RESEARCH ARTICLE

Inhibitors of hyaluronan export from hops prevent osteoarthritic reactions

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Scope: An early reaction in osteoarthritic chondrocytes is hyaluronan overproduction followed by proteoglycan loss and collagen degradation. We recently found that hyaluronan is exported by the ATP-binding cassette transporter multidrug resistance associated protein 5 (MRP5) in competition with cGMP and that some phosphodiesterase 5 inhibitors also inhibited hyaluronan export. These inhibitors also prevented osteoarthritic reactions in cartilage. In an effort to identify the improved inhibitors directed primarily toward MRP5, we analyzed the flavonoids

Methods and results: Prenylflavonoids from hop xanthohumol, isoxanthohumol and 8-prenylnaringenin inhibited MRP5 export at lower concentrations than phosphodiesterase 5 activity. They were analyzed for their effect on IL-induced osteoarthritic reactions in bovine chondrocytes. Xanthohumol was the superior compound to inhibit hyaluronan export, as well as proteoglycan and collagen loss. It also prevented the shedding of metalloproteases into the culture medium. It directly inhibited MRP5, because it reduced the export of the MRP5 substrate fluorescein immediately and did not influence the hyaluronan synthase activity.

Conclusions: Xanthohumol may be a natural compound to prevent hyaluronan overproduction and subsequent reactions in osteoarthritis.

Keywords:
Hyaluronan export / MRP5 / Osteoarthritis / Prenylflavonoids / Xanthohumol

1 Introduction

Many pathological disturbances are accompanied by hyaluronan overproduction including tumor metastasis [1] and osteoarthritis [2, 3]. Hyaluronan is a space filling component, because it has an enormous hydration volume. It displaces other macromolecules from the site of swelling at the cell membrane and expands the tissue, when it is extruded [4]. The sequence of the osteoarthritic reactions can be mimicked in tissue culture of bovine cartilage explants activated by ILs. Hyaluronan overproduction is one of the first changes that disintegrate the compact assembly of articular cartilage [5, 6]. The induction of hyaluronan export is followed by proteoglycan loss and collagen degradation. Collagen degradation is mediated by metalloproteases that are produced by activated chondrocytes and can only diffuse to their targets, when the compact assembly of the tissue is lost. Over the past decades, therapeutic interventions were concentrated on the development of protease inhibitors which indeed inhibited collagen degradation, but could not be transferred into therapeutic drugs because ofmassive side effects [7]. Second, the protease inhibitors acted at the end of the osteoarthritic reaction cascade and may thus be less effective.

It is thus desirable to evaluate inhibitors of hyaluronan overproduction for their effect on these diseases. Recently, we discovered that hyaluronan is exported from fibroblasts and chondrocytes by the ATP-binding cassette transporter multidrug resistance associated protein 5 (MRP5), which is competitively inhibited by cGMP, which is also an endogenous substrate [5, 6, 8–10]. Analogs of cGMP have intensively been studied as phosphodiesterase 5 (PDE5) inhibitors and some of them were also inhibitory for MRP5 transport. In

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Abbreviations: HEK, human embryonic kidney; **MRP5,** multidrug resistance associated protein 5; **PDE5,** phosphodiesterase 5

order to reduce cellular and physiological reactions mediated by elevated levels of intracellular cGMP, we searched for more specific MRP5 inhibitors and considered natural sources in order to take advantage of the evolutionary drug design and avoid expensive and laborious high-throughput screening. It was our intention to identify the compounds that exert a high and specific MRP5 and a low PDE5 inhibitory activity. Initially we considered icariin lead compound, because it is a natural PDE5 inhibitor [11]. Then we focussed our attention on related plant flavonoids and found that prenylflavonids from hops inhibited hyaluronan export. Their efficacy was analyzed on bovine cartilage explants activated by ILs for osteoarthritis reactions.

2 Materials and methods

2.1 Materials

Bovine articular cartilage was from a local slaughterhouse. Hyaluronan-binding protein was from Calbiochem, hyaluronan (Healon®) was a gift from Genzyme, Cambridge, MA. Xanthohumol, isoxanthohumol and 8-prenylnaringenin were from Alexis Biochemicals. Additional chemicals were from Sigma-Aldrich Chemical. The PDE5 assay kit was from BPS Bioscience.

2.2 General methods

The cytotoxicity of the drugs [12] and proteoglycans [13] was determined as described. The determination of the IC_{50} values on the PDE5 was performed according to the instructions of BPS Bioscience. The cytotoxicity was measured by the Alamar blue assay which determined the cell viability by reduction of resazurin with NADH [14].

