

Aurothiomalate as Preventive and Chain-Breaking Antioxidant in Radical Degradation of High-Molar-Mass Hyaluronan

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Dedicated to Professor *Radomír Nosál'*, MD, DSc, on the occasion of his 70th birthday

The potential anti- or pro-oxidative effects of a disease-modifying antirheumatic drug, aurothiomalate, to protect high-molar-mass hyaluronan against radical degradation were investigated along with L-glutathione – tested in similar functions. Hyaluronan degradation was induced by the oxidative system Cu^{II} plus ascorbate known as the *Weissberger's* oxidative system. The time- and dose-dependent changes of the dynamic viscosity of the hyaluronan solutions were studied by the method of rotational viscometry. Additionally, the antioxidative activity of aurothiomalate expressed as a radical-scavenging capacity based on a decolorization 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was inspected. At the higher concentrations tested, L-glutathione showed excellent scavenging of ·OH and peroxy-type radicals, however, at the lowest concentration applied, its pro-oxidative effect was revealed. The effects of aurothiomalate on hyaluronan degradation were similar to that of L-glutathione, however, at the lowest concentration tested, no significant pro-oxidant effect was observed.

Introduction. – Hyaluronan (HA; *Fig. 1*) is a linear unbranched polysaccharide consisting of repeating disaccharide units of β -1,4-D-glucuronic acid (GlcUA) and β -1,3-*N*-acetyl-D-glucosamine (GlcNAc). HA is omnipresent in the vertebrate connective tissues, particularly in the umbilical cord, synovial fluid (SF), vitreous humor, dermis, cartilage, *etc.*, where its molecular size can reach the values of up to 10⁷ Da [1]. The turnover of HA is extremely rapid: it is estimated that, in the 70-kg human body consisting *ca.* 15 g of HA, it is 5 g per day. In an apparently inert tissue such as cartilage, the *t*_{1/2} of HA is *ca.* one to three weeks, while this value in SF of healthy humans is *ca.* 12 h.

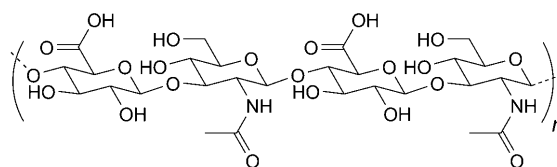


Fig. 1. The structure of hyaluronan, the acid form

The importance of high-molar-mass HA in maintaining the unique rheological properties of joint SF is well-established. In rheumatoid arthritis (RA), the HA macromolecules within the joint SF are degraded, and the concentration of HA fragments with lower molar mass in RA patients is elevated [2]. There is a clear evidence that low-molar-mass HA has different activities than the native high-molar-mass biopolymer. Large-sized matrix HA is space-filling, anti-angiogenic, and immunosuppressive, whereas the intermediate-sized HA macromolecules, comprising 25–50 disaccharide units, are inflammatory, immunostimulatory, and highly angiogenic; these hyaluronan fragments appear to function as endogenous danger signals [3].

Thiols play an important role as biological reductants (antioxidants) preserving the redox status of cells and protecting tissues against damages caused by the elevated reactive oxygen/nitrogen species (ROS/RNS) levels by which oxidative stress might be indicated [4]. L-Glutathione (GSH; L- γ -glutamyl-L-cysteinyl-glycine; Fig. 2,a), a ubiquitous endogenous thiol, maintains the intracellular reduction–oxidation (redox) balance and regulates signaling pathways during oxidative stress/conditions. GSH is mainly cytosolic in the concentration range of *ca.* 1–10 mM; however, in the plasma as well as in SF, the range is only 1–3 μ M [5][6]. This unique thiol plays a crucial role in antioxidant defence, nutrient metabolism, and in regulation of pathways essential for the whole body homeostasis. Depletion of GSH results in an increased vulnerability of the cells to oxidative stress [7].

