Free-radical degradation of high-molar-mass hyaluronan induced by Weissberger's oxidative system: Potential antioxidative effect of bucillamine

Mária Baňasová [1], Vlasta Sasinková [2], Raniero Mendichi [3], Tomáš Perečko [1], Katarína Valachová [1], Ivo Juránek [1], Ladislav Šoltés [1]

1 Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, Slovakia
2 Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia
3 Istituto per lo Studio delle Macromolecole, CNR, Milan, Italy

Correspondence to: Mária Baňasová, MSc.
Institute of Experimental Pharmacology and Toxicology,
Slovak Academy of Sciences, Dúbravská cesta 9, 84104 Bratislava, Slovakia.
TEL: +421-259410669; FAX: +421-254775928; E-MAIL: maria.banasova@savba.sk

Submitted: 2012-09-01 Accepted: 2012-11-15 Published online: 2012-12-26

Key words: **FT-IR; hyaluronan; rotational viscometry; SEC-MALS**

Neuroendocrinol Lett 2012; **33**(Suppl.3):151–154 **PMID:** 23353859 NEL330912A22

Abstract

OBJECTIVES: Hyaluronan (HA), one of the main components of extracellular matrix, is a glycosaminoglycan composed of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid linked by β-(1→4) and β-(1→3) glycoside bonds. High-molar-mass HA was used as a model for studying its oxidative degradation. In the present paper protective effects of bucillamine against the free-radical degradation of HA were investigated. The HA fragments generated were characterized as well.

METHODS: To induce free-radical-mediated degradation of high-molar-mass HA under aerobic conditions, we applied Weissberger's oxidative system, comprising biogenic compounds in relevant pathophysiological concentrations, i.e. 100 μM ascorbate plus 1 μM Cu(II). Time-dependent decreases of dynamic viscosity of the HA solutions were recorded by rotational viscometry. Electron donor behaviors of bucillamine were studied by a standard ABTS test method and a chemiluminescence (CL) assay. Ability of incorporation of generated bucillamine thiyl radicals into the biopolymer was verified by Fourier-transform infrared spectroscopy (FT-IR) and size exclusion chromatography with a multi-angle light scattering photometer (SEC-MALS).

RESULTS: Decrease of HA viscosity reflected HA degradation. The drug tested was applied in two arrangements: to prevent •OH radical generation (1) and ROO• type radicals propagation (2). Bucillamine, which acted as an efficient •H donor, is also a proper electron donor, as proved by ABTS and CL assays. FT-IR and SEC-MALS methods showed that the drug tested did not incorporate into the biopolymer chains.

CONCLUSION: Bucillamine significantly protected high-molar-mass HA against free-radical degradation *in vitro*, and supposedly this positive action of the drug may be involved in its beneficial effect observed in clinical practice.

ORIGINAL ARTICLE

Abbreviations:

ABTS	- is a standard test method based on 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
CL	- chemiluminescence assays
DRI	- differential refractometer
DTGS	- deuterated triglycine sulfate
FT-IR	- Fourier-transform infrared spectroscopy
HA	- hyaluronan
HRP	- horseradish peroxidase
MMD	- molar-mass distribution
Mw	- weight average of molar-masses
R_g	- radius of the macromolecule gyration
SEC-MALS	- size exclusion chromatography with a multi-angle light scattering photometer

INTRODUCTION

Hyaluronan (HA, Figure 1), a unique glycosaminoglycan composed of disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine, is synthesized in the plasmatic membrane of various cells of vertebrates. The molar-mass of HAs can reach up to 10^7 Da. HA is characterized by an extraordinarily high rate of turnover. A 70 kg individual contains ≈15 g of HA and one third of this amount turns over daily. The HA half-life is varied, in cartilage it is 3 weeks, in the skin 2 days, in synovial fluid of healthy subjects 12 hours, in blood 2–5 minutes.

Bucillamine (Figure 1, *N*-(2-mercapto-2-methyl-propionyl)-L-cysteine) is a derivative of the amino acid cysteine. This drug is prescribed for treatment of chronic inflammations (Horwitz 2003).

The aims of the study are divided into three groups:

- The first objective is the investigation of •H donor behavior of bucillamine against •OH radicals which induce HA degradation.
- The second objective is the investigation of electron donor behaviors of bucillamine by ABTS decolorization assay and by chemiluminescence in cell free system.
- The third objective is verification of possible incorporation of bucillamine generated thiyl radicals into biopolymer chains.