2.3 Culture of bovine chondrocytes in alginate beads

Chondrocytes were suspended in an alginate solution (1.2% in 0.9% NaCl) at a cell density of 4×10^6 cells/mL and pressed though a 22G syringe dropwise (3 drops) into a sterile solution of 102 mM $CaCl_2$ that had been added in the wells of a microtiter plate. This treatment leads to the formation of alginate beads containing chondrocytes. Osteoarthritic reactions were induced in chondrocytes growing in alginate beads by incubation with the medium containing 10 ng/mL of IL-17 for 6 days, and in the cartilage explants with 10 ng/mL of IL-17 and 5 ng/mL of IL-1α. The supernatants were collected and stored at −20°C for later analysis. The explants were weighed and extracted with 1 mL of 4 M guanidinium hydrochloride for 72 h at 4°C. The proteoglycan concentrations were determined in the extracts.

2.4 Hyaluronan synthase activity

The hyaluronan synthase activity was determined on a cell membrane fraction [8]. Chondrocytes were grown to confluency and stimulated by the addition of fetal calf serum to a final concentration of 15%. After incubation for 5 h, the cells were washed with cold PBS, harvested with the aid of a rubber policeman, sedimented at $1500 \times g$ for 5 min and suspended in 30 mL of ice-cold PBS. The cells were transferred into a Parr-cell disruption bomb, exposed to a nitrogen pressure of 900 psi for 15 min and disrupted by nitrogen cavitation [15] and the particulate fraction was obtained by centrifugation at $40\,000 \times g$ for 20 min. The sediment was suspended in 50 mM Tris-malonate pH 7.0 at a protein concentration of 200 μg/mL and were mixed with an equal volume of the substrate for hyaluronan synthesis that contained 8 μM UDP-[^{14}C]GlcA, 166 μM UDP-GlcNac, 4 mM dithiothreitol, 20 mM $MgCl_2$ in 50 mM Tris-malonate pH 7.0 and incubated at 37°C for 4 h in the presence of increasing concentrations of multidrug resistance inhibitors. Hyaluronan synthesis was stopped by adding a solution of 10% SDS to a final concentration of 1%. The mixtures were applied to descending paper chromatography that was developed with ethanol/aq. 1 M ammonium acetate pH 5.5 (13:7) as solvent. After 18 h the radioactivity of [^{14}C] hyaluronan at the origin was determined.

2.5 Inhibition of hyaluronan synthesis

The chondrocyte containing beads in the wells of the microtiter plate were washed with PBS and incubated with RPMI-media and increasing concentrations of the flavonoids for another 6 days at 37°C. The beads were washed with PBS and solubilized with a solution of 125 μl 55 mM Na-citrate in 0.9% NaCl, pH 6.05 for 10 min at 37°C. The cells were sedimented at $2000 \times g$ for 5 min and the supernatants were supplemented with 12.5 μL of 200 μg/mL papain in 0.1 M Na-acetate pH 5.53, 50 mM EDTA, 0.9% NaCl, 5 mM cysteine and also incubated for 20 h at 37°C. Aliquots were taken for determination of the hyaluronan concentration by the following ELISA-like assay [16]. Briefly, the wells of a 96-well Covalink-NH-microtiter plate (NUNC) were coated with 100 μl of a mixture of 100 mg/mL of hyaluronan (Healon®), 9.2 μg/mL of *N*-hydroxysuccinimide-3-sulfonic acid and 615 μl/mL of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide for 2 h at room temperature and overnight at 4°C. The wells were washed three times with a solution of 2 M NaCl, 41 mM $MgSO_4$, 0.05% Tween-20 in 50 mM PBS pH 7.2 (buffer A) and once with 2 M NaCl, 41 mM $MgSO_4$, in PBS pH 7.2. Additional binding sites were blocked by incubation with 300 μL of 0.5% bovine serum albumin in PBS for 30 min at 37°C. Calibration of the assay was performed with the standard concentrations of hyaluronan ranging from 15 ng/mL to 6000 ng/mL in equal volumes of culture medium, as used

for measurement of the cellular supernatants. A solution (50 μL) of the biotinylated hyaluronan-binding fragment of aggrecan (Applied Bioligands, Winnipeg, Canada) in 1.5 M NaCl, 0.3 M guanidinium hydrochloride, 0.08% bovine serum albumin, 0.02% NaN_3, 25 mM phosphate buffer pH 7.0 was preincubated with 50 μL of the standard hyaluronan solutions or cellular supernatants for 1 h at 37°C. The mixtures were transferred to the hyaluronan-coated test plate and incubated for 1 h at 37°C. The microtiter plate was washed three times with buffer A and incubated with 100 μL/well of a solution of streptavidin–horseradish–peroxidase-conjugate (Amersham) at a dilution of 1:100 in PBS, 0.1% Tween-20 for 30 min at room temperature. The plate was washed five times with buffer A and the color was developed by incubation with a 100 μL/well of a solution of 5 mg *o*-phenylenediamine and 5 μL 30% H_2O_2 in 10 mL of 0.1 M citrate-phosphate buffer pH 5.3 for 25 min at room temperature. The adsorption was read at 490 nm in an ELISA-reader. The concentrations in the samples were calculated from a logarithmic regression curve of the hyaluronan standard solutions.