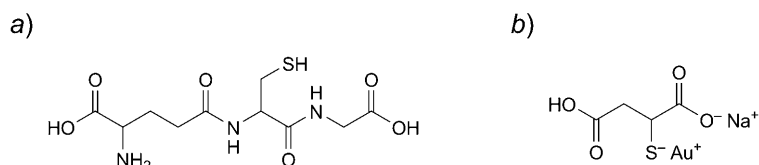


Fig. 2. a) L-Glutathione b) and sodium aurothiomalate

Gold salt thiol-based drugs, are a part of the group of disease-modifying antirheumatic drugs (DMARDs), which have been used in the treatment of RA for more than 50 years; however, the mechanism of their action is not yet fully known in all details [8–10]. There are available gold(I)–thiol drugs, such as aurothioglucose or aurothiomalate (Fig. 2,b) that are administered to patients intramuscularly, whereas auranofin is effective *via* oral administration [9–11]. Unlike DMARDs, such as cyclosporin A, hydroxychloroquine, leflunomide, methotrexate, and sulfasalazine, it was shown that aurothiomalate inhibits cyclooxygenase-2 expression and subsequent prostaglandins production [12]. Au^I–Thiol drugs inhibit lysosomal enzymes, such as β -glucuronidase, acid phosphatase, and protein kinase C, which are known to participate in inflammatory reactions in the joints [8][13]. *Schuhmann et al.* [14] concluded that the adverse effects of Au^I salts are caused by Au^{III} ions which must be generated *in vivo* through oxidation of Au^I ions by ROS, such as HOCl in the phagocytic cells – monocytes and macrophages. Au^{III} Ions can irreversibly oxidize proteins and, thus, may be considered also as reactive intermediate metabolites [8]. *Otiko et al.* [15], by the method of ¹H spin-echo Fourier transform NMR, showed that, when added to the

suspensions of red cells, aurothiomalate intracellularly bound GSH. When added to red cell lysates, a specific binding of gold to cysteine moiety of GSH along with release of thiomalate has been observed. Vuorio *et al.* [16] studied the effects of aurothiomalate on the quantity and quality of HA synthesized by normal and RA fibroblast cultures. They observed that the molecular size of the biopolymer fragments produced by RA fibroblasts were shifted towards normal HA molar-mass values. Stuhlmeier [17] found out that aurothiomalate decreased HA release from fibroblasts by the drug interference with HA synthase 1.

To our knowledge, no reports devoted to direct effects of aurothiomalate on the HA degradation under the conditions simulating those occurring at oxidative stress has appeared so far. Thus, the presented study is focused on investigation of the ability of the two above-mentioned thiol compounds, namely aurothiomalate and GSH, to act as either anti- and/or pro-oxidant monitoring the kinetics of the free-radical degradation of high-molar-mass HA. As an effective HO[•] radical inducer, the system comprising ascorbate *plus* Cu^{II}, the so-called Weissberger's oxidative system, was established. Along with monitoring the dynamic viscosity of HA solution by the method of rotational viscometry, the standardized decolorization ABTS assay was applied to classify both aurothiomalate and GSH to confirm their radical-scavenging capacities.

Results and Discussion. – The HA sample used, P9710-2A, contained residual transition metal cations, namely, according to the Certificate of Analysis, 4 ppm of Cu and 13 ppm of Fe. The content of Cu ions in the HA sample solution used (2.5 mg/ml) was thus equal to 0.16 μM , while that of Fe ions was 0.5 μM .

First, the effect of the addition of CuCl₂ solution alone to the HA solution was inspected. As evidenced (data not shown), during all the measurement period (5 h), no degradation of HA macromolecules can be detected. In contrast, a slight increase of the solution dynamic viscosity (η) was observed. As already started, this augmentation of the solution η value is caused by the orientation of HA polymer chains during spindle rotation, *i.e.*, by a phenomenon called rheopexy [18].

