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Free-radical degradation of high-molecular-weight hyaluronan induced by ascorbate *plus* cupric ions. Testing of bucillamine and its SA981-metabolite as antioxidants

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ABSTRACT

High-molecular-weight hyaluronan (HA) samples were exposed to free-radical chain-degradation reactions induced by ascorbate in the presence of Cu(II) ions – the so-called Weissberger's oxidative system. The concentrations of both reactants [ascorbate, Cu(II)] were comparable to those that may occur during an early stage of the acute phase of joint inflammation. The time-dependent changes of the viscosity of the HA solution in the absence of the substance tested were monitored by rotational viscometry. However, when the anti- or pro-oxidative effects of the antioxidants/drugs were investigated, their dose-dependency was also examined. Additionally, the anti-oxidative activities of these substances were screened by the well-established ABTS and DPPH decolorization assays. The actions of the disease-modifying anti-rheumatic drugs, namely bucillamine and D-penicillamine, were compared to those of L-cysteine and of SA981, the oxidized metabolite of bucillamine.

The results indicated that bucillamine was the most efficient scavenger of hydroxyl- and/or peroxyl-type radicals, even at the lowest drug concentration. In contrast, SA981 demonstrated no scavenging activity against the aforementioned free radicals. D-Penicillamine and L-cysteine showed a dual effect, i.e. a pronounced anti-oxidative effect was, after a given time period, followed by a significant pro-oxidative effect.

1. Introduction

Hyaluronan (Fig. 1a; HA) is a unique glycosaminoglycan (GAG) composed of regularly alternating units of *N*-acetyl-D-glucosamine and D-glucuronic acid linked by β-(1 → 4) and β-(1 → 3) linkages. HA is widely distributed in vertebrates. Its molecular size can reach values of up to 10^7 Da [1]. A 70 kg individual has approximately 15 g of HA. This GAG is characterized by an extraordinarily high rate of turnover; one third of its total amount turning over daily. High-molecular-weight HA, whose physiological level within synovial fluid (SF) is 2–3 mg/mL [2], accounts for its important viscoelasticity.

Degradation of high-molecular-weight HA occurring under inflammation and/or oxidative stress is accompanied by impairment and loss of its viscoelastic properties [3]. Low-molecular-weight HA was found to exert different biological activities compared to the native high-molecular-weight biopolymer. HA chains of 25–50 disaccharide units are inflammatory, immunostimulatory, and highly angiogenic. HA fragments of this size appear to function as endogenous danger signals, reflecting tissues under stress [4,5].

Bucillamine [Fig. 1b, *N*-(2-mercapto-2-methylpropionyl)-L-cysteine] is classified as a disease-modifying anti-rheumatic drug (DMARD). It is prescribed in Japan and South Korea for the treatment of rheumatoid arthritis (RA). *In vitro* studies demonstrated positive effects of bucillamine, such as attenuation of various types of reperfusion injury, inhibition of T cell proliferation, cytokine production, *etc.* Some animal studies suggest that bucillamine may attenuate damage during myocardial infarction, cardiac surgery, and other acute inflammatory disorders.

Several investigations have demonstrated the efficacy of bucillamine, administered orally for the treatment of RA. The drug was taken daily by patients in doses ranging from 100 to 300 mg/day, but occasionally as high as 600 mg/day. Serum measurements confirmed good oral bioavailability of bucillamine [6,7]. However, as

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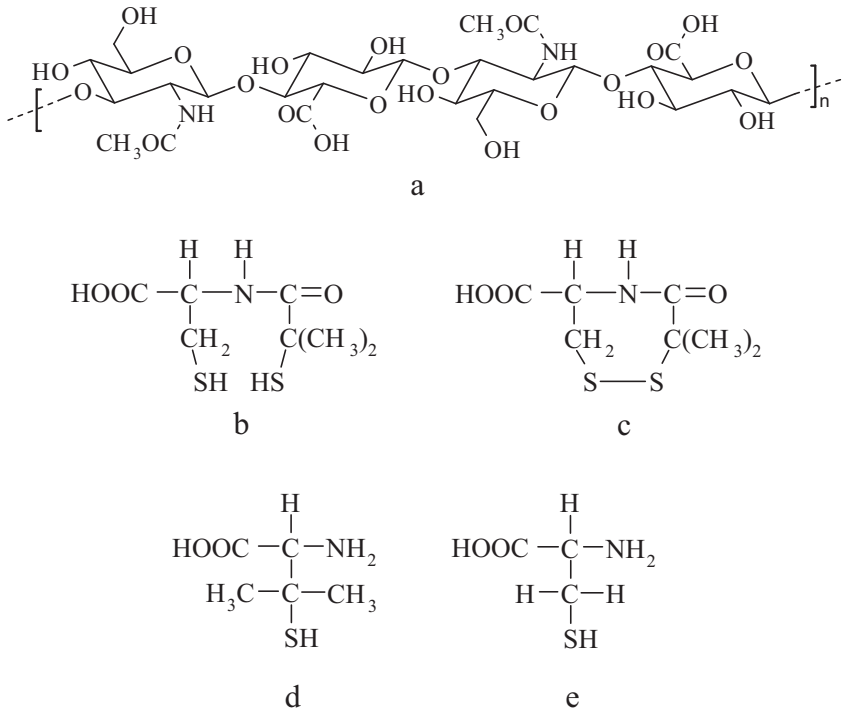


Fig. 1. (a) Hyaluronan, the acid form; (b) bucillamine; (c) SA981; (d) D-penicillamine; and (e) L-cysteine.

already shown, the concentration of the main bucillamine metabolite – SA981 (cf. Fig. 1c) – significantly exceeds that of the parent drug both in serum and in SF of patients treated for RA [8].