2.6 Determination of proteoglycans

Cartilage explants were weighed (average wet weight: 20 mg) and incubated in the absence and presence of 10 ng/mL of IL-17 and 5 ng/mL of IL-1α and the inhibitors at various concentrations for 5 days. The tissues were extracted with 1.5 mL of a solution of 4 M guanidinium hydrochloride, 0.1 M ε-aminohexanoid acid, 5 mM benzamidine, 10 mM *N*-ethylmaleinimide, 0.5 mM PMSF for 3 days at 4°C. The solution was centrifuged for 5 min at 10 000 × *g* and the proteoglycans were determined in the supernatant by the Alcian blue method as described [13].

2.7 Determination of the proteoglycan synthesis

Chondrocytes were cultured in alginate beads as described above and supplemented with 25 μL of [^{35}S]sulfate (0.5 mCi/mL) for 24 h. The beads were washed three times with 102 mM $CaCl_2$ to remove unincorporated radioactivity and dissolved in 55 mM sodium citrate. Proteoglycans were isolated by the Alcian blue precipitation method [17] and aliquots were used for the determination of radioactivity.

2.8 Zymography of matrix proteases

Bovine cartilage explants were cultured in serum-free DMEM for 5 days in the presence or absence of 10 ng/mL of IL-17 and 5 ng/mL of IL-1α and 12.5 or 50 μM of xanthohumol, isoxanthohumol, 8-prenylnaringenin or icariin. The protein concentrations of the culture media were determined and equal amounts of proteins were directly applied to a 7.5% SDS-polyacrylamide gel that contained 0.1% gelatin. After electrophoresis, the gel was washed twice with 2.5% Triton X-100 for 30 min, three times with water for 10 min and a solution of 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnSO_4$, pH 8.0 for 5 days at 37°C. The gel was stained with Coomassie blue.

2.9 Determination of collagen synthesis

Chondrocytes were cultured in alginate beads for 1 week with 10% fetal calf serum in Dulbecco's medium. The medium was changed and supplemented with 1 mM cysteine, 1 mM pyruvate, 60 μg/mL β-aminopropionitrile and 25 μg/mL ascorbic acid and the cells were incubated for 24 h. The medium was replaced with serum-free medium containing the above supplements, 10 ng/mL of IL-17 and 5 ng/mL of IL-1α, the inhibitors and [^{14}C]proline (2 μCi/mL), and the cells were incubated for 24 h. The beads were washed three times with 0.9% NaCl, $CaCl_2$ (116 mg/L) for 30 min to remove unincorporated radioactivity, dissolved in 500 μL of 55 mM sodium citrate, and the radioactivity was determined.

2.10 Export inhibition of the MRP5 substrate fluorescein

The effect of the prenylflavonoids on the MRP5 export rate was measured with fluorescein diacetate. MRP5 overexpressing human embryonic kidney (HEK) 293 cells were incubated in the 96-well microtiter plates with 100 μL of DMEM in the presence of 4 μM fluorescein diacetate and 10 μM of the prenylflavonoids. At different time periods, the fluorescence of the medium was determined at 485 and 528 nm by a Synergy HT reader (Biotek Instruments).

2.11 Histochemical staining of collagen in cartilage

Cartilage explants were incubated with and without a mixture of 10 ng/mL of IL-17 and 5 ng/mL of IL-1α for 14 days. The addition of the cytokines was repeated daily. The tissues were fixed with 3.7% paraformaldehyde for 24 h, imbedded in paraffin and stained by the van Gieson method [18].

2.12 Zymography of matrix metalloproteases

Bovine chondrocytes in alginate beads were cultured in serum-free DMEM for 3 days in the absence or presence of IL-17 (25 ng/mL) and 12.5 μM or 50 μM of the inhibitors xanthohumol, isoxanthohumol, 8-prenylnaringenin and icariin. The protein concentrations of the culture media were determined by the Lowry method [19] and equal amounts of proteins were directly applied to a 7.5% SDS-polyacrylamide gel that contained 0.1% gelatin. After electrophoresis, the gel was

washed twice with 2.5% Triton X-100 for 30 min, three times with water for 10 min and a solution of 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnSO_4$, pH 8.0 for 5 days at 37°C. The gel was stained with Coomassie blue.

