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Isoflavonoids with inhibiting effects on human hyaluronidase-1 and norneolignan clitorienolactone B from *Ononis spinosa* L. root extract

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ABSTRACT

Human hyaluronidase-1 (Hyal-1) is one of the main enzymes in the homeostasis of hyaluronic acid (HA), the main polysaccharide of extracellular matrix. Development of specific Hyal-1 inhibitors might be a promising target for improved wound healing, tissue regeneration, and looking at renal function for diuresis. By using surface-displayed Hyal-1 on *Escherichia coli* F470 cells, HA as substrate and stains-all method for quantification of undegraded HA, the respective enzyme activity can be determined easily. Based on the traditional use of extracts from the roots from *Ononis spinosa* L. (Restharrow root) as a weak diuretic to achieve flushing of the urinary tract and as an adjuvant in minor urinary complaints the herbal material was selected for bioactivity guided fractionation for compounds with Hyal-1 inhibition activity. Hot water and hydroalcoholic extracts showed moderate inhibiting effects (IC_{50} 1.36 resp. 0.73 mg/mL) while dichloromethane extract exerted an IC_{50} of 190 µg/mL. Bioassay guided fractionation of the dichloromethane extract yielded four isoflavonoids with anti Hyal-1 activity: onogenin **1**, sativanone **2**, medicarpin **3** and calycosin-D **4** with inhibition rates of 25.4, 61.2, 22.4 and 23.0%, respectively at test concentration level of 250 µM. The norneolignan clitorienolactone B **5**, the first time described for the genus *Ononis*, was inactive. The IC_{50} of sativanone, the most active compound was determined with 1501 µM, which was better than that of the positive control glycyrrhizinic acid (177 µM). Thus, a possible explanation for diuretic properties of *Ononis spinosa* L. root extract may be postulated from the results so far obtained.

1. Introduction

Human hyaluronidase-1 (Hyal-1) is an enzyme strongly involved in the regulation of the extracellular matrix by balancing the deposition and potential degradation of hyaluronic acid (HA) in many tissues, especially in the extracelluar matrix. High molecular weight HA (> 20 kDa) regulates many physicochemical effects in the tissue as e.g. water-binding, regulation of osmotic pressure, anti-inflammatory effects by influencing the migration of leucocytes and macrophages and induction of growth factors for epithelial cells, which again influences cell proliferation, and differentiation [1]. In contrast low molecular weight HA (< 20 kDa) promotes angiogenesis, inhibits apoptotic processes, stimulates inflammation, exerts reduced water-binding capacity [2–4] and leads to increased urine excretion in the kidneys. Depolymerization of HA to low molecular weight HA occurs by hyaluronidases (Hyal) [2] of which Hyal-1 and Hyal-2 are the main enzymes involved in the HA metabolism of human tissue. Hyal-2, mainly located at the plasma membrane, produces polymers of a molecular weight of up to about 20 kDa. Hyal-1, mainly located in lysosomes and plasma, degrades HA down to oligomers with a degree of polymerization of ≥4. It has been reported that cellular degradation of HA starts at the plasma membrane by Hyal-2, followed by internalization of the break down products via HA scavenging receptor CD44 into lysosomes [5]. In the lysosomes, the final depolymerization towards low molecular weight oligosaccharides by Hyal-1 is observed. The inhibition of Hyal by specific inhibitors might be a promising target for improved wound healing and tissue regeneration. Also renal fluid regulation in kidney cells is at least in part controlled by the specific HA turnover [6]. Inhibitors of HA synthesis in rats reduces the ability of the kidney to respond with an appropriate diuretic response upon hydration [6]. In contrast,

Abbreviations: HA, Hyaluronic acid; Hyal, human hyaluroidase; Urinary tract infections, UTI

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inhibitors of Hyal increase renal fluid excretion, which means they induce diuretic effects.

In phytotherapy some medicinal plants are used traditionally for renal disorders, especially for treatment of uncomplicated urinary tract infections (UTI) or flushing of the urinary tract for elimination of renal gravel. Despite the fact that some of these herbal materials are claimed to exert weak diuretic effects details on the respective underlying effects on the renal system or the definition of molecular or cellular targets are more or less unknown. Consequently, the following proof of concept study was initiated to investigate selected herbal materials for Hyal-1 inhibition and to identify lead structures with significant inhibiting activity against this enzyme.

The roots from *Ononis spinosa* L. (Fabaceae), also called Restharrow roots, are used for flushing of the urinary tract, especially in cases of inflammation and renal gravel, and as an adjuvant in treatment of bacterial infections of the urinary tract [7]. Also the European Medical Agency stated a potential use of the herbal preparation to increase the amount of urine to achieve flushing of the urinary tract as an adjuvant in minor urinary complaints [8].

Pharmacodynamic properties of Restharrow extracts are based on moderate diuretic activity determined within in vivo rat experiments [9]. Antiinflammatory effects are described under in vitro conditions for a methanolic extract of *O. spinosa* roots and for the pterocarpan medicarpin, isolated from the extract by inhibition of leukotriene and 5-lipoxygenase formation [10]. In the Carrageenan-induced rat paw edema assay, a methanolic extract of *O. spinosa* roots reduced significantly edema formation after i.p. application [11]. The phytochemical composition of *O. spinosa* is reviewed in detail in the ESCOP monograph [7], listing as main compounds the isoflavones trifolirhizin, formononetin together with its 7-O-β-D-glucoside-6″-malonate and 7-O-β-D-glucoside (syn. ononin), biochanin A-7-O-β-D-glucoside and the pterocarpan medicarpin; additionally, the root contains triterpenes, notably α-onocerin, phytosterols, especially β-sitosterol, deoxybenzoines, especially ononetin, phenolic acids, tannins, minerals, and about 0.02% of essential oil with *trans*-anethole, carvone, menthol as main compounds.

Some of these compound classes found in the roots of *O. spinosa* are known to have anti-hyaluronidase activity; especially flavonoids and terpenoids have been shown to exhibit anti-hyaluronidase activity against testicular hyaluronidase [12,13]. Also certain triterpene saponins can exert activity against Hyal-1 [14]. In addition, the diuretic activities of some plants have been attributed to the presence of flavonoids. For instance, oral administration of flavonoids for one week elicited significant increase in water and solute renal excretion revealing reduction in tubular reabsorption of water and accompanying anions in rats and dogs [15]. However, the mechanism of action has remained largely unknown.

Following preliminary screening of activity of *O. spinosa* this herbal material was selected for bioactivity guided fractionation and isolation of possible lead compounds with Hyal-1 inhibition activity.

2. Results

2.1. Bioactivity guided fractionation

From a pre-screening of traditionally used medicinal plants documented for use in UTI or for irrigation of the renal system the hydro-alcoholic extract from the roots of *O. spinosa* had been found to have a moderate inhibition of Hyal-1. Subsequently six different extracts with different polarity (hot water, MeOH, EtOH-water 1:1, EtOH, acetone-water 7:3, dichloromethane) were obtained from Restharrow roots and investigated for potential Hyal-1 inhibition and subjected to a bioassay guided fractionation (Fig. 1).

Testing against Hyal-1 was performed using an cell-based autodisplay assay described recently with slight modifications [14]. *E. coli* F470 cells with the pAK009 constitutive expression system for Hyal-1 were incubated for 5 min with variable concentrations of the test fractions; glycyrrhizinic acid served as positive control (IC_{50} 177 μM). For quality control *E. coli* F470 host cells without Hyal-1 expression system were used in parallel to exclude unspecific cell activating or inhibiting effects. Hyal-1 activity was monitored by its degrading properties against HA over a period of 5 min. While the polar extracts had a moderate inhibitory activity against the enzyme, best activity was found for the CH_2Cl_2 extract (IC_{50} 0.19 mg/mL, Fig. 1). This indicated that the most active principles were essentially non-polar; mainly isoflavonoids and terpenoids (e.g. α-onocerine) were expected to be part of this extract. Fractionation of the CH_2Cl_2 extract on silica gel yielded 8 main fractions in increasing order of polarity (OD_A to OD_H) (Fig. 1). The fractions were screened for anti-Hyal-1 inhibitory activity at a concentration level of 1 mg/mL. OD_B and OD_G showed high inhibition rates of 86 and 92%, respectively, and were used for further fractionation. According LC-MS ODG contained the triterpene α-onocerin as one of the major compounds. Bioassay with the pure compound (2.26 mM) indicated that α-onocerin did not influence Hyal-1 activity (data not shown).

Interestingly, there was no defined relationship between the polarity of the extracts and the anti-hyaluronidase activity.

Using preparative HPLC on RP18 stationary phases compounds **1**, **2** and **3** were obtained from OD_B which were later identified as onogenin, sativanone and medicarpin. At 250 μM **2** showed a significant effect on Hyal-1 with 61.2% inhibition at a concentration of 250 μM and an IC_{50} of 150.7 μM (Fig. 2). From OD_G four subfractions were obtained on RP18 stationary phase, with OD_{GB} being active. A subsequent chromatography of OD_{GB} yielded the active compound **4**. From the non-active fraction OD_{GC} compound **5** was isolated. Fig. 1 summarizes the bioactivity-guided isolation and the respective Hyal-1 inhibiting effects.

2.2. Identification of compounds *1, 2 and 3*

Compounds **1**, **2** and **3** were identified from subfraction OD_B by comparison of physicochemical data with previously isolated and characterized compounds from the roots of *O. spinosa* [16]. For unambiguous identification the respective retention times in UHPLC, the UV spectroscopic data as well as UHPLC-MS-MS data were used. Fig. 3 displays the UHPLC chromatogram from OD_B and the respective analytical data obtained by comparison with the respective reference compounds. The three isoflavonoids were identified as onogenin **1**, sativanone **2** and medicarpin **3**. The respective structural features are displayed in Fig. 4.

2.3. Structure elucidation of compounds *4 and 5*

The isolated compound **4** showed UV maxima at λ = 248.6 nm and 289.0 nm, similar as described for 7-hydroxy-isoflavones with hydroxylated and methoxylated B ring [17–19]. HR-ESI-MS indicated *m/z* $[M + H]^+$ of 285.0787, being in accordance with the structure of a dihydroxylated and monomethoxylated flavone/isoflavone (calculated *m/z* $[M + H]^+$ 285.0763). UHPLC spiking experiments of **4** together with compounds of similar molecular weight which have been described for *O. spinosa* (maackiain, biochanin A and calycosin) did not prove the same retention behaviour.

From the ESI-MS/MS (positive mode) the characteristic degradation of isoflavonoids was observed. The retrocyclization of the C-ring through retro Diels-Alder reaction formed the A^+ fragment with $[M + H]^+$ at *m/z* 137 [16]. Also the gradual degradation of the molecule through successive loss of CO, $[M + H\text{-}CO\text{-}CO]^+$ was observed with the fragment at *m/z* 169. Also, a ion at *m/z* 253 from $[M + H\text{-}CH_4O]^+$ was observed. The presence of the methoxy group was confirmed by *m/z* 270 corresponding to $[M + H\text{-}CH_3]^+$ [20].

The ^1H NMR spectrum of **4** displayed aromatic signals at δ 8.07 ppm (1H, d, *J* = 8.8 Hz H-5), δ 6.91 ppm (1H, d, *J* = 2.2 Hz H-8), and δ 7.00 ppm (1H, *dd*, *J* = 2.2, 8.8 Hz H-6). The multiplicity of the aromatic