

Free-radical degradation of high-molar-mass hyaluronan induced by ascorbate *plus* cupric ions: Testing of stobadine and its two derivatives in function as antioxidants

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The authors dedicate this paper to the relatives of our co-author Dr. Vladimír Šnirc who died tragically on May 30, 2011.

Abstract. Stobadine-2HCl and its two hydrophilic derivatives SM1dM9dM10-2HCl and SME1i-ProC2-HCl were tested in the function of antioxidants on hyaluronan (HA) degradation induced by the Weissberger oxidative system [ascorbate *plus* Cu(II)]. As a primary method, rotational viscometry was applied, where the substance tested was added before or 1 h after the initiation of HA degradation. The most effective scavengers of •OH and peroxy-type radicals were recorded to be stobadine-2HCl and SME1i-ProC2-HCl, respectively. The most effective scavenger, determined by applying the ABTS assay, was stobadine-2HCl.

Key words: ABTS assay — Glycosaminoglycans — Hexahydropyridoindole derivatives — Rotational viscometry — Weissberger oxidative system

Abbreviations: ABTS, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; GAG, glycosaminoglycan; HA, hyaluronan; HHPI, hexahydropyridoindole; SF, synovial fluid; THPI, tetrahydropyridoindole.

Introduction

Hyaluronan (HA, Figure 1) is a glycosaminoglycan (GAG) found especially in extracellular matrix of vertebrate organisms. It is distinct from other GAGs in that it is a non-sulfated, non-protein bound and non-branching polymer of repeating dissacharides of d-glucuronic acid and N-acetyl-d-glucosamine linked *via* alternating β-1,4 and β-1,3 glycosidic bonds (Stern 2003; Puré and Assoian 2009). HA is widely distributed in vertebrate connective tissues, particularly in umbilical cord, synovial fluid (SF), vitreous humor, dermis, cartilage, and intervertebral disc (Kongtawelert and Ghosh 1989). In SF, high-molar-mass HA is in the physiological concentration 2–3 mg/ml. It

provides there the necessary lubrication for the joint and serves as a shock absorber, reducing friction of the moving bones and diminishing wear of the joint (Kogan et al. 2007; Šoltés et al. 2007a).

In normal tissues, molar-mass of HA can reach several million Daltons (Puré and Assoian 2009). By the length of its chain, large HA polymer has spacefilling function and is anti-angiogenic and immunosuppressive. Intermediate-size polymer, either synthesized *de novo* or generated by enzymatic degradation, comprising 25–50 disaccharides, is pro-inflammatory, immunostimulatory, and highly angiogenic, and it appears to function as endogenous stress signal (Stern et al. 2007).

The turnover of HA is extremely rapid. It is estimated that of 15 g of HA in a 70 kg human body, 5 g turn over daily. The half-life of HA in the circulation is from 2 to 5 min. In the epidermis it is 1 to 2 days, in SF several hours, and in cartilage approximately 1 to 3 weeks (Šoltés et al. 2005; Stern et al. 2007).

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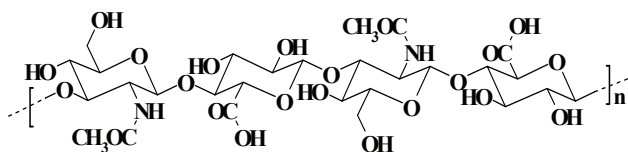


Figure 1. Hyaluronan – acid form.

Under inflammatory conditions of arthritic diseases, such as osteoarthritis or rheumatoid arthritis, high-molar-mass HA is degraded by reactive oxygen species, which reduce the viscosity of SF and impair its lubricant and shock absorbing properties, leading to dysfunction of the joint and severe pain (Yamazaki et al. 2003; Kogan et al. 2007).

The pyridoindoles, 2,3,4,5-tetrahydro- (THPI) and 2,3,4,4a,5,9b-hexahydro-1*H*-pyrido[4,3*b*]indole (HHPI), rank among substances used in the treatment of cardiovascular and neuronal disorders. Some of these drugs (Figure 2) are used in clinical practice such as antihistaminics, e.g. mebhydroline (Waitzinger et al. 1995), antipsychotics, e.g. a racemic substance – carbidine (Yakhontov and Glushkov 1983), antiemetics, e.g. alosterone (Schoenfeld 2005).