3 Results

3.1 Search for the natural hyaluronan export inhibitors

We compared the inhibitory profile of several natural flavonoids toward hyaluronan export by MRP5 from human fibroblasts and PDE5 in a cell-free assay. Table 1 shows the IC_{50} values for the inhibition of hyaluronan export from chondrocytes and for PDE5. The references indicate data obtained from the cited literature. The comparison indicated that the prenylflavonoids from hops fulfilled this requirement best. The inhibitory effect of these compounds was also tested on IL-activated bovine chondrocytes that are known to express the hyaluronan exporter MRP5-like fibroblasts [5] and compared to the related prenylflavonoid icariin, which also inhibits PDE5. Figure 1A shows that all prenylflavonoids inhibited hyaluronan export from chondrocytes in a concentration-dependent manner. The prenylflavonoids except icariin inhibited hyaluronan secretion into the media of IL-activated cartilage explants (Fig. 1B).

3.2 Specificity of the prenylflavonoids

A convenient assay of the export activity of MRP5 and its related homolog MRP4 [8] is the measurement of fluorescein in the culture supernatant of cells exposed to fluorescein diacetate. This non-fluorescent precursor is taken up by cells, converted to fluorescein by cytosolic esterases and exported by MRP5 or MRP4. The inhibitory activity of the prenylflavonoids was measured on MRP5 and MRP4 overexpressing HEK cells [10]. Figure 2 shows that the prenylflavonoids xanthohumol, isoxanthohumol and 8-prenylnaringenin inhibited, whereas icariin activated export. In contrast, the prenylflavonoids did not influence the export of fluorescein by MRP4 at concentrations of 50 μM (data not shown).

The toxicities were tested on bovine chondrocytes by the Alamar blue assay, and the prenylflavonoids were not toxic up to concentrations of 100 μM (data not shown). They were also analyzed for an effect on the hyaluronan synthase activity on membranes isolated from human fibroblasts, and they did not influence the synthase up to concentrations of 100 μM (Fig. 3). In addition, their influence of the expression of MRP5 in bovine chondrocytes was analyzed by Western blotting, and they did not alter the expression up to concentrations of 100 μM (data not shown). In conclusion, these results indicated that the prenylflavonoids xanthohumol, isoxanthohumol and 8-prenylnaringenin have a higher inhibitory activity toward MRP5 than to PDE5.

3.3 Inhibition of proteoglycan loss

It is known that hyaluronan overproduction leads to proteoglycan loss from the cartilage [5, 6]. Therefore, we determined whether this loss could be prevented by the prenylflavonoids. Cartilage explants were incubated for 6 days in the presence and absence of IL-17, an inducer of osteoarthritic reactions [20], and increasing the concentrations of the prenylflavonoids. Proteoglycans were determined in the culture supernatants and in the cartilage extracted with guanidinium hydrochloride. Figure 4A shows that IL-17 reduced the proteoglycan content in the cartilage and Fig. 4B shows the elevated concentrations in the culture medium. The best protection from proteoglycan loss was observed for xanthohumol, whereas icariin was the least active one.

In a separate experiment, the effect of the inhibitors on the proteoglycan synthesis rate was determined. Cartilage explants were incubated with [^{35}S]sulfate in the presence of the prenylflavonoids over a concentration range of 12.5–50 μM for 24 h and the radioactivity incorporated into proteoglycans was determined. [^{35}S]Sulfate incorporation into proteoglycans at inhibitor concentrations of 50 μM was 82, 89, 98 and 94% for xanthohumol, isoxanthohumol, 8-prenylnaringenin and icariin, respectively. Thus, the inhibitors had little influence on proteoglycan synthesis.

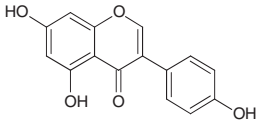
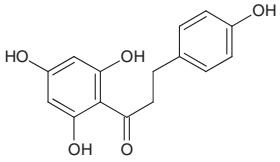
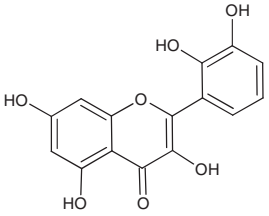
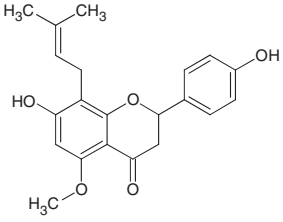
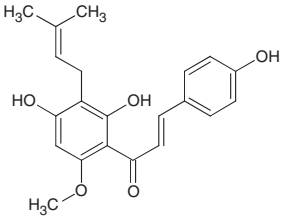
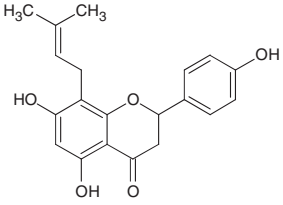
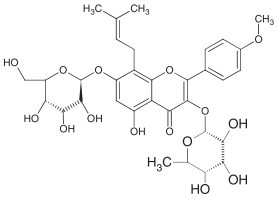
3.4 The prenylflavonoids reduce collagen degradation