To study the uninhibited/inhibited hyaluronan degradation as a consequence of the action of initiating species – the HO[•] radicals – the Weissberger's oxidative system (Cu^{II} *plus* ascorbate; *Scheme*) was applied.

As the primary variability, the concentration of Cu^{II} ions was set in the reaction vessel by CuCl₂ solution addition resulting in the value of 1.0 μM . By the way, the total concentration of Cu^{II} ions (1.0 + 0.16 μM) exceeded significantly that of Fe^{II} ions, present in the intact sample, *i.e.*, 0.5 μM , and thus, on working with this rather high level of Cu^{II} ions, the Weissberger's oxidative system should be dominative. As the second variability, the concentration of ascorbate was set to 10, 50, and 100 μM to achieve ascorbate/Cu^{II} ions concentration ratios equal to 10 : 1, 50 : 1, or 100 : 1, respectively. As can be seen in *Fig. 3*, curve 1, the oxidative system composed of 1.0 μM Cu^{II} ions *plus* 10 μM ascorbate was completely ineffective to induce the HA degradation. Moreover, a phenomenon of a step-by-step growing rheopexy of the biopolymer was observed.

Applying the ascorbate concentration of 50 μM (*Fig. 3*, curve 2), the value characteristic in patients suffering from RA, the gradual decrease of the dynamic viscosity of HA solution was observed. This fast drop of the η value during the first 90 min was followed by significant retardation of the HA degrading process. The

Scheme. Generation of H_2O_2 by Weissberger's System from Ascorbate and Cu^{II} Ions under Aerobic Conditions (adapted from Fisher and Naughton [19])

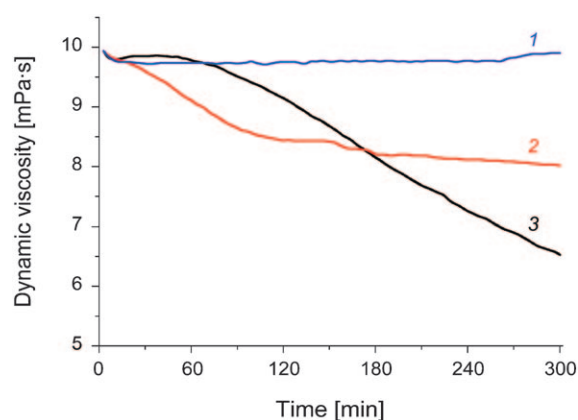
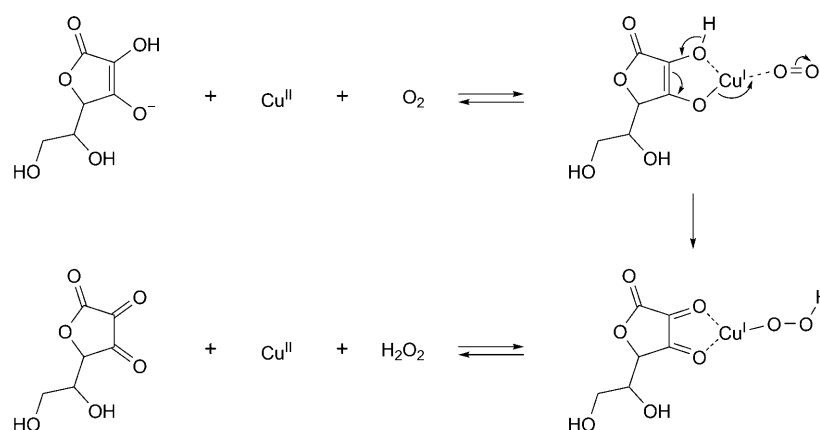


Fig. 3. Effect of the application of the pro-oxidative Weissberger's system (ascorbate + Cu^{II} ions) on the hyaluronan degradation testing various ascorbate concentrations in μM : 10 (1), 50 (2), or 100 (3), respectively. $CuCl_2$ Solution concentration: 1.0 μM .