At present, THPI and HHPI substances are administered in treating Alzheimer's, Parkinson's, and Huntington's diseases (Ivashchenko et al. 2010), sclerosis multiplex, sudden cerebral and vascular episodes, states of ischemia, diabetes mellitus, and chronic inflammation (Hung 2008). To date, the THPI derivatives are the topic of more than two thousands of clinical and experimental studies and about 500 patents devoted to these groups of substances have been pending (Ivachtchenko et al. 2010).

In the Institute of Experimental Pharmacology and Toxicology of the Slovak Academy of Sciences the original substances from the group of HHPI derivatives have been developed for more than two decades. One of them, stobadine (Fig. 3) – an optical enantiomer of carbidine (Figures 2), is a cardioprotective and antihypoxic agent (Kittová et

al. 1985), which exerted beneficial effects in the 1st and 2nd stage of clinical tests (Šoltés et al. 2000). The antioxidative effect of stobadine-2HCl is related to the presence of the indoline –NH– group that directly participates in the reaction with an unpaired electron of a reactive radical (Staško et al. 1990; Steenken et al. 1992; Kagan et al. 1993; Račková et al. 2002). Protective effects of stobadine-2HCl on ascorbate *plus* Cu(II)-induced HA degradation were published by Rapta et al. (2010), whereas this substance was shown to be a remarkable scavenger of $\cdot\text{OH}$ and peroxy-type radicals in a dose-independent manner.

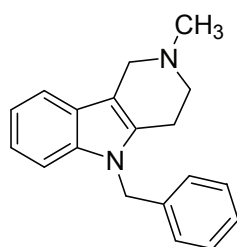
Stobadine derivatives were demonstrated to be effective antioxidants, particularly the hydrophilic derivatives SMe1EC2-HCl and SM1M3EC2-HCl, investigated in the function of $\cdot\text{OH}$ and peroxy-type radical scavengers. The results of rotational viscometry showed, however, that these derivatives had less protective effects against free radical-mediated HA degradation compared to stobadine-2HCl. According to our previous investigations of these derivatives (Rapta et al. 2010), we concluded that both are far less effective in scavenging $\cdot\text{OH}$ and peroxy-type radicals compared to stobadine-2HCl.

The aim of the presented study was to investigate the antioxidative effects of two HHPI derivatives, namely SM1dM9dM10-2HCl and SMe1i-ProC2-HCl, and compare those effects with that of stobadine-2HCl.

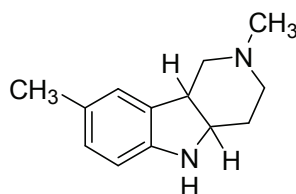
Materials and Methods

Chemicals

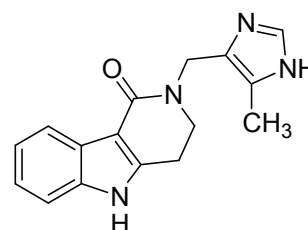
The high-molar-mass hyaluronan sample P9710-2A (weight average of molar masses $M_w = 808.7$ kDa; number average of molar masses $M_n = 496.1$ kDa; Šoltés et al. 2007b) was obtained from Lifecore Biomedical Inc. (Chaska, MN, U.S.A.). The declared contents of transition metals in the HA sample were 4 ppm Cu and 13 ppm Fe.



Mebhydroline



Carbidine



Alosterone

Figure 2. Structural scheme of pyridoindole derivatives.

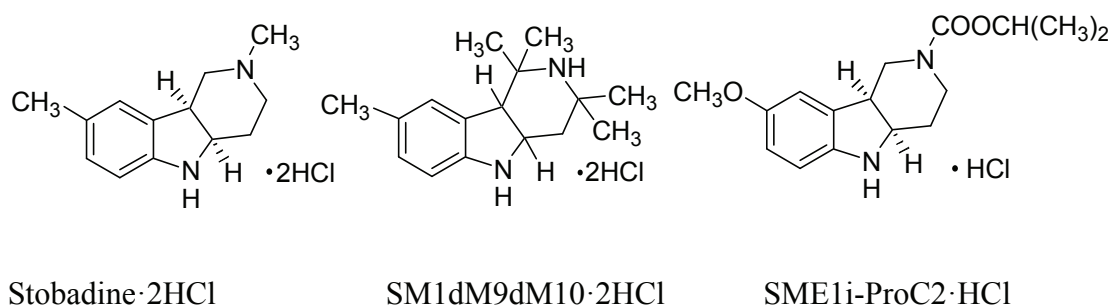


Figure 3. Structural scheme of pyridoindole – HHPI derivatives.

