CHEMISTRY and PHYSICS of MODERN MATERIALS

Processing, Production and Applications

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CHAPTER 6

PRACTICAL HINTS ON TESTING VARIOUS HEXAHYDROPYRIDOINDOLES TO ACT AS ANTIOXIDANTS

KATARINA VALACHOVA, MARIA BANASOVA, LUBICA MACHOVA, IVO JURANEK, STEFAN BEZEK, and LADISLAV SOLTES

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6.1 INTRODUCTION

Inflammation of synovial joints is accompanied by a decrease in the viscosity of synovial fluid (SF), in which hyaluronan (HA, called also hyaluronic acid; Fig. 1) is the macromolecular component, which imparts the SF viscosity. It has been hypothesized that reactive oxygen species (ROS), produced by (infiltrated) neutrophils, may be responsible for the degradation of HA macromolecules within the SF of patients suffering from rheumatoid arthritis (RA). *In vitro* studies underline that of the various individual ROS, hydroxyl radicals – •OH are the most degradative against the HA chain [1].

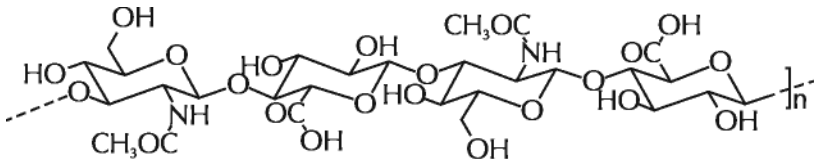
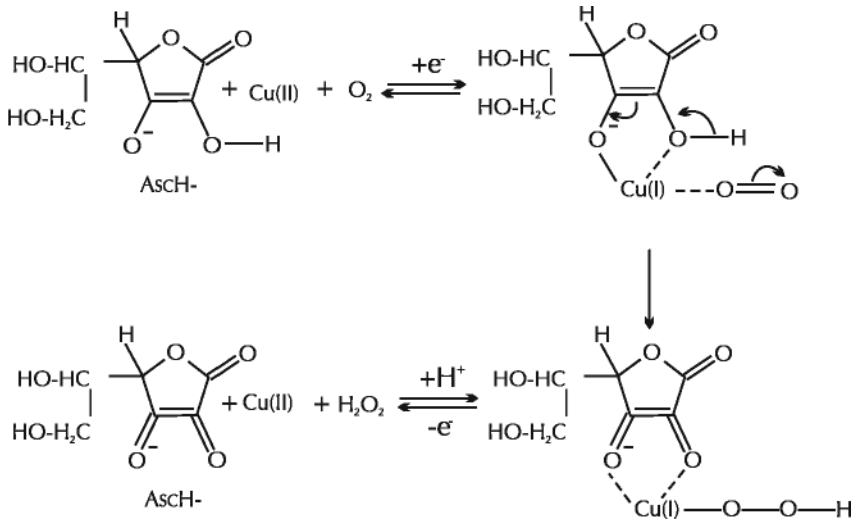


FIGURE 1 Hyaluronan − acid form.

Several *in vitro* generators of •OH radicals investigated, exploit primarily the system comprising hydrogen peroxide and reduced transition metal ions, mostly Fe(II) – the Fenton reactants. One fact has however been criticized on applying the Fenton's generator of •OH radicals, namely that the experiments at which H_2O_2 solution is applied like a "bolus" are really far from pathophysiological conditions, which are actually involved also in inflammation of synovial joints. *In vivo*, the process of generation of hydrogen peroxide as a pre-cursor of further ROS should run continually and the *in situ* "born" H_2O_2 molecules should be stepwise converted to •OH radicals. Of such continual generators of hydrogen peroxide and/or hydroxyl radicals we would like to call attention to the so called Weissberger's biogenic oxidative system – WBOS (cf. Scheme 1 [2−7].

$AscH^-$ and DHA denote ascorbate anion and dehydroascorbate.



SCHEME 1 Chemistry of Weissberger's biogenic oxidative system: Hydrogen peroxide is generated by oxidation of ascorbate at catalytic action of Cu(II) ions (adapted from Ref. [8]).

6.1.1 WBOS – CONDITION SETTINGS

Taking into account the well known fact that in most human tissues, including that of SF, the concentrations of ascorbate never exceed the value of 200 μM [9] (in mean ≈ 100 μM), settings of ascorbate concentration in the WBOS to 100 μM is comprehensible. The second variable in the WBOS, namely the level of Cu(II) ions, can fall to a few μM /l (4.33 μM – as determined in *post mortem* collected SF from subjects without evidence of connective tissue disease [10]. Yet, as reported by Naughton et al. [11], the level of copper ions in the SF ultrafiltrates of RA patients equals 0.125 ± 0.095 μM. Therefore, the application of 0.1 μM of cupric ions (in the form of e.g., $CuCl_2$) as the second variable in the WBOS could well model pathophysiological conditions. Thus it can be claimed that the Cu(II) concentration equaling 0.1 μM along with 100 μM of ascorbate is a proper setting, especially valuable to model the situation within SF during the early stage of acute phase inflammation of synovial joints [12]. Under such [Cu(II)]:[ascorbate] setting, it is evident (cf. Scheme 1) that within

one single reaction cycle 0.1 μmol H_2O_2 is yielded, which by action of e.g. the intermediate Cu(I)-complex is altered to 0.1 μmol of $^{\bullet}OH$ radicals. One fraction of these radicals reacts *in statu nascendi* with the present (target) HA macromolecules, while another fraction is scavenged by the ascorbate excess according to the reaction.

$$AscH^{-} + {}^{\bullet}OH \rightarrow Asc^{\bullet -} + H_2O \quad (1)$$

where $Asc^{\bullet -}$ denotes ascorbyl anion radicals, which disproportionate immediately, yielding ascorbate and DHA.