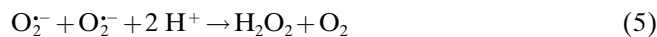
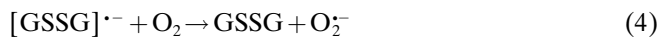
decrease of the HA dynamic viscosity from its initial value of 9.93 to 8.01 $mPa \cdot s$ was, however, classified as not sufficiently effective for our further studies.

As evident from the curve 3 in Fig. 3, by increasing the ascorbate concentration to 100 μM , a reaction lag phase evidenced up to 60 min was continued by a decrease of the dynamic viscosity of the HA solution achieving a final value of 6.53 $mPa \cdot s$ within 5 h. As a simple and the most plausible explanation of the degradative lag phase observed (Fig. 3, curve 3), one should take into account the following facts: *i*) according to the Scheme, H_2O_2 generated can decompose both by the intermediate Cu^I complex and the trace Fe^{II} present within the reaction system; *ii*) within the very early time interval (the initiation of the chain radical reaction), the $HO\cdot$ radicals are generated by the Fenton

reaction: $\text{Cu}^{\text{I}}/\text{Fe}^{\text{II}} + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{\text{II}}/\text{Fe}^{\text{III}} + \text{OH}^- + \text{HO}^\bullet$; *iii*) however, due to the presence of a rather high excess of uncomplexed ascorbate (AscH^-), it is the reactant which can scavenge the most of the HO^\bullet radicals leading to ascorbyl radical species – $\text{AscH}^- + \text{HO}^\bullet \rightarrow \text{Asc}^{\bullet-} + \text{H}_2\text{O}$. These tenets, *i–iii*, and especially the last one have been unequivocally established by using the EPR spectroscopy [20]. At the later time interval (> 60 min), however, as confirmed by the EPR, the HO^\bullet radicals generated ‘overdose’, the actual scavenging effectivity of ascorbate and HO^\bullet radicals can thus start the reaction with the HA polysaccharide chain leading to a HA-derived C-macroradical. The time interval between 60 and 300 min (*Fig. 3*, curve 3), which is classifiable as a continual stepwise decrease of the solution dynamic viscosity, most probably represents a chain radical process during which the HA macromolecules are decayed leading to shorter polymeric fragments.

The high-molar-mass HA can be classified as a regular copolymer of the type $[\text{-GlcUA-GlcNAc-GlcUA-GlcNAc-}]_n$. By abstracting H^\bullet radical from the HA macromolecule by the action of HO^\bullet radical, a HA-derived C-macroradical is formed. However, under aerobic conditions, the phase of the chain reaction, known as propagation, during which a dioxygen molecule reacts with the HA-derived C-macroradical, leads to peroxy-type radical species. The peroxy radicals continue the radical reaction by random trapping of H^\bullet radicals from neighboring HA macromolecules to forming hydroperoxyls, and novel HA-derived C-macroradicals are generated. Since the cascade of the propagation reactions continually proceeds, the smaller-sized biopolymer fragments are formed. The process is accompanied by lowering the solution dynamic viscosity. The radical chain reaction, which is usually characterized by the four individual steps (initiation, propagation, transfer, and termination) can be stopped by addition of a free-radical scavenger. When such a scavenging compound is loaded into the HA solution before applying the *Weissberger’s* oxidative system, the scavenger may be tested in its function as a preventive antioxidant while, on applying the tested compound during the propagation phase of the HA degradation, its function may be tested as a chain-breaking antioxidant.