It is known that hyaluronan overproduction leads to collagen degradation from the cartilage [5, 6]. Initial experiments with IL-17 alone indicated that this treatment was insufficient to induce detectable collagen degradation. Therefore, cartilage explants were incubated with a mixture of the osteoarthritis inducing chemokines IL-1α, IL-1β and IL-17 with 12.5 and 50 μM of the prenylflavonoids for 14 days and collagen was stained by the van Giesson method. Figure 5 shows that particularly xanthohumol was effectively protecting the cartilage from collagen loss.

3.5 The prenylflavonoids reduce the action of gelatinases

It is known that chondrocytes produce gelatinases particularly if activated by ILs [21, 22]. The enzymes diffuse out of the tissue and are found in the culture supernatants [5]. We analyzed the effect of the prenylflavonoids on gelatinases released from chondrocytes grown in alginate beads and incubated in the absence and presence of IL-17 and the prenylflavonoids. Enzymes in the media were analyzed by gel zymography. Figure 6 shows three bands with molecular weights of 86, 66 and 62 kDa. The upper band was MMP9, because it reacted with monoclonal MMP9 antibodies [5]. The lower two bands comigrated with an authentic sample

Table 1. Inhibition of PDE5 and hyaluronan export from bovine chondrocytes by MRP5

Compound	Structure	Inhibition of hyaluronan export IC_{50} (μM)	Inhibition of PDE5 IC_{50} (μM)	Natural source	Reference
Genistein		44	739	Soybeans	[43]
Phloretin		130	No inhibition	Apples	[44]
Quercetin		12	>100	Many plants	[43]
Isoxanthohumol		15	>50	Hops	
Xanthohumol		8	>50	Hops	
8-Prenylnaringenin		15	>50	Hops	
Icariin		25	59	Horny goat weed	[11]

Bovine chondrocytes were incubated in alginate beads and hyaluronan export and the PDE5 activity was determined as described in Section 2.

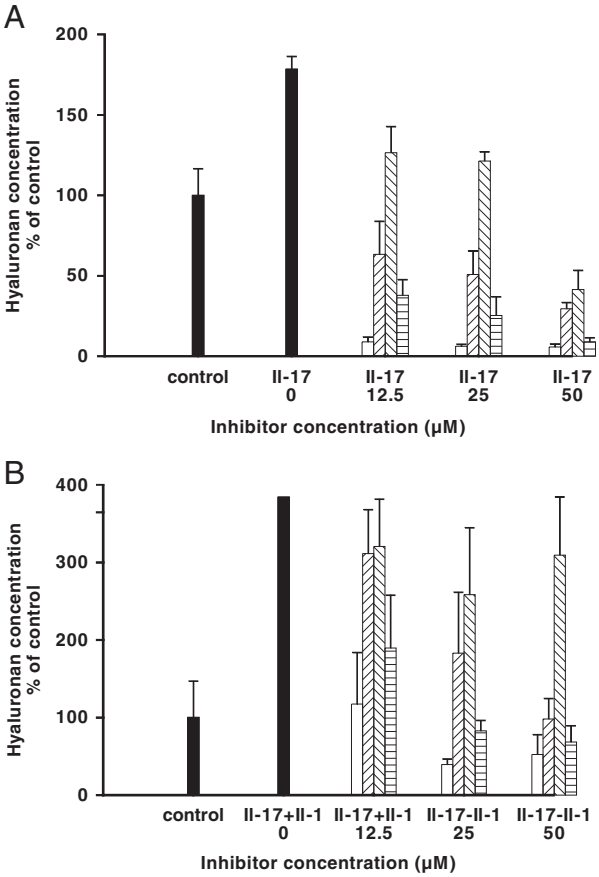


Figure 1. Inhibition of hyaluronan export from bovine chondrocytes and explants of bovine cartilage. Bovine chondrocytes in alginate beads (A) were incubated in the absence and presence of IL-17 and increasing concentrations of xanthohumol (open bars), isoxanthohumol (left hatched bars), icariin (right hatched bars) and 8-prenylnaringenin (vertical hatched bars) for 6 days and the hyaluronan concentrations were determined in the supernatant. The concentrations were related to 100% of the control. Bovine cartilage explants (B) were similarly incubated, weighed and hyaluronan concentrations were determined. The concentrations were related to 100% of the control. The error bars indicate the SD of four determinations.

of pro-MMP2 and MMP2 gelatinases. IL-17 enhanced the release of the gelatinase, and particularly xanthohumol and 8-prenylnaringenin inhibited the MMP release.

