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EFFECTS OF [(1-PHENYL-5-FORMYL-1*H*-IMIDAZOL-4-YL)THIO]ACETIC ACID ON THE ANTIOXIDANT STATUS OF THE LIVER AND KIDNEY IN RATS WITH TETRACHLOROMETHANE POISONING

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The effects of [(1-phenyl-5-formyl-1*H*-imidazol-4-yl)thio]acetic acid on oxidant-antioxidant homeostasis in rats with tetrachloromethane poisoning were studied. This compound was found to normalize most parameters of the oxidant-antioxidant balance in the liver and kidneys of animals with toxic hepatitis. These results point to the ability of compounds of this type to suppress free-radical lipid oxidation and oxidative protein modification processes and to activate the antioxidant protection system in animal tissues.

Keywords: imidazole, [(1-phenyl-5-formyl-1*H*-imidazol-4-yl)thio]acetic acid, tetrachloromethane hepatitis, antioxidant activity.

Impairments to the balance of the oxidant-antioxidant system lead to activation of free-radical processes and the peroxidation of polyunsaturated fatty acids in the human body, which are accompanied by more than a hundred different pathological states [1]. For this reason, use of antioxidants in the complex pharmacotherapy of many types of diseases is obligatory. Pharmacologically active substances of this type regulate the occurrence of free-radical transformations, protecting cells from damage due to free radicals, thus significantly decreasing the risk of developing diseases [2 – 4]. The arsenal of antioxidants is significant, though their use in some cases is limited because of a variety of adverse events. This fact leads to increased interest in the search for biologically active compounds with antioxidant properties as candidate drugs. A potential route to the creation of such drugs is provided by targeted synthesis of multifunctional compounds whose structures contain multiple groups with antioxidant activity, including imidazole derivatives [5 – 8].

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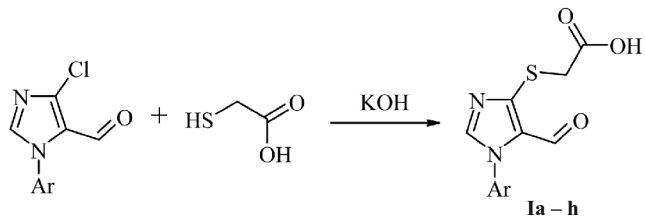
EXPERIMENTAL CHEMICAL SECTION

We have previously synthesized a series of original imidazole derivatives functionalized with a thioacetic acid residue at position 4 and a formyl group at position 5 (compounds **Ia-h**) [9]. A scheme was proposed for preparing these, based on the use of available 1-aryl-5-formyl-4-chloro-1*H*-imidazoles [10], which on heating with thioglycolic acid in ethanol for 2 h in the presence of potassium hydroxide form [(1-aryl-5-formyl-1*H*-imidazol-4-yl)thio]acetic acids (**Ia-h**), whose structures were confirmed by a set of physicochemical analysis methods [9].



Ia – h

R = Ph (a), 3-FC_6H_4 (b), 4-FC_6H_4 (c), 4-ClC_6H_4 (d), 2-MeC_6H_4 (e), 3-MeC_6H_4 (f), 4-MeC_6H_4 (g), 1-$C_{10}H_7$ (h).

Primary screening for antioxidant activity among the compounds synthesized here showed that at concentrations

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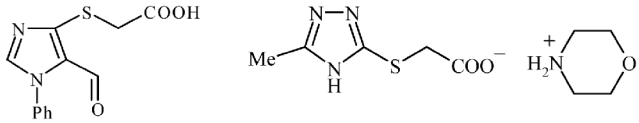

Ia **II**

Structural formula of [(1-phenyl-5-formyl-1*H*-imidazol-4-yl)thio]-acetic acid (**Ia**) and thiotriazoline (**II**).

of 10^{-1} to 10^{-3} M, all had in vitro activity suppressing the intensity of Fe^{2+}-ascorbate-induced free-radical lipid oxidation (FRLO) [9]. Taking this into account, [(5-formyl-1*H*-imidazol-4-yl)thio]acetic acid derivatives are interesting objects for further study of this type of activity *in vivo*. Further investigation of the effects of 5-formylimidazolylthioacetic acids on the operation of the antioxidant defense system in rats in conditions of tetrachloromethane hepatitis led to selection of the most active compound – [(1-phenyl-5-formyl-1*H*-imidazol-4-yl)thio]acetic acid (compound **Ia**). The reference compound was thiotriazoline (**II**) – an officinal azole derivative with antioxidant activity [11, 12], the closest structural analog to the compound studied here.