Analytical purity grade NaCl and $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ were purchased from Slavus Ltd. (Bratislava, Slovakia); L-ascorbic acid and potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$; *p.a.* purity, max 0.001% nitrogen) were the products of Merck KGaA (Darmstadt, Germany); 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS; *purum*, >99%) was from Fluka (Steinheim, Germany). Stobadine·2HCl and its two derivatives 2,2,3,3,8-pentamethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3b]indolinium dihydrochlorid (SM1dM9dM10·2HCl) and 2-isopropoxycarbonyl-8-methoxy-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3b]indolinium monohydrochloride (SME1i-ProC2·HCl) were prepared at the Institute of Experimental Pharmacology and Toxicology (Bratislava, Slovakia) by the classical Fischer indole synthesis using 4-methylphenylhydrazine as a precursor (Štolc et al. 2010). Deionized high-purity grade water, with conductivity of $\leq 0.055 \mu\text{S}/\text{cm}$, was produced by using a water purification system of Thermo Scientific TKA (Niederelbert, Germany).

ABTS assay

The standard ABTS assay was applied as already reported (Re et al. 1999; Cheng et al. 2006). Briefly, the aqueous solution of $\text{ABTS}^{\bullet+}$ cation radical was prepared 24 h before the measurement at room temperature as follows: ABTS aqueous stock solution (7 mM) was mixed with $\text{K}_2\text{S}_2\text{O}_8$ 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 of the volume 250 μl was added to 2.5 μl of the ethanolic solution of stobadine·2HCl, SM1dM9dM10·2HCl or SME1i-ProC2·HCl. The concentration range of tested substances was 101–0.808 mM. Absorbance of the sample mixtures was recorded at 734 nm in the 6th min after mixing the reactants.

The measurements were performed quadruplicately in 96-well Greiner UV-Star microplates (Greiner-Bio-One GmbH, Germany) with Tecan Infinite M 200 reader (Tecan AG, Austria). The calculated values of IC_{50} , expressed as mean $\pm$ SEM, are absolute.

Solutions

The HA sample solutions 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 L-ascorbic acid (16 mM), stobadine·2HCl, and its two derivatives (16 mM), cupric chloride (16 mM diluted to a 16 μM solution) were also prepared in 0.15 M aqueous NaCl.

Hyaluronan oxidative degradation

Oxidative degradation of HA was induced by the Weissberger system comprising L-ascorbic acid (100 μM) and CuCl_2 (0.1 μM). 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 the reaction mixture was left to stand for 7.5 min at room temperature after 30-s stirring. Then, 50 μl of L-ascorbic acid solution (16 mM) were added to the HA solution and stirred again for 30 s. The solution mixture was then immediately transferred into the viscometer teflon cup reservoir.

Procedures to investigate effects of stobadine·2HCl and its two derivatives were as follows:

- 1) 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 (16 mM) were added to the solution and stirred again for 30 s. Finally, 50 μl of L-ascorbic acid solution (16 mM) were added to the HA solution and stirred for 30 s. The solution 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 tested substance to scavenge $\text{OH}^\bullet$ radicals, i.e. to act as a preventive antioxidant (Šoltés et al. 2006).
- 2) In the second experimental setting a similar procedure as that described in (1) was applied, however, after leaving the

reaction mixture for 7.5 min at room temperature, 50 μl of L-ascorbic acid solution (16 mM) were added. After a 1-h stirring of the reaction mixture, finally 50, 200 or 500 μl of the substance solution (16 mM) were added to the HA solution followed by 30-s stirring. 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 tested substance to scavenge peroxy-type radicals, i.e. to act as a chain-breaking antioxidant (Valachová et al. 2010a).

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, 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 recorded at $25.0 \pm 0.1^\circ\text{C}$ 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} .

Results

Table 1 presents the absolute IC_{50} values of the substances tested by the ABTS assay. As evident, the IC_{50} value of stobadine-2HCl was found to be about 2- or 12-times lower compared to its derivatives SME1i-ProC2-HCl or SM1dM9dM10-2HCl, respectively. This indicated that stobadine-2HCl has the highest ABTS $^{+\bullet}$ cation radical scavenging capacity among the compounds tested.