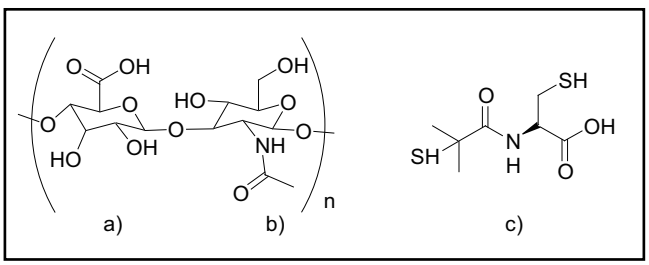

Fig. 1. Chemical structure of hyaluronan: a) D-glucuronic acid, **b)** *N*-acetyl- D-glucosamine, **c)** bucillamine.

MATERIALS AND METHODS

Biopolymer and chemicals

High-molar-mass HA sample P0207-1 (Mw = 970.4 kDa) was purchased from Lifecore Biomedical Inc., Chaska, MN, U.S.A. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was from Sigma–Aldrich Chemie (Germany), horseradish peroxidase (HRP) from Merck (Germany), hydrogen peroxide from Chemapol (Praha, Czechoslovakia). Further chemicals used were identical to those reported previously (Valachová *et al.* 2011, Baňasová *et al.* 2012).

Rotational viscometry and study of uninhibited and inhibited hyaluronan degradation

Standard experimental conditions used were as already published (Valachová *et al.* 2010). The dynamic viscosity of the reaction mixture (8 ml) containing HA (20 mg), ascorbate (100 μM) and Cu(II) ions (1 μM) in the absence and presence of bucillamine (100 μM) was monitored by a Brookfield LVDV-II+PRO digital rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.) at 25.0 ± 0.1°C at a shear rate of 237.6 s^{-1} for 5 h in a reservoir-spindle couple made of Teflon® (Valachová *et al.* 2008, 2011). The drug was introduced into the reservoir vessel at the start of reaction or 1 h after the reaction onset. At the end of experiment, the whole reaction mixture was poured into 20 ml ethanol, centrifuged, and the precipitate was dried in a desiccator. Dry polymers were further studied by FT-IR and SEC-MALS.

ABTS assay

Standard experimental conditions were used as published (Valachová *et al.* 2011, Baňasová *et al.* 2012).

Chemiluminescence in cell free system

Chemiluminescence of bucillamine (100 μM) was initiated with 50 μl hydrogen peroxide (100 μM) in the presence of 50 μl HRP (2 U/ml) and 50 μl luminol (10 μM). The experiment was made in triplicate. All chemiluminescence responses were measured in a microtiter plate computer-driven luminometer Immunotech LM-01T (Immunotech, Czech Republic).

Fourier-transform infrared spectroscopy

FT-IR spectra were measured with Nicolet 6700 (Thermo Fisher Scientific, USA) spectrometer equipped with DTGS detector and Omnic 8.0 software. The spectra were collected in the middle region from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}, the number of scans was 128. Diamond Smart Orbit ATR accessory was applied for measurement in solid state.

SEC-MALS

The molar mass distribution (MMD) of samples was determined by a modular multi-detector SEC-MALS system. The SEC system consisted of an Alliance 2695

HPLC separation module from Waters (Milford, MA, USA). MALS comprised two on-line detectors: a multi-angle light scattering photometer (MALS Dawn DSP-F) from Wyatt (Santa Barbara, CA, USA) and a 2414 differential refractometer (DRI) from Waters, used as a concentration detector. The setup of this multi-detector SEC system was serial in the following order: Alliance-MALS-DRI. The wavelength of the MALS laser was 632.8 nm. The light scattering signal was detected simultaneously at fifteen scattering angles ranging from 14.5° to 151.3°. The experimental methodology for a reliable use of the SEC-MALS chromatographic system was described in detail (Wyatt 1993; Mendichi *et al.* 1998; 2002). The experimental conditions of the SEC-MALS system were as follows: two Shodex (KB806 and KB805) columns from Tosoh Bioscience (Stuttgart, Germany); mobile phase: 0.20 M NaCl; flow rate, 0.5 ml/min; temperature: 35 °C; injection volume: 150 µl; injected polymer concentration: 0.4–1.0 mg/ml.

RESULTS AND DISCUSSION

Rotational viscometry

Free-radical degradation of high-molar-mass HA was induced by Weissberger´s system, 1 µM Cu(II) *plus* 100 µM ascorbic acid, generator of 100 µM •OH radicals (Valachová *et al.* 2008, 2010). Under these conditions the dynamic viscosity of the HA solution decreased during five hours significantly (Figure 2, curve 0). However, as evident from Figure 2, addition of 100 µM bucillamine either before the reaction onset (1) or 1 h after the initiation of HA degradation (2) totally inhibited degradation of HA macromolecules.

Bucillamine in its chemical structure has two –SH groups, which freely donate •H radicals. In this way bucillamine acts as a scavenger of •OH and ROO• type radicals: thus this drug can be classified as a proper preventive and chain-breaking antioxidant.