6.1.2 WBOS UTILITY

For testing the efficiency of a substance in function as preventive antioxidant and/or scavenger of the generated $^{\bullet}OH$ radicals (the substance H atom donating property) one should take into account that on applying a potentially "perfect/absolute inhibitor" of HA degradation induced by the $^{\bullet}OH$ radical, the experimental curve should copy the gray one (cf. Appendix, Fig. 1). On the contrary, the substance with nil $^{\bullet}OH$ radical trapping properties (donor of no H atom) should yield a curve, which superimposes that of the black one (cf. Appendix, Fig. 1). Results of measurements of time dependencies on the HA solution dynamic viscosity falling within the region between the gray and black curves relate to greater or lower efficiency of the test substance to act as a preventive antioxidant.

Since the primary/initiation step of the HA reaction with $^{\bullet}OH$ radical should yield a reactive *C*-type macroradical (hereafter denoted as $A^{\bullet}$), under aerobic conditions the $A^{\bullet}$ macroradicals should react with dioxygen yielding a peroxyl-type macroradical (hereafter denoted as $AOO^{\bullet}$).

$$HA + {}^{\bullet}OH \rightarrow A^{\bullet} + H_2O$$
$$A^{\bullet} + O{=}O \rightarrow AOO^{\bullet}$$

and the $AOO^{\bullet}$ macroradical immediately starts the propagation phase of the free-radical chain degradation of HA macromolecules. This fact could, however, be exploited for testing substances acting as chain-breaking antioxidants.

For testing the efficiency of a substance in function as a chain-breaking antioxidant and/or a scavenger of the propagated $AOO^{\bullet}$

radicals, one should take into account that a "perfect/absolute chain-breaker" of the propagation phase of the free-radical HA degradation should result in an experimental curve, as represented by the gray one (cf. Appendix, Fig. 2). On the other hand, the substance with nil chain-breaking properties (donor of no H atom) should yield a curve, which superimposes that of the black one (cf. Appendix, Fig. 2). Results of time-dependent measurements of dynamic viscosity of the HA solution falling within the region between the gray and black curves relate to a greater or lower efficiency of the test substance to act as a chain-breaking antioxidant.

Both spectrophotometric methods, namely the ABTS and DPPH decolorization assays, cannot be classified as absolute methods [13]. By the reduction action of the test substance, the color indicator ($ABTS^{\bullet+}$ or $DPPH^{\bullet}$) is converted to the final compound ABTS or $DPPH^{-}$ according to the reactions

$$ABTS^{\bullet+} + e^{-} \rightarrow ABTS \qquad \text{(one electron reduction)}$$
$$DPPH^{\bullet} + e^{-} \rightarrow DPPH^{-} \qquad \text{(one electron reduction)}$$

By measuring the reaction kinetics, one can classify the test substance as either fast or slowly acting reductant. Measurements at a pre-selected time interval after the reaction onset show that the substance reduction property can be coined, e.g., as an IC_{50} value. Although both assays are still often exploited, their impact is questionable since neither the $ABTS^{\bullet+}$ nor $DPPH^{\bullet}$ indicator represents any biomolecule, and is not even found in any biological system.

Vast amount of hexahydropyridoindoles with different physico-chemical properties have been synthesized so far [14−16]. Of them, all five derivatives, namely the dihydrochlorides of – stobadine (**1**) and SM1dM9dM10 (**2**) and the monohydrochlorides of – SME1i-ProC2 (**3**), SM1M3EC2 (**4**), and SMe1EC2 (**5**) (see Appendix, Fig. 3), which undergone pharmacodynamic studies are examined and reported in this paper from the point of view of their H atom donating as well as reductive properties.

6.2 RESULTS AND DISCUSSION

6.2.1 WEISSBERGER'S BIOGENIC OXIDATIVE SYSTEM

Scheme 1 implicates the statement that, e.g., at the ratio of the reactants [Cu(II)]:[ascorbate] = 0.1/100 the reaction cycle will be repeated