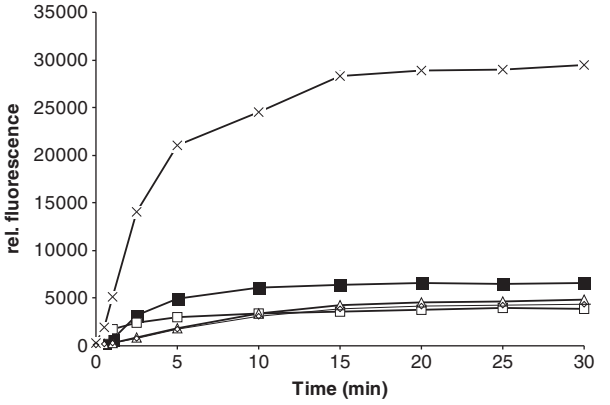


Figure 2. Fluorescein export by MRP5. Fluorescein is an MRP5 substrate and a useful marker of export activity. MRP5 overexpressing HEK cells were incubated with non-fluorescent fluorescein diacetate, which is intracellularly cleaved to the MRP5 substrate fluorescein in the absence (■) or presence of icariin (x), xanthohumol (◇), isoxanthohumol (□) or 8-prenylnaringenin (Δ). The SD of three determinations was below 2%. *p*-values calculated for control *versus* the treated cultures by ANOVA on the areas-under-the-curve for the last 5 values were below 0.0001 and post-hoc *t*-tests showed that the differences were significant.

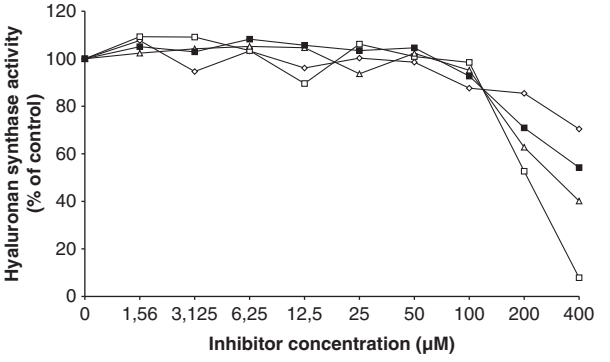


Figure 3. Effect of prenylflavonoids on the hyaluronan synthase. A membrane fraction was isolated from chondrocytes and incubated with radioactive substrates UDP-[^{14}C]GlcA and UDP-GlcNac for hyaluronan synthesis and increasing concentrations of icariin (◇), xanthohumol (■), isoxanthohumol (Δ) or 8-prenylnaringenin (□). After 4 h the mixtures were applied to paper chromatography and the radioactivity of [^{14}C]hyaluronan at the origin was determined.

4 Discussion

There is an urgent need for specific inhibitors of hyaluronan production to interfere with several pathological disturbances. Most inhibitors known so far were unspecific, because they are analogs of cGMP and developed as PDE5 inhibitors. The prenylflavonoids xanthohumol, isoxanthohumol and 8-prenylnaringenin did not inhibit PDE5, but hyaluronan export by MRP5 from fibroblasts and chondrocytes with IC_{50} values between 8 and 15 μM. They inhibited the export of the MRP5 substrate fluorescein from MRP5 overexpressing HEK cells, but not from MRP4 overexpressing HEK cells, whereas icariin inhibited PDE5, activated fluorescein export by MRP5 and inhibited hyaluronan export from fibroblasts and chondrocytes. Icariin differs from xanthohumol, isoxanthohumol and 8-prenylnaringenin by the presence of two sugar moieties (glucose and rhamnose) and an additional double bond. This structural difference may explain the altered action profile.

Theoretically, the prenylflavonoids could have shown the same effect, if they altered the activity or expression of the

