin. CL was measured 5 min after the start of reaction.

Oxidative metabolism of granulocytes was studied in heparinized samples of rat whole blood (20 U/ml). We measured luminol-induced or lucigenin-induced (10 mM) CL under zymosan stimulation (2 mg/ml) [2]. The test compounds were added simultaneously with zymosan. The maximum CL response was recorded.

The effectiveness of the test compound in these 4 systems was expressed as a percentage of the corresponding value without preparation. The half-inhibition concentration (IC_{50}) was calculated by interpolation of the dose—response dependences. They were obtained by studying the compounds in at least five effective concentrations.

RESULTS

All compounds with the hydroxyl and/or thiosulfonate group had antioxidant and antiradical properties (Table 1). The test compound gained prooxidant capacity after methylation of functionally active OH group in S-10. Addition of this compound to the experimental system promoted oxidation of ethyl oleate and LDL and stimulated the production of ROS in other systems. Studying the oxidation of ethyl oleate and LDL showed that AOA of preparations increased with increasing the degree of OH group masking. Thiosulfonates (TS-10, TS-13, and TS-17) were more potent than the corresponding sulfonates (S-10, S-13, and S-17), which was probably associated with antioxidant properties of bivalent sulfur in the thiosulfonate fragment. AOA of compounds with 2 tert-butyl substituents in the *ortho*-position was higher that that of structurally similar potassium phenosan.

The study of radical production by stimulated granulocytes and experiments in a xanthine-xanthine oxidase system and SIN-1 degradation revealed no relationship between the inhibitory effect of

TABLE 1. AOA of Phenol Compounds (IC_{50}, μM)

Compound	Cu^{2+}-induced oxidation of ethyl oleate (induction period, min)	Oxidation of blood LDL		Granulocyte respiratory burst		O_2^- (xanthine-xanthine oxidase system)	$ONOO^-$
		Cu^{2+}	Fe^{2+}	with luminol	with lucigenin		
S-10	10	Dose-dependent stimulation					
TS-10	13	11 800	$>1\times10^6$	74	141	1285	16.5
S-9	38	2770	2650	214	245	2878	5.9
TS-9	53	398	381	27	219	577	2.9
S-13	59	43	32	1170	1600	1064	10.1
TS-13	64	30	18	525	1800	1214	9.7
S-17	200	15	3.6	631	724	207	24.7
TS-17	258	10	1.5	123	68	1044	10.7
Potassium phenosan	93	13	1.8	800	1400	1170	21.3

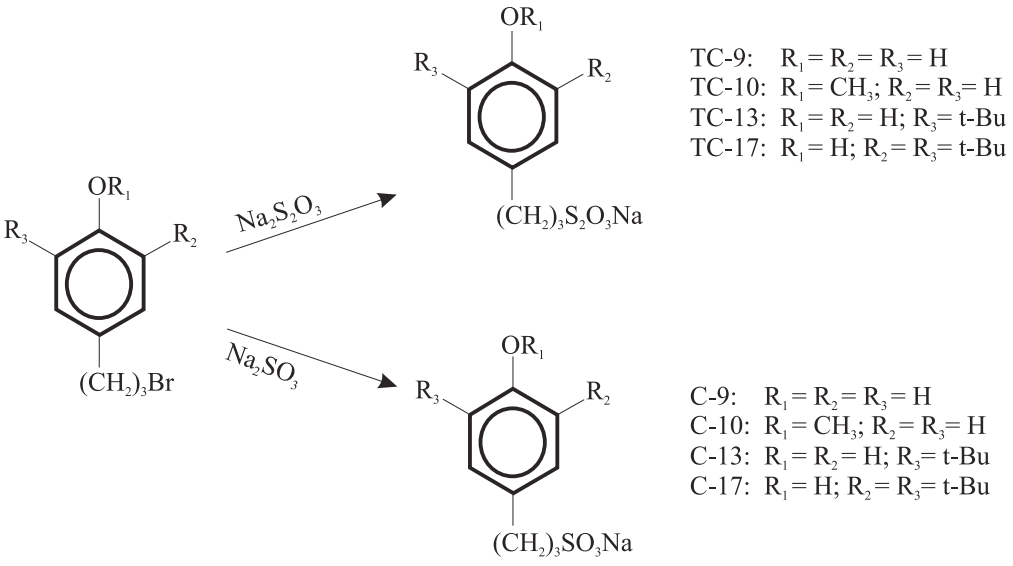

Fig. 1. Synthesis of S-alkyl thiosulfonates (TS-9, TS-10, TS-13, and TS-17) and S-alkyl sulfonates (S-9, S-10, S-13, and S-17).

the test compounds and masking of the OH group (Table 1). In systems of luminol-induced CL (granulocytes and SIN-1), thiosulfonates were more active than the corresponding sulfonates. These features were probably related to inactivation of H_2O_2 and peroxynitrite with the thiosulfonate fragment. Published data show that these compounds inhibit luminol luminescence [5].

These data and results of a QSAR study show that antioxidant properties of phenol compounds under model conditions depend strongly on masking of the phenol OH group and nature of the ionogenic fragment in the *p*-propyl substituent.

The proposed combination of *in vitro* methods allows us to perform a complex differential study for antioxidant properties of the test compound and to reveal the determinant functional groups. This is an essential but insufficient approach for the development of new antioxidant agents. The existence of strong antiradical properties *in vitro* does not necessarily reflect the *in vivo* effectiveness of the test compound for free radical disorders in animals and humans.

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