1000-times and at 100% efficacy of all elementary reaction steps the products will be DHA and H_2O_2 – both in the amount of 100 μmol. This statement is naturally incorrect since the product generated, i.e., hydrogen peroxide is decomposed yielding •OH radicals due to the presence of the reactant Cu(II) reduced to Cu(I)-intermediate [17]. According to reaction (1), however, during the early stage of the reaction cycles, there is a high molar surplus of ascorbate within the system, and thus most of the generated •OH radicals will be immediately scavenged and the exclusively detectable "radical product" in the reaction mixture will be $Asc^{\bullet-}$ – the ascorbyl anion radicals. This implicit conclusion was proved by EPR measurements of the aqueous system comprising $CuCl_2$ (0.1 μmol), ascorbic acid (100 μmol), and the spin-trapping agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; 250 mmol) [18]: During the first approximately 60 min of the reaction of WBOS components, the exclusive EPR signal detected was that belonging to ascorbyl anion radical ($Asc^{\bullet-}$; Fig. 2a). The •DMPO-OH adduct was detectable as late as 1 h after the reaction initiation, i.e., after disappearance of the EPR signal of ascorbyl anion radical, pointing to the depletion of ascorbate in the reaction mixture monitored. Figure 2b shows an explanatory chart of the time courses of the integral EPR signals of $Asc^{\bullet-}$ anion radical and the •DMPO-OH adduct.

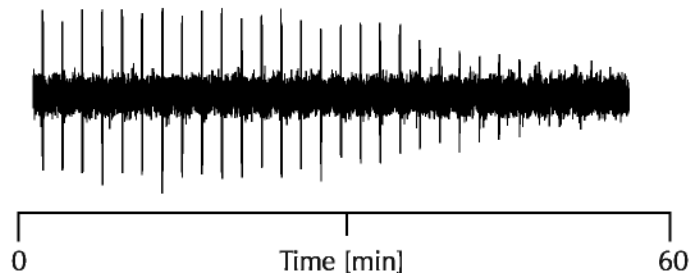


FIGURE 2(A) Time course of EPR spectra of the aqueous mixture containing $CuCl_2$ (0.1 μmol), ascorbic acid (100 μmol), and spin trapper DMPO (250 mmol) at room temperature – adapted from Šoltés et al. [18].

The record illustrates the scans of the $Asc^{\bullet-}$ anion radical evidenced in time from 0.5 to 56 min.

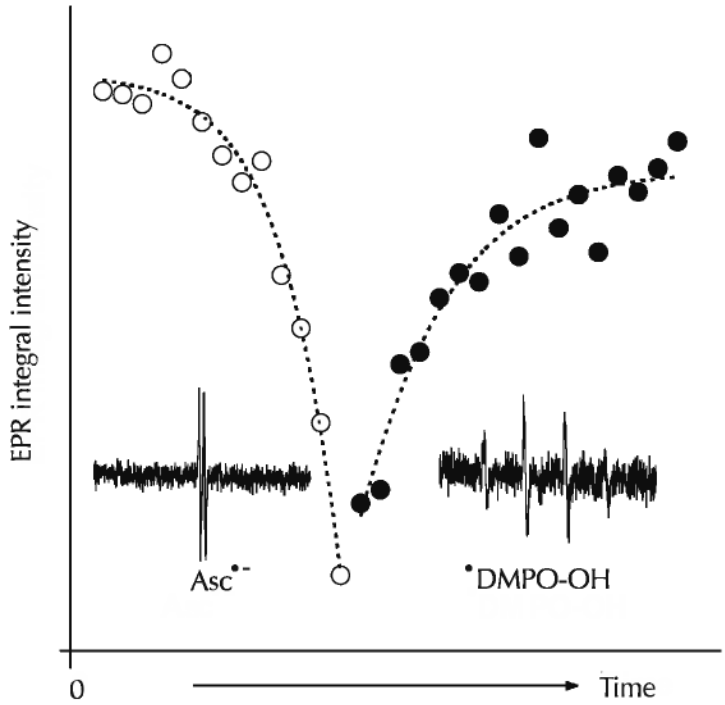


FIGURE 2(B) Illustrative representation of the time dependences of the integral EPR signals of $Asc^{\bullet-}$ anion radical (○) and the •DMPO-OH adduct (●) – adapted from Šoltés *et al*. [18].

In the figure, both the EPR spectrum of the ascorbyl anion radical $Asc^{\bullet-}$ and that of the •DMPO-OH aduct are depicted.

6.2.2 HYALURONAN FREE-RADICAL DEGRADATION

As can be deduced from the above-mentioned observations (cf. Figs. 2a and 2b), by applying WBOS, •OH radicals should be generated after a certain time delay, i.e., after the "consumption" of a certain amount of ascorbate, which acted as a scavenger of hydroxyl radicals. Yet, in spite of such an expectation, after ascorbic acid addition to the HA solution

containing copper ions, the degradation of the biopolymer macromolecules starts practically immediately (cf. Appendix, Figs. 1 and 2, black curves). Dynamic viscosity *vs*. time relationship of the solution monitored decreases gradually and the initial dynamic viscosity (η) value = 9.93 mPa×s (corresponding to M_w = 808.7 kDa) decreases in time and in the 5th h its value equals 6.30 mPa×s (corresponding to $M_w \approx 420$ kDa [19]).