The two already established experimental designs to investigate the HA degradation by the *Weissberger’s* oxidative system [21][22] were preferentially used on investigating both the preventive and chain-breaking antioxidative properties of the DMARD, *i.e.*, aurothiomalate. The results represented in *Fig. 4,a*, show that the tested drug, aurothiomalate, at the lower concentrations tested (1 and 10 μM), unequivocally retarded or, at the higher concentrations tested (50 and 100 μM), even fully inhibited the HA degradation by the applied *Weissberger’s* oxidative system. For comparison, similar experimental design and concentrations were tested by using a well-known preventive and chain-breaking antioxidant, L-glutathione (*Fig. 4,b*). On the basis of the results shown in *Fig. 4,a*, it can be claimed that the DMARD drug, aurothiomalate, acted dose-dependently as a preventive antioxidant. Although the results represented in *Fig. 4,b*, could, at the first inspection, lead to a conclusion that GSH (10, 50, and 100 μM) is an excellent scavenger of HO^\bullet radicals, which usually initiate HA degradation, one should take into account that, when applying the smallest drug concentration (1 μM ; *Fig. 4,b*, curve 1), a pro-oxidative effect can be observed. The following *Reactions 1–5* can shortly explain the well-known phenomenon that is the L-glutathione disulfide anion radical $[\text{GSSG}]^{\bullet-}$ formation under aerobic conditions:



As described above, the product of *Reaction 5* should generate an additional excess of HO^\bullet radicals: $\text{Cu}^{\text{I}}/\text{Fe}^{\text{II}} + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{\text{II}}/\text{Fe}^{\text{III}} + \text{OH}^- + \text{HO}^\bullet$.

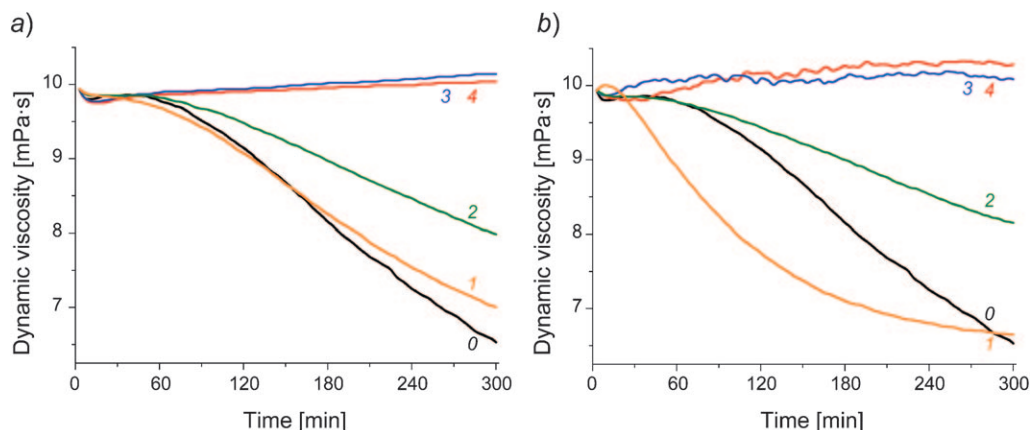


Fig. 4. Comparison of the effect of a) aurothiomalate and b) L-glutathione on the hyaluronan degradation induced by the system composed of $1.0 \mu\text{M}$ CuCl_2 plus $100 \mu\text{M}$ ascorbate. The thiols were added before the reaction onset. The concentrations of aurothiomalate and L-glutathione in μM : 0 (0), 1 (1), 10 (2), 50 (3), 100 (4).

Fig. 5,a, represents the results of investigation of the chain-breaking antioxidative properties of the DMARD, aurothiomalate. It should be admitted that the tested drug admixed into the sample mixture 1 h after the reaction onset, where residual trace HO^\bullet radicals are still present during the propagation phase, may react primarily with HA-derived peroxy radicals and, possibly, also with newly generated C-macroradicals. As evidenced from the results shown in Fig. 5,a, the higher aurothiomalate concentrations (50 and $100 \mu\text{M}$) almost completely inhibited the radical chain degradation of HA macromolecules. However, the lower drug concentrations (1 and $10 \mu\text{M}$) were ineffective. Yet, it should be pointed out that, contrary to a pro-oxidative effect caused by GSH ($1 \mu\text{M}$; clearly evident in Fig. 5,b, coded 1), apparently less pro-oxidative phenomenon has been observed on applying aurothiomalate ($1 \mu\text{M}$; Fig. 5,a, curve 1).