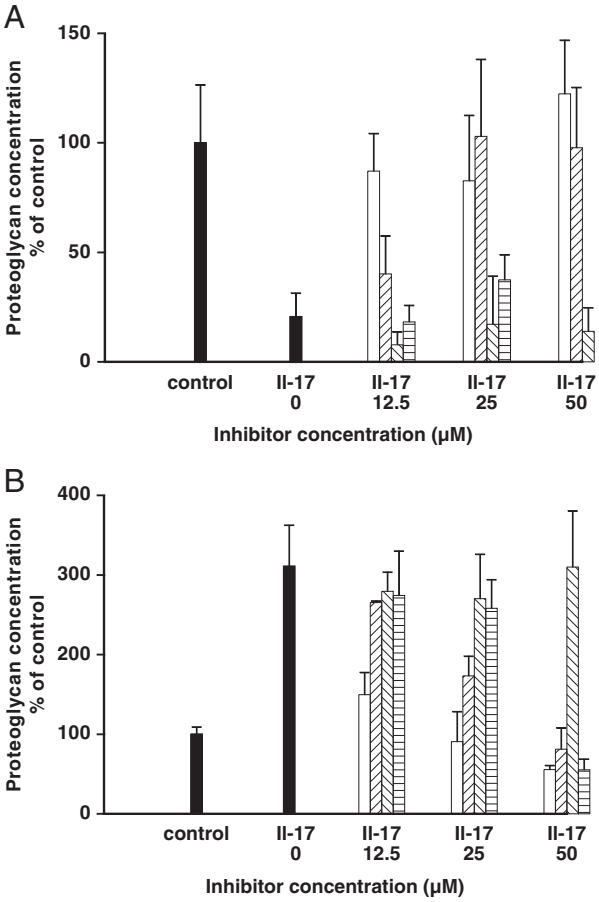


Figure 4. Inhibition of proteoglycan loss. Bovine cartilage explants were weighed and incubated in media (control) or in media containing an osteoarthritis inducing mixture of IL-17 in the presence of increasing concentrations of the prenylflavonoids. The proteoglycan concentrations were determined after 3 days in the guanidinium hydrochloride solubilized explants (A) and in the culture media (B). The concentrations were related to 100% of the control. The error bars indicate the SD of four determinations.

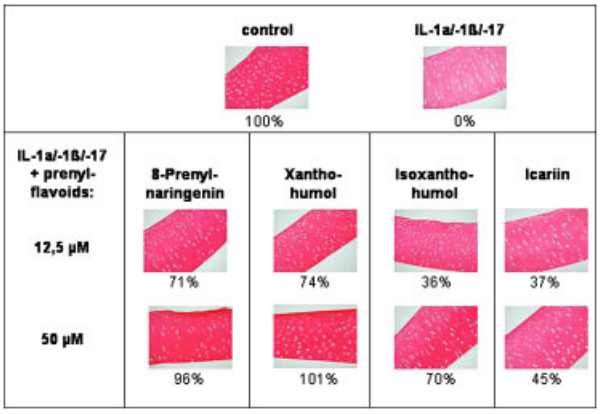


Figure 5. Inhibition of collagen degradation. Cartilage explants were incubated with and without a mixture of IL-1α, IL-1β and IL-17 for 14 days in the presence of 12.5 or 50 µM 8-prenylnaringenin, xanthohumol, isoxanthohumol or icariin. Collagen was visualized by the van Giesson stain. The staining intensity was quantified using the Scion image software and the results were calculated as percentage of protection from the IL-treated image.

hyaluronan synthase or the expression of MRP5. However, these possibilities were unlikely, because the hyaluronan synthase activity, as measured by incorporation of radioactive glucuronic acid in membrane preparation, was not effected, and because of the immediate effect on the fluorescein efflux test, as inhibition of MRP5 expression should take a longer period.

The paradoxical phenomenon that icariin inhibited hyaluronan export from chondrocytes and fibroblasts, but activated fluorescein export from MRP5 overexpressing HEK cells, could be caused by the different experimental setup. Fluorescein export was measured immediately after addition of the prenylflavonoids and icariin activated the MRP5 export, whereas hyaluronan export could only be measured after a certain amount of hyaluronan had accumulated in the media. Thus, icariin had the opportunity to inhibit PDE5 and raising the cGMP concentration which then inhibited hyaluronan export. In contrast, xanthohumol, isoxanthohumol and 8-prenylnaringenin appeared to inhibit MRP5 export directly.

Their efficacy was tested on osteoarthritic reactions. In osteoarthritis, hyaluronan overproduction is an early cellular reaction and precedes protease activation that leads to collagen degradation and cartilage destruction [21, 23, 24]. In previous publications we showed that hyaluronan overproduction led to increased diffusion of proteolytic enzymes through the lacunae of the cartilage matrix, which in turn caused the loss of proteoglycans and degradation of collagen from osteoarthritic cartilage [5, 6]. Our results showed that the prenylflavonoids from hops reduced hyaluronan export, protected cartilage from proteoglycan loss and inhibited the release of metalloproteases into the medium and collagen degradation at micromolar concentrations. In contrast, icariin inhibited only hyaluronan export from isolated fibroblasts and chondrocytes, but not proteoglycan loss or collagen degradation.