To elucidate the above-mentioned observations it is necessary to note here the following: Under the experimental conditions used within the solution mixture monitored (pH ≈ 6.0–6.5), the HA macromolecules (2.5 mg/ml) are highly ionized since the pK_a values of the D-glucuronic acid residues equal 3.12 [20]. The anions of the D-glucuronic acids (6.25 mmol) – the structural elements of HA macromolecule – naturally form salts with the (counter) cations of copper ions (0.1 μmol). Moreover, as reported [21], hyaluronic acid binds reversibly cupric ions (the binding constant = 3.0×10^3 l/mol [22]). Yet plausibly, due to the really high molar ratio of [$-COO^-$]:[Cu(II)] = 62,500, the copper cations will be dispersed throughout the chain of HA macromolecule, randomly forming a relatively sporadic population of Cu(II)-bond micronuclei. Under aerobic conditions the pre-formed micronuclei of the HA-Cu(II) complex may react with ascorbate generating thus *in situ* hydrogen peroxide, which decomposes and forms the highly reactive •OH radical. The latter, *in statu nascendi*, reacts with the chain of the HA macromolecule, yielding the A• macroradical. Hence, the substance whose role is to (preventively) inhibit the generation of •OH radicals, must diffuse as close as possible to the micronucleus of the HA-Cu(II) complex and either decomposes the molecules of H_2O_2 to inert components [23] or effectively donates the atom H from its molecule. In case that the given substance is an efficient H atom donor, the radical formed from the substance should be ineffective to re-initiate the reactions' cascade of the free-radical chain degradation of HA macromolecules. The latter condition mentioned is very well fulfilled by applying L-glutathione (GSH) [7,24,25], the endobiotic substance, which donates H atom really freely yielding a weakly reactive glutathiyl radical (GS•). Within the organism, G.S• radicals re-

combine rapidly to glutathione disulfide – GSSG (called also "oxidized" glutathione).

6.2.3 THE POTENCY OF SUBSTANCES 1, 2, 3, 4 OR 5 TO ACT AS PREVENTIVE ANTIOXIDANTS

On inspecting the experimental results represented in Fig. 3, panel A, one can state that from all five substances tested substance **1** is classifiable as a really efficient preventive antioxidant. In the concentrations used (100, 400, and 1000 μM) substance **1** inhibited the free-radical chain degradation of HA macromolecules almost completely (85.0, 82.2, and 78.1%) during the first hour. Yet even at the high potency of substance **1** to act as a preventive antioxidant, it was not efficient enough to inhibit the degradation of HAs (Fig. 3, panel B). The percentage of the inhibition of HA degradation at 5 h was practically independent of substance **1** concentration used and ranged between the values 24.4–31.3% (cf. Fig. 3, panel B, black line). From the point of view of the potency of the substances tested to act as preventive antioxidants, exclusively the action of substance **3** is valuable enough to be taken into account (cf. Fig. 3, panel A): Substance **3**, although less effective than **1** at 1 h, demonstrated a concentration dependent inhibitory action at 5 h with values of 23.2, 39.4, and up to 53.8% at the concentrations 100, 400, and 1000 μM (cf. Fig. 3, panel B). The remaining three substances, i.e., **2**, **4**, and **5**, concerning their potency to prevent free-radical chain degradation of HA macromolecules, were much less effective as compared to the efficiency of substances **1** and **3**. The registered negative values of the percentages of the inhibition of degradation of the HA macromolecules, evidenced markedly on testing the action of substance **4** (cf. Fig. 3, panels A and B, concentrations 100 and 400 μM), indicate that its radical formed within the reaction mixture might, most plausibly, initiate by itself a cascade of free-radical chain reactions. According to the above-mentioned facts, it can be concluded that substances **1** and **3** are much more relevant for application as preventive scavengers of HA degradation induced by WBOS.

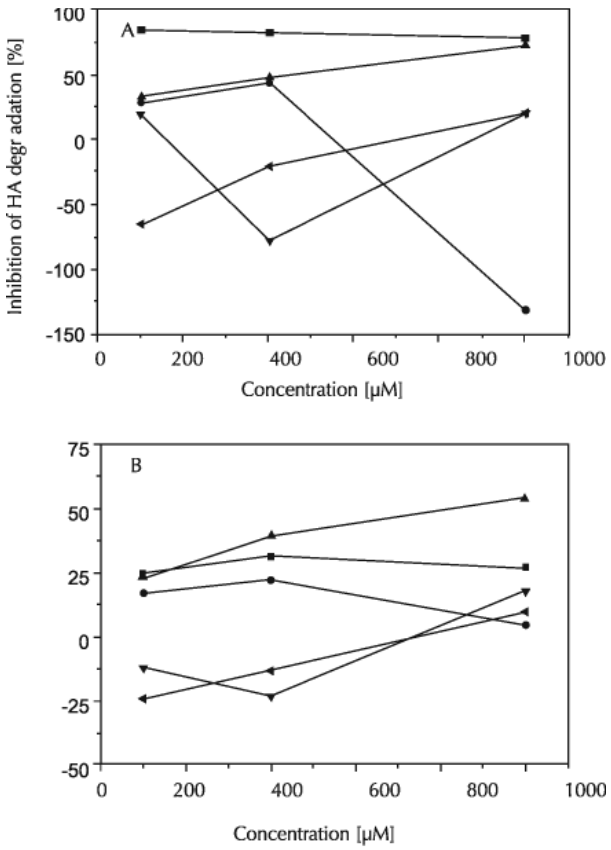


FIGURE 3 Percentage of HA degradation inhibition at 1 h (panel A) or 5 h (panel B) after the reaction onset inhibited by the substance tested to function as a preventive antioxidant.