To support the results achieved on the radical-chain HA degradation study by rotational viscometry, the free-radical scavenging capacity of both compounds; aurothiomalate and GSH, were tested using the well-established standard 2,2'-

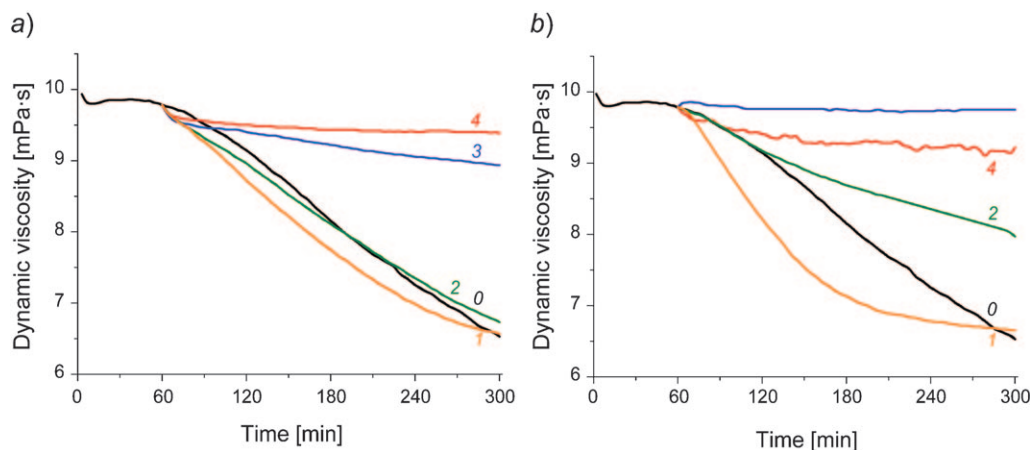


Fig. 5. Comparison of the effect of a) aurothiomalate and b) L-glutathione on the hyaluronan degradation induced by the system composed of $1.0 \mu\text{M}$ $\text{CuCl}_2 + 100 \mu\text{M}$ ascorbate. The thiols were added 1 h after the reaction onset. The concentrations of aurothiomalate and L-glutathione in μM : 0 (0), 1 (1), 10 (2), 50 (3), 100 (4).

azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) decolorization method. The results from this antioxidant test show that aurothiomalate is a faster $\text{ABTS}^{\bullet+}$ scavenger than that of GSH (Fig. 6). Aurothiomalate test evidenced a prompt and fast decrease of the $\text{ABTS}^{\bullet+}$ concentration, followed by a continuous, time-dependent, slower concentration decrease of ABTS radical cation (Fig. 6, a). On the basis of the