Table 1. The absolute IC_{50} values of stobadine-2HCl and its two derivatives determined by the ABTS assay

Substance	IC_{50} (μM)
Stobadine-2HCl	12.6 ± 0.24
SME1i-ProC2-HCl	27.6 ± 0.46
SM1dM9dM10-2HCl	155 ± 2.2

Values are means $\pm$ SEM ($n = 4$).

Figure 4 shows typical recordings of free-radical-mediated degradation of the high-molar-mass HA by the oxidative system ascorbate *plus* Cu(II) monitored by rotational viscometry (curves 0). Addition of stobadine-2HCl to the reaction mixture (time = 0 min) led to a dose-independent (100, 400, 1000 μM) total inhibition of HA degradation initiated by $\bullet\text{OH}$ radicals up to approx. 60 min. Then, a gradual decrease of HA dynamic viscosity was observed (Fig. 4A, curves 100, 400, 1000). When applying stobadine-2HCl to the reaction mixture after 1 h, it was shown to be an effective scavenger of peroxy-type free radicals. This fact was observed again up to approx. 60 min from the measurement onset. Then, however, a dose-independent gradual decrease of HA dynamic viscosity was recorded for stobadine-2HCl tested at lower concentrations (Fig. 4B, curves 100, 400). At the highest concentration (Fig. 4B, curve 1000) it exerted a somewhat more pronounced protection against the peroxy-radical-mediated HA degradation.

The results of SM1dM9dM10-2HCl action are depicted in Figure 5. At the lower concentrations applied (100 and 400 μM) the compound exhibited a significant protective effect against the $\bullet\text{OH}$ radical-induced degradation of HA for approx. 90 and 60 min, respectively. Further, a moder-

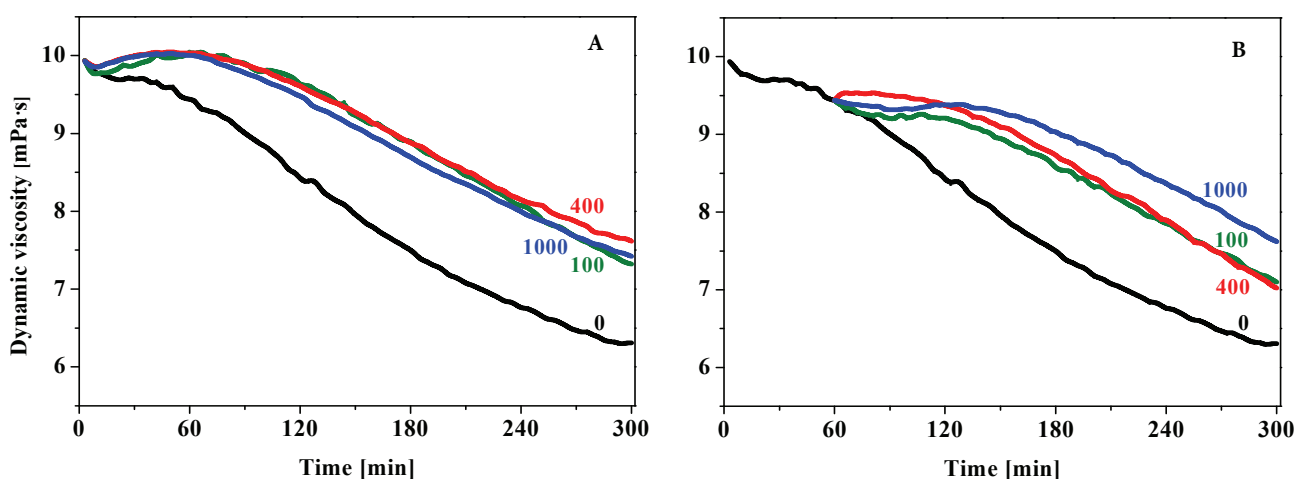


Figure 4. Effect of stobadine-2HCl on HA degradation induced by the oxidative system containing $0.1 \mu\text{M}$ CuCl_2 + $100 \mu\text{M}$ ascorbic acid. Stobadine-2HCl (0, 100, 400, and 1000 μM) added in the oxidative system before the start (A) or after 1 h (B) of HA degradation. Concentration of the HA solution was 2.5 mg/ml .