6.2.4 SUBSTANCE 1, 2, 3, 4 OR 5 POTENCY TO ACT AS CHAIN-BREAKING ANTIOXIDANTS

Figure 4 shows the percentage of the inhibition of HA degradation at 5 h by the substance applied into the vessel 1 h after the reaction onset. At such an experimental setting, again substance **1** demonstrated the "greatest" efficacy, namely 23.2% at 100 μM. Simultaneously, the percentage of the inhibition of degradation of HAs by applying substance **1** indicates a weak positive concentration dependency with the value of inhibition

equaling 38.3% at 1000 μM. The efficacy of substance **1** at 1000 μM was slightly exceeded on applying substance **5** (51.6%). The latter substance (**5**) was the only one, which unambiguously demonstrated a significant concentration-dependent inhibitory action (cf. Fig. 4, line **5**).

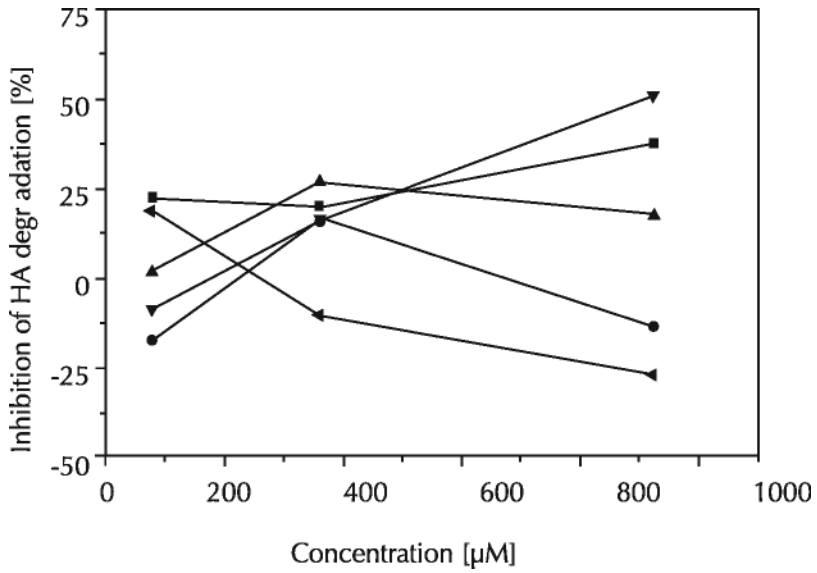


FIGURE 4 Percentage of inhibition of HA degradation at 5 h after the reaction onset inhibited by the substance tested to function as a chain–breaking antioxidant.

6.2.5 REDUCTIVE PROPERTY OF SUBSTANCES 1, 2, 3, 4 OR 5

A simple order of substances **1**–**5** based on the determined values of IC_{50} recorded in Table 1 shows that the order of the efficacy of the substances tested by both ABTS and DPPH assays is not identical. While the data obtained by ABTS decolorization assay resulted in the efficacy of the substances in the order: **4** > **1** > **5** > **3** > **2**, the order of the substances determined by the DPPH assay was: **3** > **5** > **4** > **2** > **1**. The most disputable result was the IC_{50} value = 122±5.2 μM determined for stobadine by the DPPH assay. The most appropriate explanation is the fact that both stobadine and substance **2** used in the DPPH assay were dihydrochlorides. Since this assay necessitates work in an absolutely non-aqueous environment – in

absolute methanol – it is questionable if ever and to what extent the reduction capability of dihydrochlorides (**1** and **2**) or monohydrochlorides (**3**, **4**, and **5**) of hexahydropyridoindoles was influenced by the absence of H^+ protons, i.e., by H_3O^+ ions. Due to this fact, it can be stated that for correct determination of the IC_{50} values of the substances investigated, the ABTS assay is more relevant compared to the DPPH assay. The latter method has been already applied to some pyridoindole derivatives [26], yet a free base of these substances was used in this assay. The results of determining the IC_{50} values by the latter assay can be therefore significantly influenced by inefficient or practically nil ionization of substances **1**, **2**, **3**, **4**, and **5** in the $[H^+]/[H_3O^+]$-deficient milieu, i.e., in non-aqueous methanol.

TABLE 1 IC_{50} values of five hexahydropyridoindoles determined by ABTS and DPPH assays.

Substance	ABTS assay [M]	DPPH assay [M]
1, Stobadine	12.6±0.24	122±5.2
2, SM1dM9dM10	155±2.2	29.4±0.85
3, SME1i–ProC2	27.6±0.46	10.6±0.51
4, SM1M3EC2	10.8±0.38	21±1.4
5, SMe1EC2	17±2.5	16.9±0.67

Values are means ± SEM; n = 4.