ABTS assay

The $ABTS^{\bullet+}$ cation radical by a one electron reduction changes its color from sea-green to colorless substance (Magalhaes *et al.* 2008), namely to ABTS according to the reaction

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

By using the ABTS assay, the bucillamine IC_{50} value equaling 4.00 µM indicates a high reducing power of this drug. This value can be compared with quercetin, the substance classified as a standard natural antioxidant. The IC_{50} value obtained from the ABTS assays for quercetin was 2.86 µM.

Chemiluminiscence in cell free system

Bucillamine addition in 100 µM concentration (Figure 3, curve 1) to reference (Figure 3, curve 0) exerted a clear prooxidative effect on chemiluminiscence in cell free system.

Fourier-transform infrared spectroscopy

As shown from detailed analyses of the FT-IR spectral records obtained for thiol-modified HA samples and intact hyaluronan (Figure 4), the region which includes also the C–S bond vibrations is at 730–570 cm^{-1} (Günzler *et al.* 2002). These vibrations usually have weak intensities and, due to the very low additive moiety (originally 100 µM of bucillamine) in modified HA samples they are hardly to be interpreted (Figure 4).

SEC-MALS

The comparison of the conformation plot of the radius of the macromolecule gyration (R_g) versus HA molar-mass ($R_g = f(M)$) of four HA samples is shown in Figure 5.

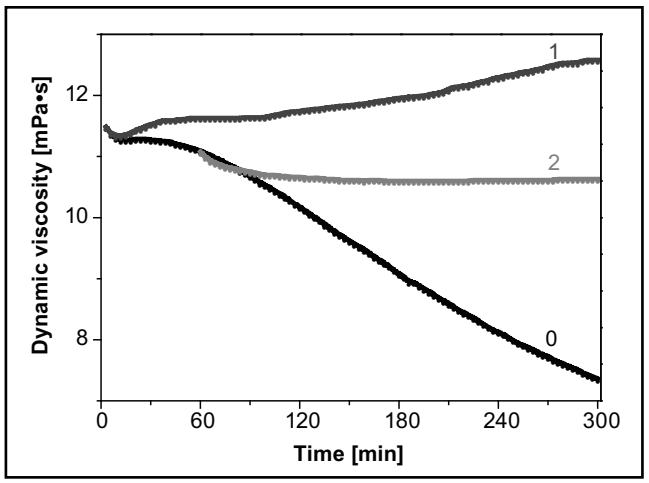

Figure 2. Effect of bucillamine on free-radical degradation of high-molar-mass HA induced by 1 µM Cu(II) *plus* 100 µM ascorbate. Reference experiment, curve coded (0) – nil addition of bucillamine. Effect of 100 µM bucillamine added into the system before the onset of HA degradation (1) and after 1h of initiation of HA degradation (2).

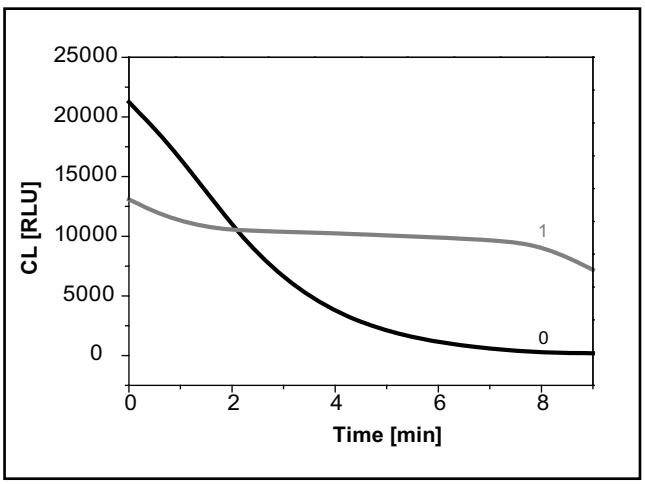

Figure 3. Effect of 100 µM bucillamine addition (1) on chemiluminesce in cell free system, (0) – reference curve, no bucillamine added.

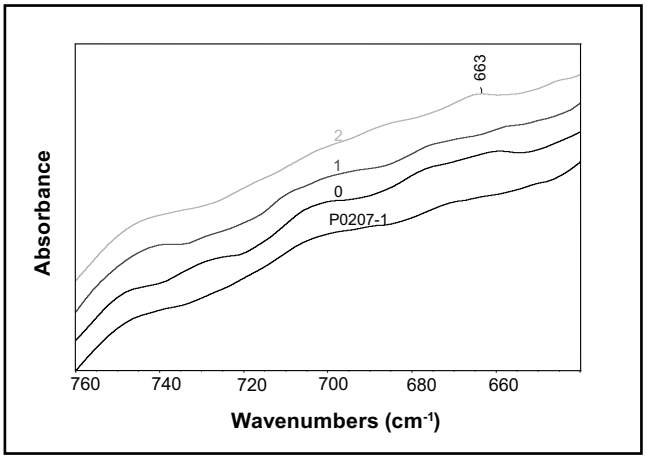

Figure 4. Records of FT-IR for HA sample P0207-1 and for the three modified HA samples (0, 1, 2).