D-Penicillamine (Fig. 1d, D-pen, β/β-dimethylcysteine), a metabolite of penicillin, is a DMARD currently used also for the treatment of Wilson's disease. Whereas the drug D-enantiomer is therapeutic, the L-enantiomer is highly toxic [9,10]. *In vitro* studies have shown that D-pen may scavenge free radicals released by activated neutrophils and also inhibit release of the enzyme myeloperoxidase; thus it reduces local tissue damage [9]. The typical daily oral dose of D-pen administered to RA patients is 0.5–2.0 g. The serum half-life of a single oral dose of the drug is 1–3 h [11].

L-Cysteine (Fig. 1e), a naturally occurring amino acid, contributes to the intracellular thiol pool and is a precursor of intracellular L-glutathione biosynthesis [6,12,13]. This amino acid, when used as a nutritional supplement, is usually taken up in the form of *N*-acetyl-L-cysteine [14]. As reported, the mean concentration of L-cysteine in normal human plasma is in the micromolar range (≈ 8 μM [15]). The availability of L-cysteine in body fluids is low, since *in vivo* L-cysteine is rapidly oxidized to cystine [12].

The aim of this contribution was to present results obtained on investigating the effects of two thiol drugs, namely D-penicillamine and bucillamine, the bucillamine oxidized metabolite SA981 and the endogenous substance – L-cysteine – on the degradation kinetics of a high-molecular-weight HA *in vitro*. As a free-radical inducer, the well-known *Weissberger*'s oxidative system [ascorbate *plus* Cu(II) ions] was applied, simulating thus the early phase of acute synovial joint inflammation. Additionally, the anti-oxidative activity of the drug was screened using the well-established ABTS and DPPH decolorization assays.

2. Materials and methods

2.1. Biopolymers

The two high-molecular-weight HA samples P9710-2A (M_w = 808.7 kDa; M_w/M_n = 1.63) and P0207-1 (M_w = 1.07 MDa; M_w/M_n = 1.60) were obtained from Lifecore Biomedical Inc., Chaska, MN, U.S.A. [16].

2.2. Chemicals

Analytical purity grade NaCl and $CuCl_2 \cdot 2H_2O$ were purchased from Slavus Ltd., Bratislava, Slovakia.

D-Penicillamine, L-cysteine, 2,2′-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS; *purum*, >99%), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were the products of Sigma–Aldrich, Steinheim, Germany. Bucillamine and SA981 were gifts of Santen Pharmaceutical Co., Osaka, Japan. Potassium persulfate (*p.a.* purity; max 0.001% nitrogen) and L-ascorbic acid were the products of Merck KGaA, Darmstadt, Germany. Ethanol (96%) and distilled methanol, both *p.a.* purity grade, were purchased from Mikrochem, Pezinok, Slovakia. Redistilled deionized high quality grade water, with conductivity of <0.055 mS/cm, was produced using the TKA water purification system from Water Purification Systems GmbH, Niederelbert, Germany.

2.3. Preparation of stock solutions

The HA sample solutions (2.5 mg/mL) were prepared in the dark at room temperature in 0.15 M aqueous NaCl in two steps: first 4.0 mL of the solvent and after 6 h 3.85, 3.80, or 3.70 mL of the solvent were added. The stock solutions of L-ascorbic acid, L-cysteine, SA981 (all as 16 mM), bucillamine, D-penicillamine (both as 32 mM), and cupric chloride (16 mM diluted to a 160 μM solution) were also prepared in 0.15 M aqueous NaCl.

2.4. Study of uninhibited/inhibited hyaluronan degradation

The procedures related to the study of the degradation of high-molecular-weight HA in the absence and presence of the antioxidant/drug tested were previously described [17–20].

2.5. Rotational viscometry

The resulting reaction mixture (8.0 mL) was transferred to the Teflon® cup reservoir of a Brookfield LVDV-II + PRO digital rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.). The recording of viscometer output parameters started 2 min after onset of the experiment. Changes in dynamic viscosity of the reaction mixture were measured at 25.0 ± 0.1 °C 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}.

2.6. ABTS and DPPH assays

The standard experimental procedures have been reported [21,22]. Briefly, the aqueous solution of $ABTS^{\bullet+}$ radical cation was prepared 24 h before the measurement at room temperature as follows: ABTS aqueous solution (7 mM) was mixed with $K_2S_2O_8$ aqueous solution (2.45 mM) in equivolume ratio. The following day, 1.1 mL of the pre-formed stock aqueous solution was diluted with 96% ethanol to a final volume of 50 mL. Of this ethanol-aqueous reagent 250 μL was added to 2.5 μL of the ethanolic solution of bucillamine, SA981, L-cysteine, or D-pen – the concentration of each was 101 mM. The absorbance of the sample mixture was measured at 734 nm 6 min after mixing the reactants.