The range of the IC_{50} values of substances **1**, **3**, **4**, and **5** from 10.8±0.38 to 27.6±0.46 μM determined by the ABTS assay indicates that these structural derivatives have similar reduction properties against $ABTS^{\bullet+}$ cation radical. The most effective reductants of $ABTS^{\bullet+}$ cation radical were substances **4** and **1**. Substance **2** cannot be included into the set of effective reductants for its high IC_{50} value (155±2.2 μM). A recent detailed examination showed that substance **2**, i.e., SM1dM9dM10, has to be tested especially carefully from the viewpoint of its reduction properties. This substance, in the process of a high-molar-mass hyaluronan degradation induced by ROS in WBOS, showed a significant pro-oxidative effect, which was especially evident in the concentration of 1000 μM (cf. Fig. 5, panels

A and B, gray curves). The pro-oxidative effect observed in substance **2** leads implicitely to two conclusions: (i) substance **2** is the least efficient reductant of $ABTS^{\bullet+}$ cation radical of the set of hexahydropyridoindoles tested and (ii) although the given substance **2** is an H atom donor, yet the radical formed from this substance might be effective in re-initiating the reactions cascade of the free-radical chain degradation of HA macromolecules. However, the latter fact is to be confirmed by some complementary experimental techniques.

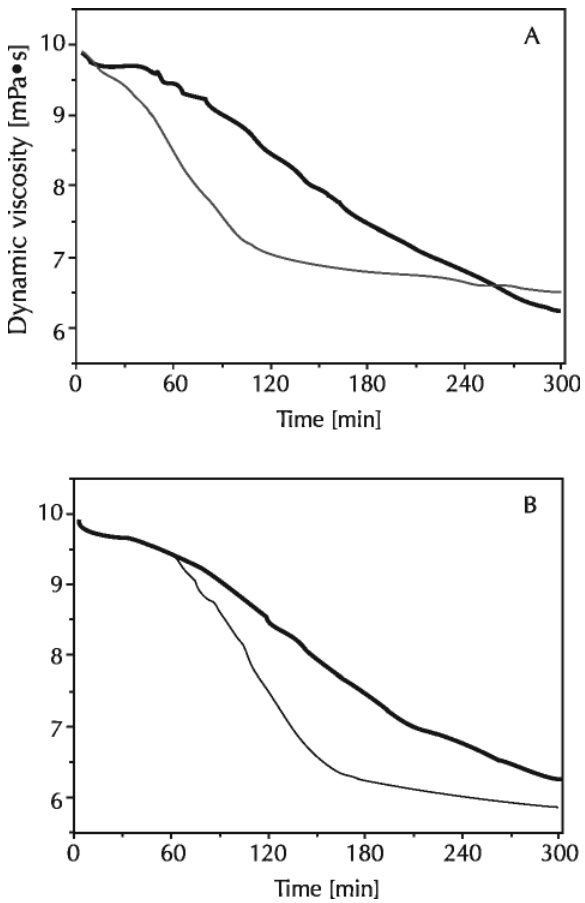


FIGURE 5 Pro-oxidative effect of substance **2** on HA degradation induced by WBOS. The substance was added to the oxidative system before the start of HA degradation (A) or after 1 h (B) in μM concentrations: 0 (black curve) and 1000 (gray curve).

6.3 CONCLUSIONS

The primary goal of the modifications of the hexahydropyridoindole chemical structure of stobadine {cis-(–)-2,3,4,4a,5,9b-hexahydro-2,8dimethyl-1-*H*-pyrido[4,3-b]indole} [27] (an enantiomer of the racemic drug carbidine [28, 29]) was to diminish adverse hypotensive effects of substance **1**, which are due to its α-adrenolytic activity [30, 31]. Since the acute toxicity of substances **2**, **3**, **4** or **5** has been much lower compared to substance **1** [15], diminishing adverse effects along with an increase of the antioxidative efficacy of the newly synthesized hexahydropyridoindoles would better meet the claims for introducing these prospective drugs into clinical practice. However, according to the above-presented observations, it is evident that any gain, if any, found on applying substances **2**, **3**, **4** or **5** did not exceed remarkably the well established antioxidative properties of substance **1** [31−33], i.e., stobadine – a cardioprotective drug [31, 34−37].

Stobadine, an amphiphilic substance with pK_{a1} = 2.9 and pK_{a2} = 7.2, can freely reach both lipoidal and hydrophilic environments in the human organism including those of (synovial) joints [38]. Due to its redox potential at neutral pH of +0.58 V, lying between that of ascorbate ($Asc^{\bullet -}$, $H^+/AscH^-$ = +0.282 V) and glutathione ($GS^{\bullet}/GS^-$ = +0.920 V) [39, 40], stobadine is a really proper reductant and H atom donor.

To avoid inappropriate applications and misinterpretation of the observations resulting from exploiting one single assay, the usage of a "battery" of assays measuring different aspects of the behavior of antioxidants has been recommended (for review see Ref. [13]). Although the ABTS and DPPH decolorization assays are the most frequently utilized, development of assays where more than one oxidative species is present in the reaction medium simultaneously should be considered inevitable. Efforts towards this direction can be appreciated by establishing the design of a standardized analytical method [41]. Within the latter method, a "cocktail" of ROS – H_2O_2, $^{\bullet}OH$, and $AOO^{\bullet}$ – acting practically simultaneously can be stated. This ROS cocktail damages the probe, i.e., the high-molar-mass hyaluronan, a process, which resembles that within the inflamed (synovial) joints.