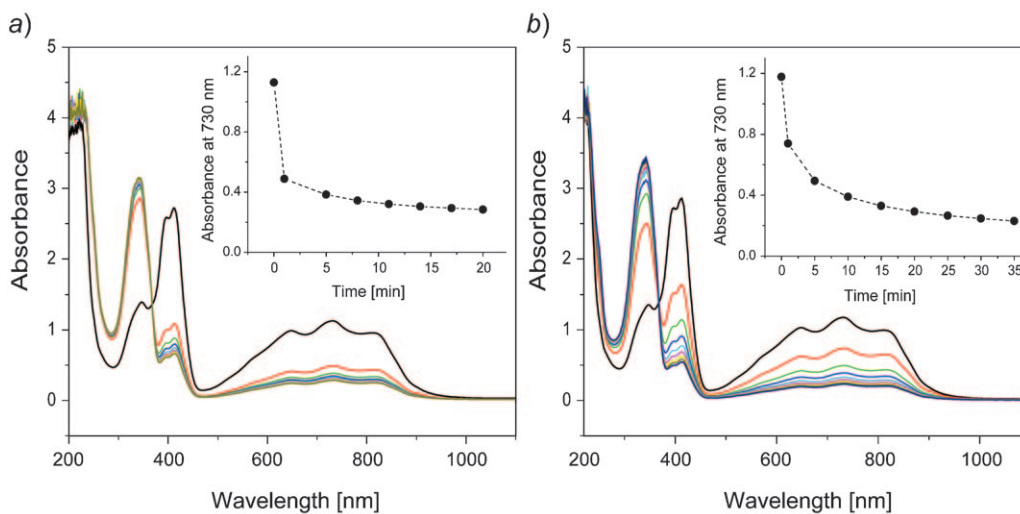


Fig. 6. The effect of ABTS radical cation scavenging by aqueous solution of a) aurothiomalate and b) L-glutathione. Insets: Time dependence of absorbance at 730 nm measured after addition of the tested compound into the $\text{ABTS}^{\bullet+}$ solution at the same experimental conditions.

results obtained, it can be concluded that both compounds show nearly identical antioxidant capacity measured after 20 min (*Table*), but in comparison to other antioxidants, namely natural polyphenols, with acute effect or endogenous GSH with effective and prolonged action, aurothiomalate exerted both properties.

Table. Scavenging Efficiency of Tested Compounds after 20 min [22]

Compound	Total oxidant scavenging capacity	Trolox®-equivalent antioxidant capacity
Aurothiomalate	75.4%	1.29 mmol/l
L-Glutathione	74.4%	1.33 mmol/l

Concluding Remarks. – The most relevant and important finding in the presented study is deduced on the basis of the results recorded in *Figs. 4,b*, and *5,b*. As evident from the graphical plots, aurothiomalate, a well-known DMARD, containing a thiol group, was shown to be an excellent scavenger of HO• radical as well as peroxy-type radicals. Aurothiomalate functions in this way simultaneously as preventive and chain-breaking antioxidant. Unlike some other compounds containing thiol group(s), *e.g.*, GSH, the trace aurothiomalate concentration levels within a tissue of patients treated with this DMARD represent, however, no danger for a start anew/restart of the sequence of reactions leading to the disulfide formation of RSSR•⁻. This species, under proper conditions, may generate superoxide anion radicals, and, subsequently H₂O₂ leads to the formation of extremely reactive HO• radicals.

Finally, from the point of view of the processes leading to the HO• radicals (for comparison, see *Reactions 1–5*), it could be stated that the application of aurothiomalate at the treatment of RA patients, during the drug clearance, accompanied with lowering its concentration level in the target tissue(s), it would be concerned as a medicament regarding practically nil potential to re-burn any free radical-damaging processes.

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Experimental Part

Material. The high-molar-mass hyaluronan sample P9710-2A ($M_r = 808.7$ kDa; M_r/M_n 1.63) used was from *Lifecore Biomedical Inc.*, Chaska, MN, USA. The declared content of transition metals in the HA sample given by Certificate of Analysis is 4 ppm of Cu and 13 ppm of Fe.

Chemicals. Anal. purity grade NaCl and CuCl₂·2 H₂O were purchased from *Slavus Ltd.*, Bratislava, Slovakia; reduced L-glutathione was from *Sigma–Aldrich Chemie GmbH*, D-Steinheim; *Tauredon® 50* (injection aurothiomalate formulation) was purchased from *Nycomed GmbH*, D-Konstanz; L-ascorbic acid and potassium persulfate (K₂S₂O₈; *p.a.* purity, max. 0.001% nitrogen) used were the products of *Merck KGaA*, D-Darmstadt; 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; *purum*, > 99%) was from *Fluka*, Germany. Deionized high-purity grade H₂O, with conductivity of ≤ 0.055 µS/

cm, was produced by using the TKA water purification system (*Water Purification Systems GmbH, D-Niederelbert*).