The prenylflavonoids from hops and in particular xanthohumol have been intensively studied and beneficial effects were found for many diseases including chronic allergic contact dermatitis [25], hepatoma carcinoma proliferation [26], hepatic inflammation and fibrosis [27] and chronic lymhocytic leukemia [28]. They are anti-inflammatory [29], antimutagenic [30], anti-invasive [31] and antigenotoxic [32]. Although xanthohumol has been shown to modulate other cellular targets such as TNF-α release [33], suppression of NF-kappaB regulated gene products [34], inhibition of topoisomerase [35] and alkaline phosphatase [36] it is also possible that some of these cellular and physiological effects are mediated by the inhibition of hyaluronan export.

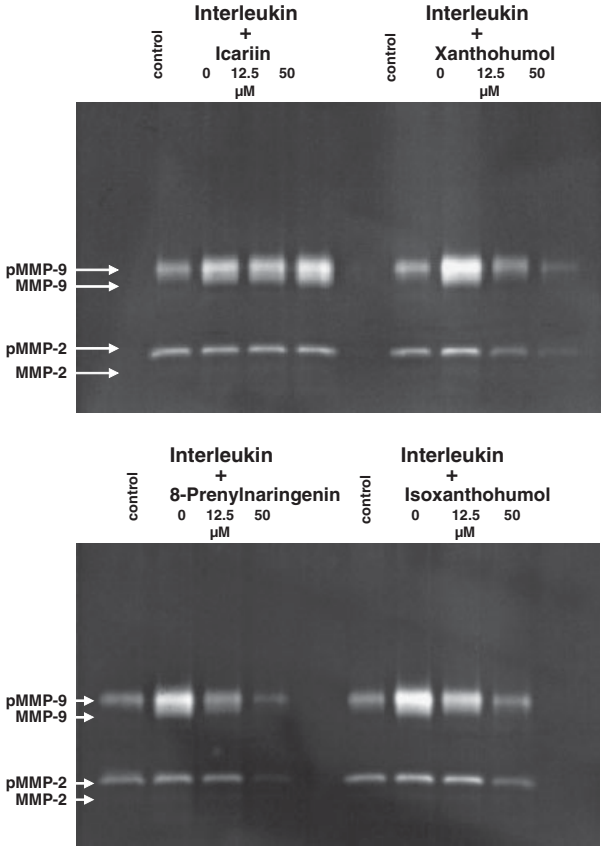


Figure 6. Inhibition of gelatinase liberation. Chondrocytes in alginate beads were incubated in the absence or presence of 25 ng/mL IL-17 and the prenylflavonoids xanthohumol, isoxanthohumol, 8-prenylnaringenin and icariin at concentrations of 12.5 and 50 µM for 3 days at 37°C. The activity of gelatin degrading enzymes released into the culture supernatant was determined by zymography.

The efficacy of xanthohumol on the inhibition of hyaluronan export by MRP5 may also extend to other diseases with hyaluronan overproduction such as metastasis, edema formation or inflammation. We showed previously that other unspecific MRP5 inhibitors reduced migration and invasion of metastatic melanoma cells [9]. On the other hand, the well-known beneficial action of xanthohumol on the diseases mentioned above could be mediated by reducing hyaluronan production.

It is interesting to note that xanthohumol can be converted to isoxanthohumol by the acidic pH of the stomach which in turn can be converted to 8-prenylnaringenin by demethylation in the liver [37]. 8-prenylnaringenin is the strongest known phytoestrogen, whereas xanthohumol is not estrogenic [38, 39]. It has been speculated that the phytoestrogens may have evolved to defend against animals that feed on the female cones and the seeds [40]. One possible target could be the inhibition of oocytes maturation that is highly dependent on hyaluronan synthesis [41, 42].

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5 References

[1] Toole, B. P., Hyaluronan: from extracellular glue to pericellular cue. *Nat. Rev. Cancer* 2004, *4*, 528–539.

[2] Nishida, Y., D'Souza, A. L., Thonar, E. J., Knudson, W., Stimulation of hyaluronan metabolism by interleukin-1alpha in human articular cartilage. *Arthritis Rheum.* 2000, *43*, 1315–1326.

[3] D'Souza, A. L., Masuda, K., Otten, L. M., Nishida, Y. *et al.*, Differential effects of interleukin-1 on hyaluronan and proteoglycan metabolism in two compartments of the matrix formed by articular chondrocytes maintained in alginate. *Arch. Biochem. Biophys.* 2000, *374*, 59–65.

[4] Prehm, P., Hyaluronan, der Gewebe-Expander: ''Weg da, jetzt komme ich''. *Biospektrum* 2008, *5*, 473–475.

[5] Deiters, B., Prehm, P., Inhibition of hyaluronan export reduces collagen degradation in IL-1 treated cartilage. *Arthritis Res. