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Influence of tiopronin, captopril and levamisole therapeutics on the oxidative degradation of hyaluronan

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ABSTRACT

The ability to protect hyaluronic acid (HA) from oxidative degradation by cupric ions and ascorbate (production of $^{\bullet}$OH and peroxy-type radicals) during acute phase joint inflammation has been investigated using the following drugs: tiopronin, captopril, and levamisole. Radical scavenging activity, *i.e.* the propensity for donation of electrons was assessed for the drugs by ABTS and DPPH assays. The kinetics of HA degradation have been measured in the presence of each drug using rotational viscometry. The results of ABTS and DPPH assays show the highest radical scavenging activity for captopril, followed by tiopronin. For levamisole, no effect was observed. Captopril and tiopronin prevented HA degradation induced by $^{\bullet}$OH radicals in a similar manner, while tiopronin was more effective in scavenging peroxy-type radicals. On the other hand, levamisole was shown to be a pro-oxidant. Recovered HA fragments were characterized using FT-IR analysis, the incorporation of a sulphur atom from captopril and tiopronin but not from levamisole into the HA molecule was demonstrated.

1. Introduction

Hyaluronan (hyaluronic acid, HA, Fig. 1a), a linear polysaccharide composed of repeating disaccharide units composed of β-D-glucosamine and β-D-glucuronic acid residues linked by (1 → 3) and (1 → 4) glycosidic bonds, has been used to study its oxidative degradation *in vitro*. In vertebrates high-molar-mass HA forms viscoelastic solutions (Collins & Birkinshaw, 2013a). HA at high molecular weight *in vivo* exhibits antiangiogenic, anti-inflammatory and immunosuppressive properties (Girish & Kemparaju, 2007; Šoltés et al., 2007). However, lower molecular weight hyaluronan demonstrates pro-inflammatory, angiogenic, and immunostimulative activities (Rychlý et al., 2006). HA is a material of increasing importance to biomaterials science and is finding applications in diverse areas ranging from tissue culture scaffolds, which has been reviewed recently (Collins & Birkinshaw, 2013b), to cosmetic materials (Vrentzos, Liapakis, Englander, & Paschalis, 2014) and cancer therapy (Shen et al., 2014). Its properties, both physical and biochemical, in solution or hydrogel form, are extremely attractive for various technologies concerned with body repair (Collins & Birkinshaw, 2013b).

HA, both *in vivo* and *in vitro*, is degraded by hyaluronidase, and/or by reactive oxygen/nitrogen species (ROS/RNS) (Bystrický, Alföldi, Machová, Steiner, & Šoltés, 2001; Stankovská et al., 2004). Studies have shown that oxidative HA degradation can be influenced by mono- and di-thiol compounds such as cysteine, cysteamine, *N*-acetylcysteine, dithioerythritol, dithiothreitol and more efficiently by glutathione along with bucillamine (Baňasová et al., 2012, 2014; Hrabárová, Valachová, Juránek, & Šoltés, 2012; Hrabárová, Valachová, Juránek, & Šoltés, 2012; Tamer, Valachova, & Soltes, 2014; Valachová et al., 2010, 2011).

Captopril and tiopronin contain a SH-group, Fig. 1b and c, which is a well-known donor of both H$^{\bullet}$ and electrons, however H$^{\bullet}$ and electron donor properties of these drugs have not been investigated to date. Captopril, 1-[(2S)-3-mercapto-2-methylpropionyl]-L-proline, has been shown to reduce anti-inflammatory properties and has been used to treat hypertension (Odaka & Mizuoki, 2000). While tiopronin, *N*-(2-mercaptopropionyl)-glycine, has been used for the treatment of rheumatoid arthritis (Mordini, Guidoni, Maestrini, Buonavia, & Lavagni, 1989). Levamisole, (6S)-6-phenyl-2,3,5,6-tetrahydroimidazo[2,1-b][1,3]thiazole, is known

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; HA, hyaluronan; DPPH, 2,2-diphenyl-1-picrylhydrazyl; SF, synovial fluid; WBOS, Weissberger's biogenic oxidative system.

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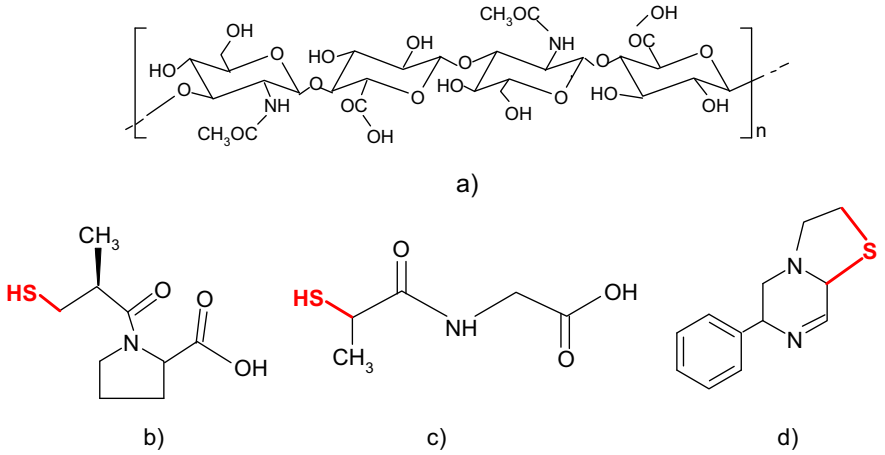

Fig. 1. Chemical structure of hyaluronan (a), captopril (b), tiopronin (c) and levamisole (d).

for its antirheumatic and anticancer properties (Mutch & Hutson, 1991). Levamisole (Fig. 1d) contains sulphur but not the —SH group.