Application of the DPPH decolorization assay in case of testing the reductive properties of salts of organic substances can lead to results, which should be reevaluated in the context of non-disociability of these

salts in the non-aqueous environment. The ABTS assay, which operates in a partially aqueous, i.e., ionic milieu, is most plausibly a proper choice to broaden the insight into the reductive (antioxidative) properties of salts of organic substances.

6.4 EXPERIMENTAL PROCEDURES

6.4.1 BIOPOLYMER AND CHEMICALS

The high-molar-mass hyaluronan sample P9710-2A used with a weight–average of the molar masses M_w = 808.7 kDa and polymolecularity value M_w/M_n = 1.63, where the M_n is the number-average of the polymer molar masses, was the product of Lifecore Biomedical Inc., Chaska, MN, U.S.A. Analytical purity grade NaCl, $CuCl_2 \cdot 2H_2O$, ethanol 96%, and methanol were purchased from Slavus Ltd., Bratislava, Slovakia; L-ascorbic acid and potassium persulfate ($K_2S_2O_8$; *p.a.* purity, max. 0.001% nitrogen) were the products of Merck KGaA, Darmstadt, Germany; 2,2´-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; *purum* >99%) was from Fluka, Steinheim, Germany; 2,2-diphenyl-1-picrylhydrazyl (DPPH) were the products of Sigma–Aldrich, Steinheim, Germany. The hexahydropyridoindoles were prepared at the Institute of Experimental Pharmacology and Toxicology, Bratislava, Slovakia. Deionized high-purity grade water, with conductivity of ≤0.055 μS/cm, was produced by using a water purification system of Thermo Scientific TKA, Niederelbert, Germany.

6.4.2 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 was added to 20 mg HA, and 3.90, 3.85, 3.70 or 3.40 ml of the solvent was added after 6 h. All stock solutions, including those of each hexahydropyridoindole (**1**, **2**, **3**, **4** or **5**; 16 mM), L-ascorbic acid (16 mM), and cupric chloride (16 mM diluted to a 16 μM solution) were also prepared in 0.15 M aqueous NaCl.

6.4.3 HYALURONAN OXIDATIVE DEGRADATION

HA degradation was induced by the WBOS comprising L-ascorbic acid (100 μmol) and $CuCl_2$ (0.1 μmol). The procedure was as follows: a volume of 50 μl of $CuCl_2$ solution (16 μM) was added to the HA solution (7.90 ml) and after 30 s stirring the reaction mixture was left to stand for 7.5 min at room temperature. Then 50 μl of L-ascorbic acid solution (16 mM) were added to the reaction mixture and stirred again for 30 s. The solution mixture (8.0 ml) was then immediately transferred into the viscometer Teflon® cup reservoir.

Procedures to investigate the H atom donating property of the substances (**1**, **2**, **3**, **4** or **5**) were as follows:

(i) The solution of $CuCl_2$ (16 μM) in the volume of 50 μl was added to the HA solution (7.85, 7.70 or 7.40 ml), which was left to stand for 7.5 min at room temperature after stirring for 30 s. Then, 50, 200 or 500 μl of the substance solution (16 mM) were added to the solution mixture and stirred again for 30 s. Finally, 50 μl of the L-ascorbic acid solution (16 mM) were added to the solution mixture and stirred for 30 s. The reaction mixture (8.0 ml) was then immediately transferred into the viscometer Teflon® cup reservoir. By adding the substance in time 0 min, i.e., before adding ascorbic acid, we investigated the capability of the substance tested to scavenge $^{\bullet}OH$ radicals, i.e., to act as a preventive antioxidant [7, 18].

(ii) In the second experimental setting, a similar procedure as that described in (i) was applied. However after leaving the solution mixture (7.90, 7.75, or 7.45 ml) for 7.5 min at room temperature, 50 μl of the L-ascorbic acid solution (16 mM) were added. After 1 h stirring of the reaction mixture, finally 50, 200 or 500 μl of the substance solution (16 mM) were added and stirred for further 30 s. The reaction mixture (8.0 ml) was then immediately transferred into the viscometer Teflon® cup reservoir. By adding the substance 1 h after admixing ascorbic acid, we investigated the capability of the substance tested to scavenge peroxy-type radicals, i.e., to act as a chain-breaking antioxidant [7, 25, 42, 43].

6.4.4 ROTATIONAL VISCOMETRY

The resulting reaction mixture (8.0 ml) was transferred into 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 the experiment onset. The changes of the η values of the reaction mixture were recorded 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}.

6.4.5 ABTS AND DPPH ASSAYS

The standard ABTS decolorization assay was applied as already reported [44−46]. Briefly, the aqueous solution of $ABTS^{\bullet+}$ cation radical was prepared 24 h before the measurements at room temperature as follows: ABTS aqueous stock 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 resulting solution was diluted with 96% ethanol to the final volume of 50 ml. The ethanol-aqueous reagent in the volume of 250 µl was added to 2.5 µl of the ethanolic solution of the substances **1**, **2**, **3**, **4**, or **5**. The concentration of each substance solution was 101−0.808 mM. The light absorbance of the sample mixture was recorded at 734 nm in the 6^{th} min after mixing the reactants.