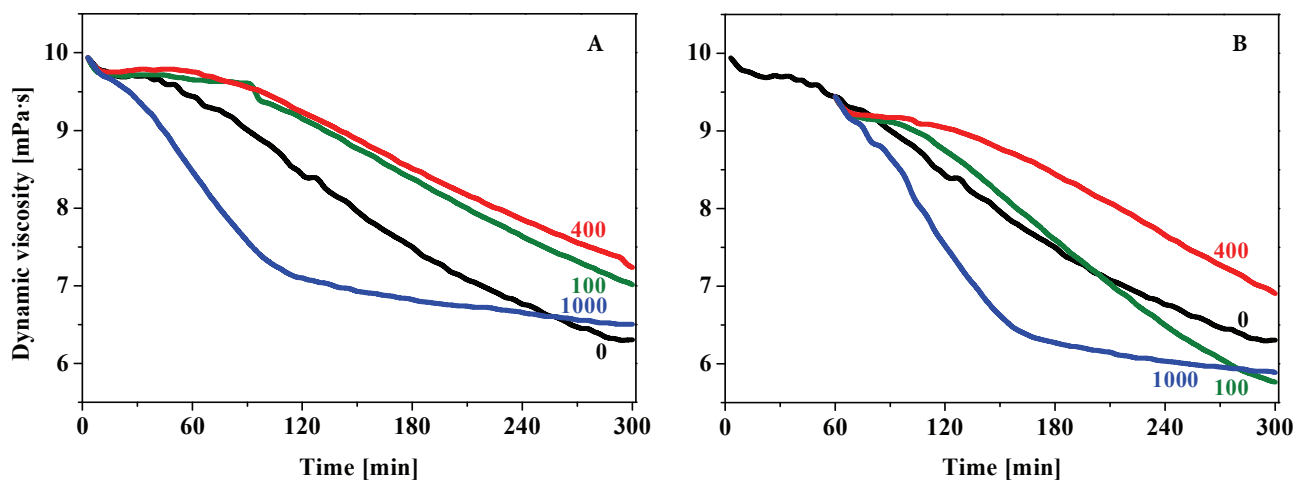


Figure 5. Effect of SM1dM9dM10-2HCl on HA degradation induced by the oxidative system containing $0.1 \mu\text{M}$ CuCl_2 + $100 \mu\text{M}$ ascorbic acid. SM1dM9dM10-2HCl (0, 100, 400, and $1000 \mu\text{M}$) added in the oxidative system before the start (A) or after 1 h (B) of HA degradation. Concentration of the HA solution was 2.5 mg/ml .

ately increased rate of HA degradation was monitored at both substance concentrations (Fig. 5A, curves 100, 400). However, further increase of the concentration ($1000 \mu\text{M}$) resulted in a rapid decrease of HA dynamic viscosity for approx. 120 min, followed by a decreased rate of HA degradation (Fig. 5A, curve 1000). Testing the substance as a scavenger of peroxy radicals showed dose-dependence, i.e. at the concentration of $400 \mu\text{M}$ SM1dM9dM10-2HCl exerted a moderate scavenging activity. On the other hand, increasing its concentration up to $1000 \mu\text{M}$ led to a significant decrease of HA dynamic viscosity values (Fig. 5B, curve 1000).

Figure 6 displays the results of adding SME1i-ProC2-HCl to the above mentioned reaction mixture inducing HA degradation. SME1i-ProC2-HCl exerted total inhibitory activity of HA degradation for a certain period of time (approx. 60 min). Then the dynamic viscosity of HA decreased moderately in a dose-dependent manner (Fig. 6A, curves 100, 400, 1000). However, practically no inhibitory effect against peroxy-type radicals was observed at the lowest concentration of the SME1i-ProC2-HCl (Fig. 6B, curve 100) compared to the reference (curve 0). On the other hand, a partial protective effect against radical-induced HA degradation was found for the substance at the higher