EXPERIMENTAL BIOLOGICAL SECTION

Studies were performed on male mongrel white rats weighing 160 – 180 g kept in standard conditions on animal-house diet. Studies of the *in vivo* antioxidant activity of compounds were performed in a standard model of oxidative stress – the rat tetrachloromethane hepatitis model [13 – 15]. The nature of morphological and biochemical changes in rats on tetrachloromethane poisoning is known [16, 17] to correspond to changes described in patients with hepatitis of various etiologies. In addition, tetrachloromethane poisoning produces significant functional impairment to the kidneys [18]. Tetrachloromethane hepatitis in rats is often used at the preclinical studies stage of investigating substances with proposed antioxidant properties as a model of oxidative stress [13, 16].

The aim of the present work was to study the effects of an original imidazole derivative synthesized by ourselves (compound **Ia**) on parameters characterizing the state of the oxidant-antioxidant system of the liver and kidney in rats with experimental tetrachloromethane hepatitis.

Toxic tetrachloromethane hepatitis was induced in rats using two (alternate days) intragastric does of CCl_4 (0.25 ml/100 g) as 50% oil solution in olive oil (Yu. I. Gubskii, 1984; N. P. Skakun, 1989) [14, 15].

Results obtained from studies of the antioxidant activity of the test compound in the *in vivo* system were compared with results from intact rats and parameters in rats given thiotriazoline **II** (Arterium, Ukraine, solution for injection, 25 mg/ml) on the background of tetrachloromethane hepatitis. Thiotriazoline is a metabolic substance with an antioxidant mechanism of action [11, 12] and, as its structure includes a triazole fragment and a thioacetic acid residue, it is the closest of the officinal azole derivatives to structural analogs of the substance of interest. The antioxidant action of thiotriazoline is mediated by the fact that it is a scavenger of reactive oxygen species (ROS), activates the main antioxidant defense enzymes, i.e., superoxide dismutase, catalase, and glutathione reductase, promotes the retention of endogenous antioxidants – α-tocopherol and glutathione – in the body, and inhibits formation of markers of the oxidative modification of proteins and lipids [11, 12].

Experimental animals were divided into four groups: group I consisted of control (intact) rats; group **II** animals were poisoned with CCl_4; group **III** animals received daily i.p. study compound **Ia** (100 mg/kg) for seven days on the background of CCl_4 poisoning; group IV consisted of rats receiving daily i.p. thiotriazoline **II** at the antioxidant dose of 100 mg/kg for seven days on the background of CCl_4 poisoning [11, 12].

All animal manipulations were performed in compliance with ethical principles for animal experiments approved by the First National Congress on Bioethics (Kiev, 2000), whose strictures are compatible with the norms of the European Convention for the Protection of Vertebrates Used in Experiments and Other Scientific Purposes (Strasbourg, 1986). Animals were decapitated under light ether anesthesia on day 8 from the beginning of the experiment. Liver and kidneys were isolated from rats in the cold and 5% homogenates were prepared in cold 50 mM Tris-HCl buffer (pH 7.4). The postnuclear supernatants of rat tissue homogenates obtained by centrifugation at 1500 rpm were used for assay of oxidation-modified proteins (OMP_{370}) using the Meshchishen method (2000), malondialdehyde by the Vladimirov and Archakov method (1972) [14]; reduced glutathione (G-SH) and the activities of the main antioxidant defense enzymes – superoxide dismutase (E.C. 1.15.1.1] (SOD), catalase [E.C. 1.11.1.6] (CAT), and glutathione peroxidase [E.C. 1.11.1.9] (GP) were estimated using previously described methods [14, 15]. In addition, the content of compounds with isolated double bonds (IDB), diene conjugates (DC), ketodiones (KD), and conjugated trienes (CT) were measured using a modification of the Volchegorskii, et al. method (1989) [15].