In DPPH decolorization assay, 2,2-diphenyl-1-picrylhydrazyl (1.1 mg) was dissolved in 50 ml of distilled methanol to generate $DPPH^{\bullet}$. The $DPPH^{\bullet}$ radical solution in the volume of 225 µl was added to 25 µl of the methanolic solution of the substances **1**, **2**, **3**, **4**, or **5** (in the concentration range of 10–0.078 mM) and in the 30^{th} min the absorbance of the sample was measured at 517 nm. All measurements by both assays were performed quadruplicately in 96-well Greiner UV-Star microplates (Greiner-Bio-One GmbH, Germany) by using the Tecan Infinite M 200 reader (Tecan AG, Austria). The calculated values of IC_{50} are expressed as mean ± SEM.

APPENDIX

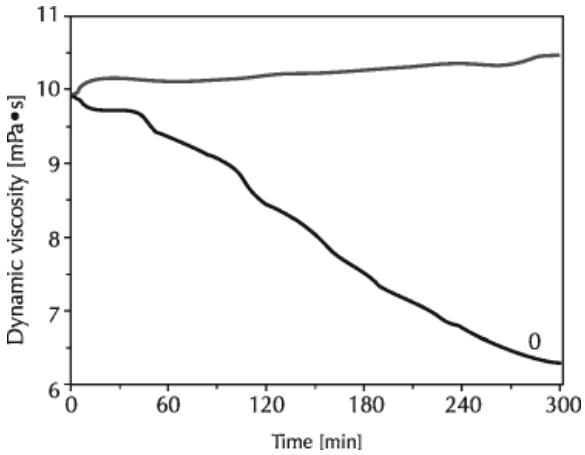


FIGURE 1 Time–dependent changes of dynamic viscosity values of the test HA solution (2.5 mg/ml).

The gray curve simulates the situation when no HA degradation occurs and the solution dynamic viscosity value rises slightly in time due to the phenomenon called rheopexy. The black curve (0) represents the real degradation of the biopolymer chains (here induced by 0.1 μmol Cu(II) *plus* 100 μmol ascorbate).

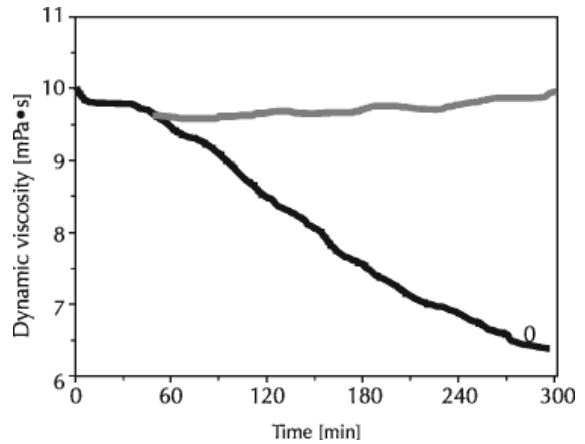


FIGURE 2 Time–dependent changes of dynamic viscosity values of the test HA solution (2.5 mg/ml).

The black curve (0) represents the real degradation of the biopolymer chains (here induced by 0.1 μmol Cu(II) *plus* 100 μmol ascorbate). The gray curve simulates the situation when the degradation of HAs initiated by the WBOS is broken–down (in the 60th minute) by addition of a "perfect/absolute chain–breaking" antioxidative substance and the solution dynamic viscosity value rises slightly in time due to the phenomenon called rheopexy.

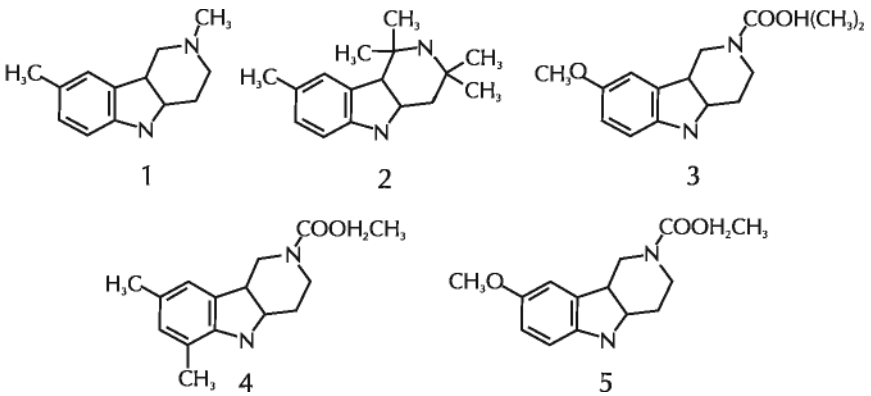


FIGURE 3 Structural formalae of stobadine (**1**), SM1dM9dM10 (**2**), SME1i-ProC2 (**3**), SM1M3EC2 (**4**), and SMe1EC2 (**5**).

KEYWORDS

- **Battery**
- **Bolus**
- **Born**
- **Cocktail**
- **Post mortem**

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