The current study determines the electron donor properties of the drugs by ABTS and DPPH assays, whilst the kinetics of oxidative degradation of HA in the presence of the drugs is investigated by rotational viscometry. The degraded HA is characterized using FT-IR analysis to demonstrate the potential incorporation of the drugs into the HA molecule.

2. Materials and methods

2.1. Materials

HA (M_w = 970.4 kDa) was purchased from Lifecore Biomedical Inc., Chaska, MN, USA. $CuCl_2{\cdot}2H_2O$ and NaCl (analytical purity grade) were purchased from Slavus Ltd., Bratislava, Slovakia; ascorbic acid and potassium persulfate from Merck KGaA, Darmstadt, Germany; 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) from Fluka, Steinheim, Germany; 2,2-diphenyl-1-picrylhydrazyl (DPPH); tiopronin and levamisole hydrochloride from Sigma-Aldrich, Steinheim, Germany; captopril from Calbiochem, a brand of EMD Chemicals Inc., an affiliate of Merck KGaA, Darmstadt, Germany, methanol and ethanol was purchased from Mikrochem, Pezinok, Slovakia. Deionised high-purity grade water, with conductivity of ≤0.055 μS/cm, was produced by using the TKA water purification system (Water Purification Systems GmbH, Niederelbert, Germany).

2.2. Preparation of stock and working solutions

The hyaluronan samples (20 mg) were dissolved in 0.15 M aqueous NaCl solution for 24 h in the dark to prevent photodegradation (Lapčík, Omelka, Kuběna, Galatík, & Kellő, 1990, Lapčík, Chabreček, & Staško, 1991).

HA sample solutions were prepared in two steps: first, 4.0 ml of 0.15 mM NaCl was added to HA to swell and after 6 h, 3.9 or 3.85 ml of 0.15 M NaCl was added, when working in the absence and presence of the drugs, respectively. Solutions of ascorbic acid, drugs (16 mM) and cupric chloride (160 μM solution) as well as their dilutions were made in 0.15 M aqueous NaCl.

2.3. ABTS and DPPH assays – determination of IC_{50} values

The first step of standard ABTS assay was preparation of the aqueous solution of $ABTS^{\bullet+}$ cation radical. The $ABTS^{\bullet+}$ is 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 next day, 1 ml of the resulting solution was diluted with distilled water to the final volume of 60 ml (Cheng, Moore, & Yu, 2006; Hrabárová, Valachová, Rapta, & Šoltés, 2010; Re et al., 1999). The aqueous reagent in the volume of 250 μl was added to 2.5 μl of the aqueous solutions of tiopronin, captopril and levamisole. The concentration of substances ranged from 0.078 to 20 mM. Absorbance (734 nm) of samples was recorded after 6 min, when $ABTS^{\bullet+}$ reacted completely with each drug.

At first, in the DPPH assay $DPPH^{\bullet}$ radical was prepared as follows: 2,2-diphenyl-1-picrylhydrazyl (1.1 mg) was dissolved in 50 ml of distilled methanol to generate $DPPH^{\bullet}$. The $DPPH^{\bullet}$ solution in the volume of 225 μl was added to 25 μl of the methanol solution of tiopronin, captopril and levamisole. The concentration of substances ranged from 0.078 to 20 mM. Absorbance (517 nm) of samples was recorded after 30 min.

In both assays the measurements were performed in the triplicate in 96-well Greiner UV-Star microplates (Greiner-Bio-One GmbH, Germany) by using the Tecan Infinite M 200 reader (Tecan AG, Austria).

2.4. ABTS and DPPH assays – kinetics of scavenging $ABTS^{\bullet+}$ and $DPPH^{\bullet}$

The $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ were prepared as mentioned above. The stock solution of each drug at concentrations 2, 1, 0.5 and 0.25 mM in the volume of 50 μl was added to 2 ml of the $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ solution. Kinetics of scavenging $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ was performed in triplicate at the wavelength 730 and 517 nm, respectively. The solutions of captopril, tiopronin and levamisole were measured during 30, 15 and 10 min, respectively. Besides aqueous solutions of glutathione used in both methods, aqueous and methanolic solutions of captopril, tiopronin and levamisole were used for the ABTS and DPPH assay, respectively.

2.5. Rotational viscometry

Degradation of high-molar-mass HA was induced *in vitro* by Weissberger's biogenic oxidative system (WBOS) comprising compounds in the respective physiological concentrations, see Fig. 2, tangibly 100 μM ascorbate *plus* 1 μM $CuCl_2$, applied under aerobic conditions. The procedure was as follows: 50 μl of $CuCl_2$ (160 μM) was added to the HA solution (7.90 ml). After stirring for 30 s, the reaction mixture was left to stand for 7.5 min at room temperature, 50 μl of ascorbic acid (16 mM) was added, and the mixture was