In the DPPH assay, the DPPH• solution (55 μM) was prepared by dissolving DPPH in distilled methanol. Then 225 μL of the above mentioned DPPH• radical solution was added to 25 μL of the methanolic solutions of bucillamine, SA981, L-cysteine, or D-pen – the concentration of each was 10 mM. The absorbance of the sample mixtures was measured at 517 nm 30 min after mixing the reactants.

In both assay methods, the substances were tested in a concentration range of 1 mM–8 μM. The measurements were performed in quadruplicate in a 96-well Greiner UV-Star microplates (Greiner-Bio-One GmbH, Germany) with Tecan Infinite M 200 reader (Tecan AG, Austria). The IC_{50} values were calculated using CompuSyn 1.0.1 software (ComboSyn Inc., Paramus, USA). Ionic fractions and log *D* values of bucillamine, SA981, L-cysteine or D-pen were calculated by free webservice: http://www.pharma-algorithms.com/webboxes/.

3. Results

3.1. ABTS and DPPH assays

The bucillamine scavenging activities, represented by the IC_{50} values of 4.00 and 8.96 μM as determined by the ABTS and DPPH assays (cf. Table 1), were greatly elevated. The drug IC_{50} values were comparable to those for quercetin, a substance typically classified as a standard natural antioxidant: 2.86 and 4.36 μM. (For determining the quercetin scavenging activity, ABTS and DPPH assays were carried out under conditions identical to those in Section 2.6.)

Table 1
IC_{50} values of the substances determined using ABTS and DPPH assays.

Substance	ABTS, IC_{50} [μM]	DPPH, IC_{50} [μM]
Bucillamine	4.00	8.96
SA981	Not detectable	Not detectable
D-Penicillamine	5.26	35.8
L-Cysteine	1300	303

3.2. Rotational viscometry

It is well documented that concentration of copper ions is significantly higher in plasma and SF of RA patients [23]. In the early phase of acute joint inflammation, levels of acute phase proteins are elevated. One of these proteins, ceruloplasmin, releases two loosely bound atoms of Cu ions. Weissberger's oxidative system ascorbate *plus* Cu(II) can be applied for *in vitro* studies simulating the conditions that occur in the early stage of joint inflammation. The initial viscosity of the solution of high-molecular-weight HA gradually decreases when a generator of e.g. •OH radicals is introduced. As documented in Fig. 2, panels A and B, curve 0, an increased rate of degradation of HA was observed. However, addition of bucillamine protected HA against free-radical degradation in a dose-dependent manner, either when the drug was applied at the experiment onset – at time 0 min – or 1 h after onset of the reaction (cf. Fig. 2, panels A and B, curve 4).

In Fig. 2, bucillamine can be observed to be not only a preventive antioxidant, but also as an efficient chain-breaking antioxidant. The latter, inhibition of the free-radical chain reaction, confirmed this property of bucillamine. This drug, containing two free –SH groups, may function both as a scavenger of free hydroxyl- and peroxyl-type radicals by inhibiting the propagation phase of the deleterious radical chain reaction. Simultaneously, bucillamine may significantly attenuate the re-initiation of the "firing up" reaction by chelating Cu ions.

The fact that bucillamine scavenges hydroxyl- and peroxyl-type radicals as well as donating one or most plausibly two hydrogen radicals (H•) results in the generation of the SA981 compound, which exhibits practically no reducing power (cf. Table 1). The results displayed in Fig. 3, panel A reflect precisely this expectation. The greater the level of SA981 added to the reaction mixture at time 0, the greater is the protection of HA from degradation initiated by •OH radicals. This effect could be attributed plausibly to abstraction

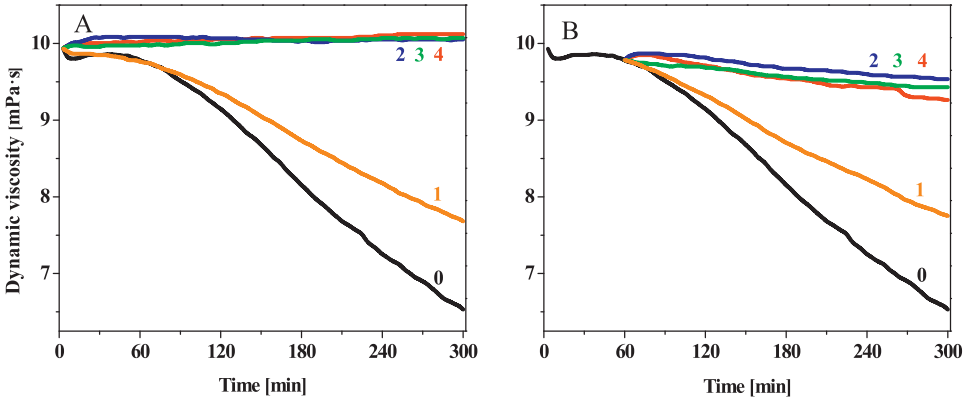


Fig. 2. Effect of bucillamine on HA degradation induced by the oxidative system containing 1.0 μM $CuCl_2$ + 100 μM ascorbic acid. Concentrations of bucillamine in the system before the start of HA degradation (A) or after 1 h (B) in μM: 0 (0); 1 (1); 10 (2); 50 (3); and 100 (4).