Preparation of Stock and Working Solns. The HA sample solns. (2.5 mg/ml) were prepared in the dark at r.t. in 0.15M aq. NaCl in two steps: first, 4.0 ml of the solvent was added to 20 mg of HA, and 3.90 or 3.85 ml of the solvent was added after 6 h. Stock solns. of L-ascorbic acid (16 mM), GSH, and aurothiomalate (0.16, 1.6, 8, and 16 mM), and CuCl₂ (16 mM diluted to a 160 µM soln.) were also prepared in 0.15M aq. NaCl.

Uninhibited/Inhibited Hyaluronan Degradation. First, HA degradation was induced by the oxidative system comprising CuCl₂ (1.0 µM) with altering concentrations of L-ascorbic acid (10, 50, and 100 µM). The procedure was as follows: a volume of 50 µl of 160 µM CuCl₂ soln. was added to the HA soln. (7.90 ml), and the mixture, after a 30-s stirring, was left to stand for 7 min 30 s at r.t. Then, 50 µl of L-ascorbic acid solns. (1.6, 8, or 16 mM) were added to the soln., and the soln. was stirred again for 30 s. The soln. was then immediately transferred into the viscometer *Teflon*[®] cup reservoir.

The procedures to investigate the thiol inhibitory effectiveness were as follows:

i) A volume of 50 µl of 160 µM CuCl₂ soln. was added to the HA soln. (7.85 ml), and the mixture, after a 30-s stirring, was left to stand for 7 min 30 s at r.t. Then, 50 µl of aurothiomalate or GSH (0.16, 1.6, 8, or 16 mM) were added to the soln. followed by stirring again for 30 s. Finally, 50 µl of L-ascorbic acid solns. (16 mM) were added to the soln., and the mixture was stirred for 30 s. The soln. was then immediately transferred into the viscometer *Teflon*[®] cup reservoir.

ii) In the second exper. setting, a procedure similar to that described in i) was applied; however, after standing for 7 min 30 s at r.t., 50 µl of L-ascorbic acid solns. (16 mM) were added, to the mixture and a 30-s stirring followed. After 1 h, finally 50 µl of aurothiomalate or GSH (0.16, 1.6, 8, or 16 mM) were added to the soln., followed by 30-s stirring. The soln. mixture was then immediately transferred into the viscometer *Teflon*[®] cup reservoir.

Rotational Viscometry. The resulting mixture (8.0 ml) was transferred into the *Teflon*[®] cup reservoir of a *Brookfield LVDV-II + PRO* digital rotational viscometer (*Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA*). The recording of viscometer output parameters started 2 min after the experiment onset. The changes of the dynamic viscosity values of the reaction mixture were measured at $25.0 \pm 0.1^\circ$ in 3-min intervals for up to 5 h. The viscometer *Teflon*[®] spindle rotated at 180 rpm, i.e., at a shear rate of 237.6 s^{-1} .

Decolorization ABTS Assay. The ABTS^{•+} radical cations were pre-formed by the reaction of an aq. soln. of K₂S₂O₈ (3.3 mg) in H₂O (5 ml) with ABTS (17.2 mg). The resulting bluish-green radical-cation soln. was stored overnight in the dark below 0°. Before experiment, the soln. (1 ml) was diluted into a final volume (60 ml) with H₂O.

Aurothiomalate (16 mM) and GSH (1 mM) were prepared as aq. stock solns. The investigated samples comprised 2 ml of the diluted ABTS^{•+} soln. with addition of 50 µl of aq. aurothiomalate or GSH.

A modified ABTS assay was used applying a UV/VIS *S2000* spectrophotometer (*Sentronic, Germany*) [22][23]. UV/VIS Spectra were recorded for up to 20 min or 35 min when working with aurothiomalate or GSH, resp.

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