To find an appropriate method for statistical evaluation of mean differences between study groups, initial assessment of the distributions of values in datasets was performed. Use of the Shapiro-Wilk test for assessment of normal distributions in datasets of size $n \leq 50$ showed that all datasets had distributions not deviating from normal ($p > 0.05$). Considering these data, use of Student's test was regarded as appropriate for drawing valid conclusions. With the aim of increasing the reliability of the conclusions, the nonparametric Mann-Whitney test was run in parallel, which gave similar results as using Student's test in terms of p. Differences were regarded as significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Poisoning of rats with tetrachloromethane is known [16 – 18] to lead to development of a syndrome consisting of hepatocyte cytolysis, cholestasis, and impairments to carbohydrate, protein, and lipid metabolism, and oxidant-antioxidant homeostasis, as in humans with hepatitis. In addition, the condition affects not only the liver, but also the kidneys [19]. The most typical pathological changes in the body in this type of poisoning are parenchymatous and fatty changes in the liver, acute toxic nephrosis, and impairments to the functions of the renal tubules throughout their length [16, 19]. Thus, tetrachloromethane poisoning produces hepatorenal syndrome, which involves both hepato- and nephrotoxic actions.

The toxic action of tetrachloromethane, according to current views on the pathogenesis of poisoning [16], is linked with the metabolic formation of radicals (of the $CCl_3^{\bullet}$ and $CCl_3O_2^{\bullet}$ types), initiation of FRLO, and formation of an excess of reactive oxygen species (ROS) and lipid peroxides (predominantly in the endoplasmic reticulum, but also in microsomes and mitochondria). In conditions of toxic tetrachloromethane hepatitis, rats show significant impairments to oxidant-antioxidant balance: FRLO and oxidative protein modification (OMP) are increased, while the effectiveness of the operation of various components of the antioxidant defense system are decreased [14].

Our results (Table 1) indicate that eight days from the beginning of the experiment, the liver of tetrachloromethane-poisoned rats showed increased contents of IDB, DC, KD, and CT (by 26.5%, 39% and 27% respectively from levels in intact controls), as well as malondialdehyde (MA) and OMP_{370} – by 57% and 35% respectively, as compared with intact rats. Thus, the contents of such primary molecular products of FRLO as DC showed the most significant increases in tetrachloromethane poisoning, as did the content of one of the main FRLO products – MA.

Single-electron reduction of oxygen is known [20] to form superoxide anion radicals, which give rise to other ROS. Its inactivation involves the enzyme SOD. The activity of this important antioxidant defense enzyme in the liver in rats on poisoning with tetrachloromethane decreased (by 35.5% compared with intact animals). The activity of the enzymes involved in the catabolism of hydrogen peroxide (one of the most aggressive oxygen species) – catalase and GP – also decreased from the levels seen in intact controls (by 32% and 30% respectively). Decreases in the activity of the key antioxidant defense enzymes in the liver may result from their oxidative modification, as these enzymes are known [14, 15] to be subject to modification involving ROS. The SOD molecule is particularly susceptible to oxidative modification because its active center contains an imidazole ring in the histidine residue. Another likely cause of the decrease in total superoxide dismutase activity (because of the greater activity of the cupper/zinc-dependent enzyme) in the liver of the rats is the intense uptake of copper ions by ceruloplasmin molecules. Ceruloplasmin (a copper-containing glycoprotein) is synthesized in the liver, enters the blood, and in conditions of oxidative stress acts as a "circulating antioxidant". Ceruloplasmin (CP) in plasma "eliminates" excess superoxide anion radicals without forming hydrogen peroxide. The literature contains data [14, 15] pointing to an inverse relationship between SOD activity and CP (in hepatitis, plasma CP activity increases and liver SOD decreases). Decreases in GP activity may be linked with both oxidative modification of the enzyme and also with G-SH deficit. GP, in contrast to catalase, uses G-SH as cofactor, and detoxifies not only hydrogen peroxide, but also the hydroperoxides of

TABLE 1. Effects of Compound **Ia** on Measures of the Oxidant and Antioxidant Systems in the Liver of Rats ($n = 6$) in Tetrachloromethane Poisoning