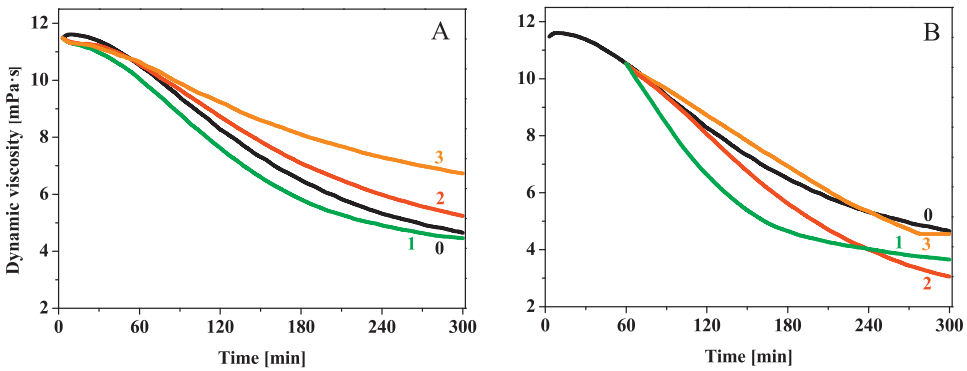


Fig. 3. Effect of SA981 on HA degradation induced by the oxidative system containing 1.0 μM $CuCl_2$ + 100 μM ascorbic acid. Concentrations of SA981 in the system before the start of HA degradation (A) or after 1 h (B) in μM: 0 (0); 100 (1); 200 (2); 400 (3).

of an H• radical from the SA981 molecule, resulting partly in scavenging of •OH radicals. Yet as shown in Fig. 3, panel B the substance SA981 did not scavenge peroxyl-type radicals even at the highest concentration applied.

For comparison, Fig. 4 demonstrates the efficacy of D-pen. In panel A the curve 2 demonstrates the inhibitory effect of the drug against HA degradation for up to 60 min. Furthermore, a significant decrease of the viscosity of the biopolymer solution was observed. On comparing the shapes of curves 1 and 2 with that of 0, a significant enhancement of the HA degrading process was detected.

Fig. 4, panel B, curve 2 demonstrates that at the highest L-cysteine concentration, 100 μM, total inhibition of HA degradation was observed for approx. 2 h, while afterwards the inhibitory process was proceeding in part, by a significant reduction of HA degradation. When, however, a lower amount of L-cysteine (50 μM) was applied, a pro-oxidative effect of this amino acid was observed (cf. Fig. 4, panel B, curve 1).

4. Discussion

4.1. ABTS and DPPH assays

The decolorization of the $ABTS^{\bullet+}$ radical cation solution or that of the DPPH• radical can be described by the chemical reactions during which a reductant provides an electron to the acceptor – the $ABTS^{\bullet+}$ radical cation or of the DPPH• radical. The reductant, which in the case of thiol-derived compounds could be described as R–SH, is oxidized as follows

$$R\text{–}SH - e^- \rightarrow R\text{–}S^{\bullet} + H^+$$

Hence the chemical structure of bucillamine (cf. Fig. 1b) establishes this drug to be a highly efficient reducing agent. In the case of providing the electrons from both –SH groups, bucillamine could "metabolize" to its oxidized form, named SA981. Yet since the SA981 compound has no further freely "decomposable" thiol group(s) in its structure, this substance has no or only a weak reducing power. As revealed by the ABTS and DPPH assays, this statement is also supported by the undetectable scavenging activity of SA981 (cf. Table 1).

The scavenging activities determined using both ABTS and DPPH assays indicate that L-cysteine can be indeed classified as a poor electron donor. The IC_{50} values determined for this amino acid significantly exceeded those found for both drugs, i.e. D-pen and bucillamine (cf. Table 1). However, it should be pointed out that the readiness of a thiol compound to provide an electron does not indicate that the substance is simultaneously a donor of the H• radical. Steric factors, degree of protonation, and some further determinants may considerably influence the availability of H• radical to be abstracted from the –SH group of the given thiol substance. Thus the readiness of the H• radical donation is another factor that should be taken into account when the given thiol substance/drug is to be used against generating free radicals, such as those of the hydroxyl- and peroxyl-types.