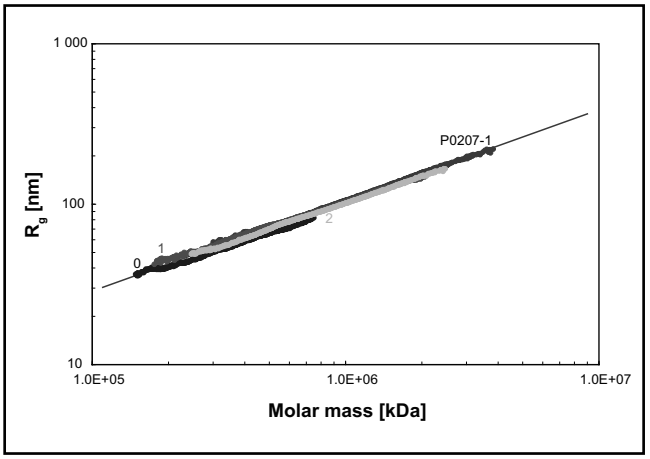

Figure 5. Comparison of conformation plot $R_g = f(M)$ of HA sample P0207-1 and three recovered polymeric samples (coded 0,1,2).

In general, the conformation plot can be in advantage exploited for deeper insight into the conformation of the polymer, branching and eventual derivatization or chemical modification of the polymer. The perfect superimposition between the conformation plot of four HA samples (Figure 5) clearly shows that only a molar-mass degradation (at different extent) is present in the 0, 1 and 2 HA samples.

ACKNOWLEDGEMENTS

Supported in part by the Slovak Grant Agency VEGA (grants No. 2/0011/11, 2/0149/12, 2/0143/09, 2/0003/10) and APVV No. 0351-10, 0052-10.

Potential Conflicts of Interest: *None disclosed.*

REFERENCES

1 Baňasová M, Valachová K, Juránek I, Šoltés L (2012). Scavenging of oxygen-derived free radicals by bucillamine and its disulfide derivative SA981: The effect likely involved in the protection of oxidative hyaluronan degradation. In: Drugs: Their action in pharmacology and toxicology, published by the Institute of Experimental Pharmacology and Toxicology, SASc., ISBN: 978-80-971042-0-7, p. 176–184.

2 Günzler H, Gremlich HU (2002). IR Spectroscopy, Wiley-VCH, ISBN 3-527-28896-1, p. 246.

3 Horwitz LD (1998). Bucillamine: A potent thiol donor with multiple clinical applications. Cardiovascular Drug Reviews. **21**: 77–90.

4 Magalhaes LM, Segundo MA, Reis S, Lima JLFC (2008). Methodical aspects about *in vitro* evaluation of antioxidant properties. Analytica Chimica Acta. **613**: 1–19.

5 Mendichi R, Giacometti Schieroni A (2002). Fractionation and characterization of ultra-high molar mass hyaluronan: 2. On-line size exclusion chromatography methods. Polymer. **43**: 6115–6121.

6 Mendichi R, Giacometti Schieroni A, Grassi C, Re A (1998). Characterization of ultra-high molar mass hyaluronan. Polymer. **39**: 6611–6620.

7 Valachová K, Hrabárová E, Gemeiner P, Šoltés L (2008). Study of pro- and anti-oxidative properties of D-penicillamine in a system comprising high-molar-mass hyaluronan, ascorbate, and cupric ions. NeuroEndocrinology Letters. **29**: 697–701.

8 Valachová K, Hrabárová E, Dráfi F, Juránek I, Bauerová K, Priesolová E, Nagy M, Šoltés L (2010). Ascorbate and Cu(II)-induced oxidative degradation of high-molar-mass hyaluronan. Pro- and antioxidative effects of some thiols. NeuroEndocrinology Letters. **31**: 101–104.

9 Valachová K, Hrabárová E, Priesolová E, Nagy M, Baňasová M, Juránek I, Šoltés L (2011). Free-radical degradation of high-molecular-weight hyaluronan induced by ascorbate *plus* cupric ions. Testing of bucillamine and its SA981-metabolite as antioxidants. Journal of Pharmaceutical and Biomedical Analysis. **56**: 664–670.

10 Wyatt PJ (1993). Light scattering and the absolute characterization of macromolecules. Analytica Chimica Acta. **272**: 1–40.