Measure, group	Control (intact rats)	CCl_4	CCl_4 + **Ia**	CCl_4 + **II**
IDB (E_{220}/g tissue)	30.24 ± 2.16	41.14 ± 2.05*	36.11 ± 1.67*#	38.17 ± 2.22*
DC (E_{232}/g tissue)	19.71 ± 1.05	32.14 ± 2.21*	24.91 ± 1.64*#	21.44 ± 1.02#
KD and CT (E_{278}/g tissue)	11.42 ± 0.05	15.62 ± 1.02*	12.64 ± 0.07^	10.81 ± 0.09#
MA (μmol/g tissue)	37.45 ± 2.64	88.23 ± 4.25*	53.41 ± 2.24*#	53.46 ± 2.14*#
OMP_{370}, ODU/g tissue	15.36 ± 0.42	23.68 ± 0.78*	20.86 ± 0.14*#^	15.80 ± 0.68#
SOD, U/mg protein	0.409 ± 0.05	0.264 ± 0.05*	0.377 ± 0.06*	0.325 ± 0.02
CAT, μmol/min.g tissue	138.69 ± 5.11	94.72 ± 11.38*	131.50 ± 6.52#	128.47 ± 4.26
G-SH, μmol/g tissue	8.66 ± 1.19	5.38 ± 0.57*	7.03 ± 0.44#^	8.28 ± 0.30#
GP, nmol/min.mg protein	193.4 ± 10.85	133.28 ± 6.40*	187.01 ± 11.93#	206.98 ± 26.03#

*significant differences compared with controls
#significant difference compared with hepatitis
^significant differences compared with thiotriazoline (**II**)

higher fatty and nucleic acids. The content of the important endogenous antioxidant – reduced glutathione – in the livers of animals with hepatitis was 38% lower than in intact rats.

Overall, our results on the oxidant-antioxidant status of rats with toxic tetrachloromethane hepatitis are consistent with published data [14, 15, 17, 21, 22]. Toxic lesions have been shown [14, 15, 21] to produce not only increases in the quantities of primary and secondary molecular products of lipid peroxidation (conjugated dienes and malondialdehyde), but also significant impairments to the functioning of the glutathione system both in the structural components of hepatocytes and in blood erythrocytes. Activation of OMP processes and suppression of the activity of the antioxidant enzymes (SOD, catalase, glutathione peroxidase, and glutathione reductase) in the liver of rats with tetrachloromethane poisoning are also noted [15, 17].

All study parameters in the liver of rats with hepatitis and treated with daily thiotriazoline (**II**) for one week normalized, apart from IDB and MA. The livers of rats given compounds **Ia** daily for the same period of time on the background of tetrachloromethane poisoning showed complete normalization of KD and CT, CAT, G-SH, and GP, while the content of compounds with IDB, DC, MA, and OMP, as well as SOD activity, showed significant changes from values in rats with hepatitis, albeit not reaching normal values.

The kidneys of tetrachloromethane-poisoned animals showed impairment to oxidant-antioxidant homeostasis analogous to the trend seen in the liver, though less marked (Table 2).

Activation of FRLO processes and biopolymer oxidation is also one of the leading mechanisms in kidney tissue damage in tetrachloromethane poisoning [18, 19, 23]. The accumulation of lipids, particularly oxidized lipids, in the renal glomeruli has a direct damaging action on the structural-functional organization of kidney tissue membranes; the accumulation of ROS within them leads to impairment to the balance of the prooxidant and antioxidant systems, with increases in FRLO activation and decreases in the activity of the antioxidant enzymes, along with depletion of the non-enzymatic component of antioxidant protection [23]. The peroxide anion radical is known [24] to trigger oxidative stress in all parts of the kidney, while hydrogen peroxide is important only in the renal cortex; at the same time, hydrogen peroxide in the cortex stimulates vasodilation of afferent arterioles.

Our results indicate that the kidneys of rats with hepatitis show activation of FRLO processes and oxidative modification of proteins: MA and OMP contents increased compared with values in intact rats by 57% and 21% respectively. The activities of the antioxidant enzymes studied here in the kidneys of rats poisoned with tetrachloromethane, as in the liver, were lower than in intact rats: SOD by 15% and GP by 25%. Catalase activity in the kidneys of rats with hepatitis also showed some decrease compared with values in animals of the control group (by 9%), though this change was not significant.

The G-SH level in the kidneys of rats with untreated hepatitis was 24% lower than that in the control group. There are several possible causes for the decrease in G-SH in rat tissues – intense utilization of reduced glutathione as a "direct-acting" antioxidant (action mediated via the free SH groups of the cysteine residue), and impairment of its synthesis in tissues and of its regeneration from the oxidized form as a result of deficit of the NADPH required for glutathione reductase to function [12, 13, 19].

The kidneys, like the liver, of rats showed positive correcting influences of compound **Ia** and thiotriazoline (**II**) on the oxidant-antioxidant balance impaired in toxic hepatitis (Table 2). Administration of compound **Ia** partially normalized measures of oxidant and antioxidant systems in the rat kidneys (the quantities of MA and OMP decreased significantly, while the levels of reduced glutathione and antioxidant enzymes completely normalized).

These results provided evidence of the ability to the imidazole derivative studied here to suppress FRLO and OMP process and stabilize the functioning of the enzymatic

TABLE 2. Effects of Compound **Ia** on Measures of the Oxidant and Antioxidant Systems in the Kidneys of Rats (n = 6) in Tetrachloromethane Poisoning

Measure, group	Control (intact rats)	CCl_4	CCl_4 + **Ia**	CCl_4 + **II**
MA (μmol/g tissue)	25.90 ± 0.51	60.72 ± 2.12*	32.36 ± 0.82*#	35.64 ± 1.43*#
OMP_{370}, ODU/g tissue	19.58 ± 0.38	24.78 ± 0.47*	14.48 ± 0.22*#	13.16 ± 0.27*#
SOD, U/mg protein	0.54 ± 0.04	0.46 ± 0.02*	0.49 ± 0.04	0.51 ± 0.03
CAT, μmol/min.g tissue	109.20 ± 6.73	98.98 ± 1.27	98.36 ± 2.17	114.00 ± 7.81
GP, nmol/min.mg protein	72.62 ± 8.34	54.29 ± 9.48*	70.07 ± 7.24	70.75 ± 9.48
G-SH, μmol/g tissue	4.82 ± 0.62	3.61 ± 0.31*	5.96 ± 0.55#	5.34 ± 0.41#

*significant differences compared with controls
#significant difference compared with hepatitis
^significant differences compared with thiotriazoline (**II**)

antioxidant defense system. Attention is drawn to the fact that treatment of rats with hepatitis with compound **Ia**, like administration of thiotriazoline (**II**), promoted activation of the glutathione system of antioxidant defense – renewal of reserves of reduced glutathione and activation of glutathione peroxidase, while the magnitude of the antioxidant effect of substance **Ia** was no less than that of thiotriazoline (**II**).

The antioxidant activity of substance **Ia** is probably due to the fact that its structure contains a thioglycolic acid residue combined with an imidazole heterocycle. This substance can evidently "trap" free radicals formed in pathological processes, exert reducing properties, help maintain the level of reduced glutathione at the level required for metabolism, and ensure its effective use by glutathione peroxidase to detoxify excess hydrogen peroxide and other hydroperoxides; it promotes increases in catalase and SOD activity. The positive influence of compound **Ia** on the activity of the antioxidant enzymes studied here may, as in the case of thiotriazoline (**II**) [11], be explained by their influences on the expression of genes encoding these enzymes. Activation of the glutathione antioxidant defense system probably occurs as a result of filling the G-SH reserves in tissues in rats (restoration of synthesis and/or regeneration from the oxidized form). The mechanism of the antioxidant influence of compound **Ia** is probably identical to the mechanism of the antioxidant action of thiotriazoline (**II**), which according to published data and our study results also has antioxidant activity, influencing SOD, catalase, and glutathione system activity [11].

Thus, in analyzing the results obtained from studies of the antioxidant activity of [(1-phenyl-5-formyl-1*H*-imidazol-4-yl)thio]acetic acid (compound **Ia**) we can note that this substance has marked antioxidant actions in *in vivo* experiments (on a model of tetrachloromethane hepatitis in rats) due to the fact that its structure contains the appropriate pharmacophore groups.

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