4.2. Uninhibited degradation of high-molecular-weight hyaluronan

Under aerobic conditions, the bicomponent system composed of ascorbate and cupric ions is generating a powerful oxidant – hydrogen peroxide [24–26]. To describe the particular processes involved, the following flow chart, which characterizes the indi-

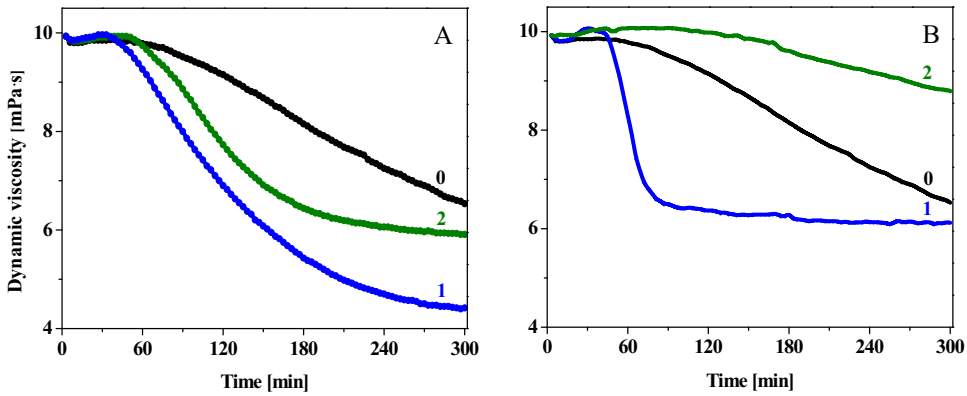
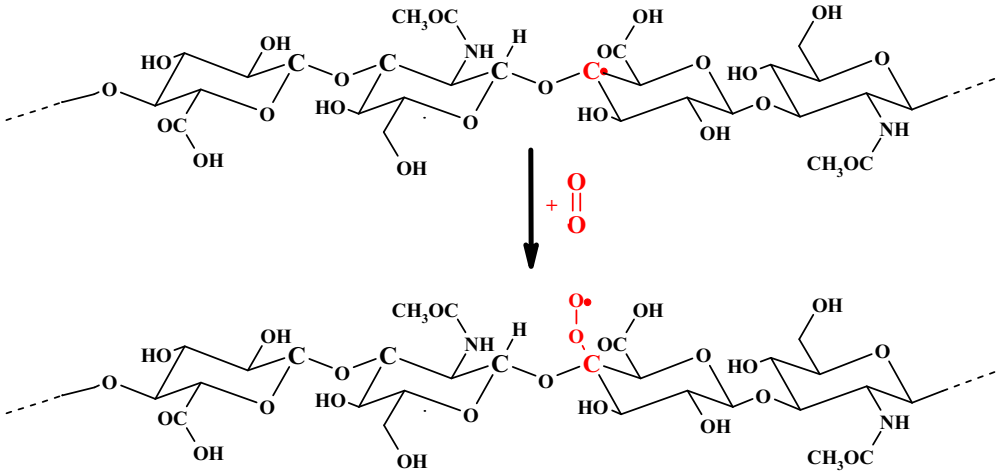


Fig. 4. Effect of D-penicillamine (panel A) or L-cysteine (panel B) on HA degradation induced by the oxidative system containing 1.0 μM $CuCl_2$ + 100 μM ascorbic acid. Concentrations of D-penicillamine or L-cysteine in the system before the start of HA degradation in μM: 0 (0); 50 (1); 100 (2).



Scheme 1. Due to its high reactivity, the hyaluronan *C*-type macroradical immediately traps a molecule of dioxygen yielding a peroxyl-type macroradical.

vidual reaction steps, represents the concerted action:

$$AscH^- + Cu(II) \rightarrow Asc^{\bullet -} + H^+ + Cu(I),$$

reduction of cupric to cuprous ions (1)

Ascorbate ($AscH^-$) donates H• radical or ($H^+ + e^-$) yielding the tricarbonyl ascorbate free radical, which in aqueous solution should be present as $Asc^{\bullet -}$ [27]. The generated unstable cuprous ions quickly undergo a charge stabilization interaction by an excess of ascorbate:

$$AscH^- + Cu(I) \rightarrow [AscH^- \cdots Cu(I)].$$

This intermediate participates in the next bi-electron reduction of the dioxygen molecule (O=O)

$$[AscH^- \cdots Cu(I)] + O{=}O + H^+ \rightarrow Asc^{\bullet -} + Cu(II) + H_2O_2,$$

hydrogen peroxide formation (2)

and the nascent H_2O_2 molecule is decomposed immediately by the "uncomplexed"/complexed Cu(I) ion(s) according to a Fenton-like reaction:

$$H_2O_2 + [Cu(I)] \rightarrow {}^{\bullet}OH + HO^- + Cu(II),$$

generation of hydroxyl radicals (3)

Finally, the following net reaction indicates the generation of extremely reactive hydroxyl radicals:

$$AscH^- + [AscH^- \cdots Cu(I)] + O{=}O \rightarrow 2Asc^{\bullet -} + Cu(II) + HO^- + {}^{\bullet}OH,$$

(net reaction)