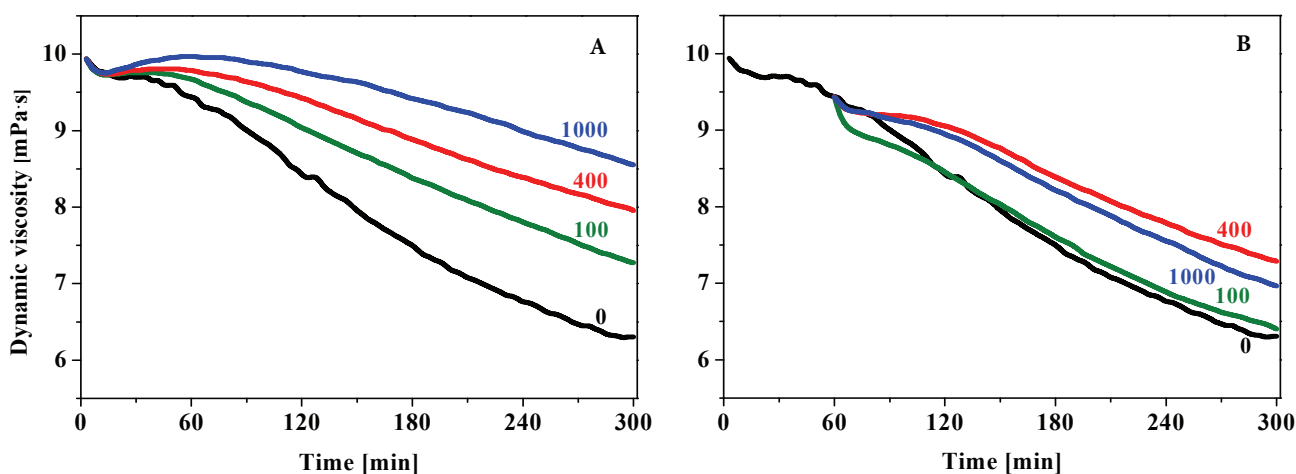


Figure 6. Effect of SME1i-ProC2-HCl on HA degradation induced by the oxidative system containing $0.1 \mu\text{M}$ CuCl_2 + $100 \mu\text{M}$ ascorbic acid. SME1i-ProC2-HCl (0, 100, 400, and $1000 \mu\text{M}$) added into the system before the start (A) or after 1 h (B) of HA degradation. Concentration of the HA solution was 2.5 mg/ml .

concentrations, i.e. 400 and 1000 μM (Fig. 6B, curves 400, 1000).

Discussion

The hexahydropyridoindole stobadine-2HCl was observed to be by far the most effective scavenger of $\text{ABTS}^{\bullet+}$ cation radical in comparison to the other substances tested. The absolute IC_{50} value of stobadine-2HCl equaled to 12.6 μM is relatively low, however, other substances tested such as D-penicillamine, arbutin or bucillamine were found to have even lower IC_{50} values: 5.26, 5.40 or 4.00 μM , respectively. Quercetin may be considered a standard $\text{ABTS}^{\bullet+}$ radical cation scavenger. Its absolute IC_{50} value represents 2.86 μM . On the other hand, the absolute IC_{50} value of SM1dM9dM10-2HCl was determined to be relatively high (155 μM). When examining L-cysteine, its IC_{50} represented the value ~ 1300 μM , which was more than 8-times higher compared to SM1dM9dM10-2HCl. For other substances such as L-glutathione and cysteamine the IC_{50} values were 24.0 and 179 μM , respectively (Valachová et al. 2010a).

The ABTS spectrophotometric assay is technically simple, which accounts for its application for screening and routine determinations. However, results obtained for the sample of interest have to be normalized since different compounds may exert different kinetic behavior. Moreover, this assay has been criticized as the $\text{ABTS}^{\bullet+}$ cation radical is not a representative of biomolecules and is not even found in any biological system. Thermodynamically, any compound that has redox potential lower than that of $\text{ABTS}^{\bullet+}$ cation radical may react with it (Magalhaes et al. 2008).

In the present study, the ABTS assay has been applied for the first time for HHPI substrates, yet antioxidative properties of stobadine-2HCl had been already investigated. The abilities of stobadine to effectively eliminate $\cdot\text{OH}$, alkoxy-, peroxy- radicals and to quench singlet oxygen have already been published (Ondriáš et al. 1989; Štefek and Beneš 1991; Štefek et al. 1992; Steenken et al. 1992; Kagan et al. 1993; Horáková et al. 1995, 1996; Štefek and Trnková 1996; Juránek et al. 2010). However, in the function of a scavenger of free-radicals generated by the Weissberger oxidative system stobadine-2HCl has been investigated already in detail by Rapta et al. (2010) for the first time. The oxidative system imitates the conditions occurring in an early stage of acute joint inflammation. Besides stobadine-2HCl, two stobadine derivatives SMe1EC2-HCl and SM1M3EC2-HCl were also tested. This is the first time these two derivatives have been tested by applying rotational viscometry and ABTS assay.