However, since at the beginning of the reaction within the system of reactants there is an ascorbate excess, the generated nascent •OH radicals are continually scavenged. According to our previous findings based on spin-trapping EPR spectroscopy [28], under the experimental conditions (Figs. 2 and 3, curve 0) shown during the earlier time intervals (≤60 min, Fig. 2 or ≤30 min, Fig. 3), monitoring the sole generation of the ascorbyl anion radicals ($Asc^{\bullet -}$) indicated that any nascent •OH radical generated is scavenged immediately. Yet at later time intervals – from approx. 30 or 60 to 300 min – clear evidence of the presence of unscavenged •OH radicals was found by the spin-trapping EPR spectroscopic method. The trace fraction of nascent •OH radicals reacts with the HA macromolecule by abstracting H• radical, resulting in the formation of a *C*-macroradical represented in Scheme 1 [29], further denoted as A•:

$$HA + {}^{\bullet}OH \rightarrow A^{\bullet} + H_2O \quad (4)$$

Under aerobic conditions, the alkyl-type macroradical – A• – reacts rapidly with the molecule of dioxygen (O_2) yielding a peroxyl-type macroradical, hereafter denoted as AOO•. The formed intermediate AOO• may react with an adjacent HA macromolecule – and in that way the radical chain reaction propagates rapidly:

$$AOO^{\bullet} + HA \rightarrow AOOH + A^{\bullet},$$

propagation of the radical chain reaction (5)

After its "collision" with an HA macromolecule (cf. reaction (5)), the generated peroxyl-type macro-radical yields a high-molecular-weight hydroperoxide (AOOH), which subsequently, mostly induced by the present Cu(I) ions {$AOOH + Cu(I) \rightarrow AO^{\bullet} + HO^- + Cu(II)$}, yields an alkoxyl-type macro-radical (AO•). This is a presumed intermediate of the main chain-cleavage resulting in biopolymer fragments. Their solution is characterized by a reduced viscosity, represented in Scheme 2 [30,31].

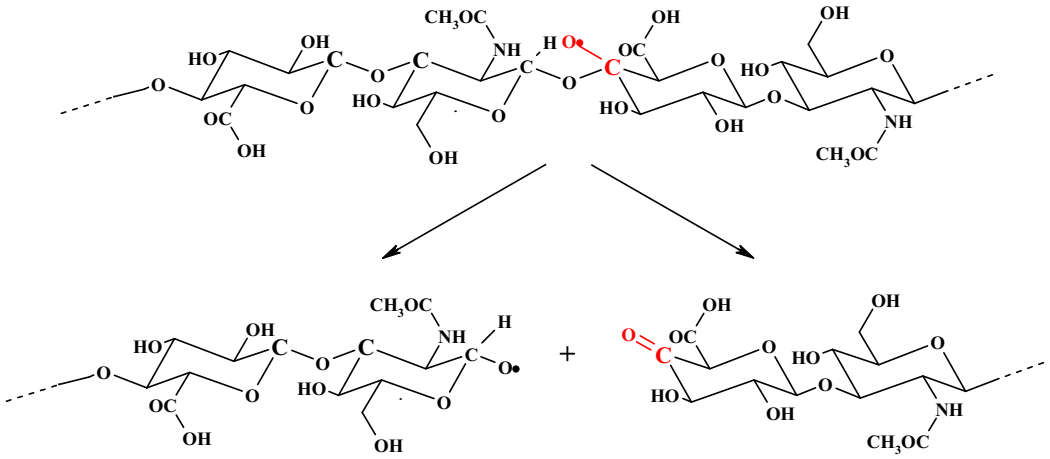
The attack of hydroxyl radicals on D-glucuronate or *N*-acetyl-D-glucosamine moieties of HA can also lead to the "opening" of rings without breaking the polymer chain [31,32].

4.3. Inhibited degradation of high-molecular-weight hyaluronan

In accord with previously presented data, it is evident that an efficient antioxidant (donor of H• radical) may retard or even totally inhibit the step of HA degradation initiation by scavenging all •OH radicals. Thus no A• type macro-radicals are generated. Such substances efficiently stop both the initiation and the propagation of the free-radical reaction and are therefore classified as preventive antioxidants. When however these substances significantly retard or even totally inhibit the free-radical reaction in its phase of propagation, they are classified as chain-breaking antioxidants.

According to this classification, it is obvious that bucillamine is both a preventive and chain-breaking antioxidant. Yet its metabolite, SA981, demonstrated no significant protective effect against HA degradation in the Weissberger oxidative system.

By taking into account the IC_{50} values determined for D-pen (cf. Table 1), a relatively high electron donating capability can be stated for this DMARD. Moreover, the chemical structure (see Fig. 1d) indicates a potential of D-pen to donate one H• radical and thus to act as



Scheme 2. Fragmentation of the alkoxy-type macroradical (AO•).

a scavenger of •OH radicals generated by the Weissberger oxidative system. The latter tenet is however only partially correct.

To explain the results presented in Fig. 4, the following sets of reactions should be considered as valid:

$$R\text{–}SH + {}^{\bullet}OH \rightarrow R\text{–}S^{\bullet} + H_2O, \quad \text{formation of a thiyl-type radical}$$

$$R\text{–}SH \rightarrow R\text{–}S^{-}, \quad \text{ionization of the thiol functional group}$$

$$R\text{–}S^{\bullet} + R\text{–}S^{-} \rightarrow [R\text{–}SS\text{–}R]^{\bullet -},$$

generation of an anion radical intermediate substance

$$[R\text{–}SS\text{–}R]^{\bullet -} + O_2 \rightarrow [R\text{–}SS\text{–}R] + O_2^{\bullet -},$$

formation of superoxide anion radical

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^{+} \rightarrow H_2O_2 + O_2,$$

dismutation reaction, generation of hydrogen peroxide

The powerful oxidant such as H_2O_2 subsequently undergoes reaction (3) generating •OH radicals. The above sets of reactions are well established for several additional thiol-derived substances, including glutathione (G–SH) yielding $[G\text{–}SS\text{–}G]^{\bullet -}$ – the oxidized glutathione anion radical [16,19,20,33–35].

Finally, according to the IC_{50} values determined for L-cysteine (cf. Table 1), practically no electron donating capability can be claimed for this amino acid. Yet in accordance with its chemical structure, this thiol-derived amino acid can be a donor of H•, yielding, analogous to D-pen, a thiol-derived free radical substance of the formula R–S•.

In conclusion, the observations of this study could stimulate further efforts to prove or disprove the anti- and/or pro-oxidative participation of thiol-derived antioxidants/drugs in free-radical HA degradation initiated by the ascorbate *plus* Cu(II) system. The opposing pro- and anti-oxidative properties of D-penicillamine may be relevant to its toxic or to its therapeutic properties.

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References

[1] R. Stern, Devising a pathway for hyaluronan catabolism: are we there yet? Glycobiology 13 (2003) 105–115.
[2] E.A. Balazs, D. Watson, I.F. Duff, S. Roseman, Molecular parameters of hyaluronic acid in normal and arthritis human fluids, I, Arthritis Rheum. 10 (1967) 357–376.
[3] B.J. Parsons, S. Al-Assaf, S. Navaratnam, G.O. Phillips, Comparison of the reactivity of different oxidative species (ROS) towards hyaluronan, in: J.F. Kennedy, G.O. Phillips, P.A. Williams, V.C. Hascall (Eds.), Hyaluronan: Chemical, Biochemical and Biological Aspects, Woodhead, Publishing Ltd., Cambridge, MA, 2002, pp. 141–150.
[4] P.W. Noble, Hyaluronan and its catabolic products in tissue injury and repair, Matrix Biol. 21 (2002) 25–29.
[5] D.C. West, I.N. Hampson, F. Arnold, S. Kumar, Angiogenesis induced by degradation products of hyaluronic acid, Science 228 (1985) 1324–1326.
[6] L.D. Horwitz, Bucillamine: a potent thiol donor with multiple clinical applications, Cardiovasc. Drug Rev. 21 (2003) 77–90.
[7] D. Mazor, L. Greenberg, D. Shamir, D. Meyerstein, N. Meyerstein, Antioxidant properties of bucillamine: possible mode of action, Biochem. Biophys. Res. Commun. 349 (2006) 1171–1175.
[8] H. Matsuno, E. Sugiyama, A. Muraguchi, T. Nezuka, T. Kubo, K. Matsura, H. Tsuji, Pharmacological effects of SA96 (bucillamine) and its metabolites as immunomodulating drugs—the disulfide structure of SA-96 metabolites plays a critical role in the pharmacological action of the drug, Int. J. Immunopharmacol. 20 (1998) 295–304.
[9] R. Munro, H.A. Capell, Penicillamine, in: Disease-Modifying Drugs Series, Br. J. Rheumatol. 36 (1997) 104–109.
[10] X. Yang, H. Yuan, C. Wang, X. Su, L. Hu, D. Xiao, Determination of penicillamine in pharmaceuticals and human plasma by capillary electrophoresis with in-column fiber optics light-emitting diode induced fluorescence detection, J. Pharm. Biomed. Anal. 45 (2007) 362–366.
[11] J.W. Coleman, A.L. Foster, J.H.K. Yeung, B.K. Park, Drug–protein conjugates XV. A study of the disposition of D-penicillamine in the rat and its relationship to immunogenicity, Biochem. Pharmacol. 37 (1988) 731–742.
[12] B. Hultberg, M. Hultberg, High glutathione turnover in human cell lines revealed by acivicin inhibition of γ-glutamyltranspeptidase and the effects of thiol-reactive metals during acivicin inhibition, Clin. Chim. Acta 349 (2004) 45–52.
[13] C. Carrasco-Pozo, M.E. Aliaga, C. Olea-Azar, H. Speisky, Double edge redox-implications for the interaction between endogenous thiols and copper ions: in vitro studies, Bioorg. Med. Chem. 16 (2008) 9795–9803.
[14] J.C. Adair, J.E. Knoefel, N. Morgan, Controlled trial of *N*-acetylcysteine for patients with probable Alzheimer's disease, Neurology 57 (2001) 1515–1517.
[15] R.A. Patterson, D.J. Lamb, D.S. Leake, Mechanisms by which cysteine can inhibit or promote the oxidation of low density lipoprotein by copper, Atherosclerosis 169 (2003) 87–94.
[16] K. Valachová, R. Mendichi, L. Šoltés, Effect of L-glutathione on high-molar-mass hyaluronan degradation by oxidative system Cu(II) plus ascorbate, in: R.A. Pethrick, P. Petkov, A. Zlatarov, G.E. Zaikov, S.K. Rakovsky (Eds.), Monomers, Oligomers, Polymers, Composites, and Nanocomposites, Nova Science Publishers Inc., New York, 2010, pp. 101–111.