The study was focused on the comparison of stobadine-2HCl derivatives, namely SM1dM9dM10-2HCl and SMe1i-ProC2-HCl, considering the following facts: by examining stobadine-2HCl and SM1dM9dM10-2HCl thor-

oughly, theoretically the higher antioxidative activity of SM1dM9dM10-2HCl compared to stobadine-2HCl may be assumed, based on the presence of two reactive centers in the SM1dM9dM10-2HCl molecule in the positions 2- and 5-. Stobadine-2HCl has α -adrenolytic, negative chronotropic and inotropic properties which account for its hypotensive effect. The ability of stobadine-2HCl to decrease blood pressure can be adverse, especially when administered in acute states (heart attack, sudden cerebral episodes, acute traumas of head and spinal cord, etc.). The administration of substance SM1dM9dM10-2HCl has several advantages. Besides the above mentioned positive effect, the substance SM1dM9dM10-2HCl has no α -adrenolytic effect (Štolc et al. 2010).

In spite of expecting a high efficacy of substance SM1dM9dM10-2HCl, its antioxidative activity, examined by the ABTS assay, was found to be significantly lower compared to stobadine-2HCl and substance SMe1i-ProC2-HCl. The results obtained by the method of rotational viscometry indicated that the preventive effect of substance SM1dM9dM10-2HCl was comparable to that of stobadine-2HCl. However, when applying substance SM1dM9dM10-2HCl at the highest concentration, a rapid pro-oxidative effect was recorded. As resulted from the ABTS assays, the antioxidative efficacy of the derivative SMe1i-ProC2-HCl was found to be comparable to that of stobadine-2HCl. The absolute IC_{50} value of SMe1i-ProC2-HCl was approximately 47% compared to stobadine-2HCl.

The results of studying HA degradation indicate that SMe1i-ProC2-HCl possesses a significant preventive antioxidative property, reflected by the inhibition of HA oxidative degradation, and at the highest concentration of 1000 μM , it completely inhibited HA degradation up to approx. 90 min. The results of rotational viscometry showed that the substances added 1 h after the initiation of HA degradation were effective chain-breaking antioxidants in the order stobadine, derivatives SM1dM9dM10-2HCl, and finally SMe1i-ProC2-HCl. Despite our expectations, the inhibitory effect of the substances stobadine-2HCl, SM1dM9dM10-2HCl, SMe1i-ProC2-HCl on HA degradation lasted only for a certain period, and was followed by a propagation phase, whose rate was similar to that recorded in the absence of the substances. The only exception was substance SMe1i-ProC2-HCl at its higher concentrations (400, 1000 μM), which led to retardation of free radical HA degradation. Unlike some well established antiinflammatory drugs, aurothiomalate and bucillamine were capable to totally inhibit free-radical-induced high-molar-mass HA degradation before or after initiating HA degradation at significantly lower concentrations, i.e. 50 and 10 μM , respectively. Such a remarkable protective effect of bucillamine could be partly related to its chelation with copper ions (Valachová et al. 2011).

From the methodological point of view, it could be stated that ABTS assay can be used in screening proper substances, yet it is desirable to carry out further screening of antioxidative activity under conditions which imitate pathophysiological conditions. One of the approaches may be rotational viscometry, which was worked out in our laboratory and has been used ever since (Orviský et al. 1994). This method was applied in testing antioxidative efficacy of substances such as naproxen, acetylsalicylic acid, melatonin, L-glutathione, D-penicillamine, Mn(II) (Šoltés et al. 2007b; Rapta et al. 2009; Valachová et al. 2009a,b; Hrabárová et al. 2010, Stankovská and Šoltés 2010; Valachová et al. 2010a,b,c). We are confident that it has a potential to be used more broadly in testing prospective compounds with antioxidative properties, which were demonstrated in the case of pyridoindoles, namely stobadine-2HCl (Juránek et al. 2010). Although, both tested hydrophilic stobadine derivatives – SM1dM9dM10-2HCl and SME1i-ProC2-HCl – are represented with diminished α -adrenolytic effect, their free-radical scavenging properties did not reach that of stobadine-2HCl.

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