[17] E. Orviský, L. Šoltés, M. Stančíková, High-molecular-mass hyaluronan—valuable tool in testing the antioxidative activity of amphiphilic drugs stobadine and vinpocetine, J. Pharm. Biomed. Anal. 16 (1997) 419–424.
[18] L. Šoltés, M. Stankovská, G. Kogan, R. Mendichi, N. Volpi, V. Sasinková, P. Gemeiner, Degradation of high-molar-mass hyaluronan by an oxidative system comprising ascorbate, Cu(II), and hydrogen peroxide: inhibitory action of antiinflammatory drugs—naproxen and acetylsalicylic acid, J. Pharm. Biomed. Anal. 44 (2007) 1056–1063.
[19] K. Valachová, P. Rapta, G. Kogan, E. Hrabárová, P. Gemeiner, L. Šoltés, Degradation of high-molar-mass hyaluronan by ascorbate plus cupric ions: effects of D-penicillamine addition, Chem. Biodivers. 6 (2009) 389–395.
[20] E. Hrabárová, K. Valachová, J. Rychlý, P. Rapta, V. Sasinková, M. Malíková, L. Šoltés, High-molar-mass hyaluronan degradation by Weissberger's system: pro- and anti-oxidative effects of some thiol compounds, Polym. Degrad. Stab. 94 (2009) 1867–1875.
[21] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radic. Biol. Med. 26 (1999) 1231–1237.
[22] Z. Cheng, J. Moore, L. Yu, High-throughput relative DPPH radical scavenging capacity assay, J. Agric. Food Chem. 54 (2006) 7429–7436.
[23] M. Yazar, S. Sarban, A. Kocyigit, U.E. Isikan, Synovial fluid and plasma selenium, copper, zinc, and iron concentrations in patients with rheumatoid arthritis and osteoarthritis, Biol. Trace Elem. Res. 106 (2005) 123–132.
[24] A.E.O. Fisher, D.P. Naughton, Vitamin C contributes to inflammation via radical generating mechanisms: a cautionary note, Med. Hypotheses 61 (2003) 657–660.
[25] A.E.O. Fisher, D.P. Naughton, Iron supplements: the quick fix with long-term consequences, Nutr. J. 3 (2004) 2.
[26] A.E.O. Fisher, D.P. Naughton, Therapeutic chelators for the twenty first century: new treatments for iron and copper mediated inflammatory and neurological disorders, Curr. Drug Deliv. 2 (2005) 261–268.
[27] G.R. Buettner, F.Q. Schafer, Ascorbate (Vitamin C), Its Antioxidant Chemistry, The Virtual Free Radical School for Oxygen Society, 2002, http://www.healthcare.uiowa.edu/corefacilities/esr/publications/buettnerpubs/pdf/Buettner-Ascorbate-Chemistry-1.pdf.
[28] L. Šoltés, M. Stankovská, V. Brezová, J. Schiller, J. Arnhold, G. Kogan, P. Gemeiner, Hyaluronan degradation by cupric chloride and ascorbate: rotational viscometric, EPR spin trapping, and MALDI-TOF mass spectrometric investigations, Carbohydr. Res. 341 (2006) 2826–2834.
[29] M.D. Rees, C.L. Hawkins, M.J. Davies, Hypochlorite and superoxide radicals can act synergistically to induce fragmentation of hyaluronan and chondroitin sulphates, Biochem. J. 381 (2004) 175–184.
[30] J. Rychlý, L. Šoltés, M. Stankovská, I. Janigová, K. Csomorová, V. Sasinková, G. Kogan, P. Gemeiner, Unexplored capabilities of chemiluminescence and thermoanalytical methods in characterization of intact and degraded hyaluronans, Polym. Degrad. Stab. 91 (2006) 3174–3184.
[31] C.L. Hawkins, M.J. Davies, Detection of intermediates formed on reaction of hyaluronic acid and related materials with the hydroxyl radical, Biochem. Soc. Trans. 23 (1995) S248.
[32] C.L. Hawkins, M.J. Davies, Direct detection and identification of radicals generated during the hydroxyl radical-induced degradation of hyaluronic acid and related materials, Free Radic. Biol. Med. 21 (1996) 275–290.
[33] L. Wlodek, Beneficial and harmful effects of thiols, Pol. J. Pharmacol. 54 (2002) 215–223.
[34] L. Šoltés, R. Mendichi, G. Kogan, J. Schiller, M. Stankovská, J. Arnhold, Degradative action of reactive oxygen species on hyaluronan, Biomacromolecules 7 (2006) 659–668.
[35] K. Valachová, E. Hrabárová, P. Gemeiner, L. Šoltés, Study of pro- and anti-oxidative properties of D-penicillamine in a system comprising high-molar-mass hyaluronan, ascorbate, and cupric ions, Neuroendocrinol. Lett. 29 (2008) 697–701.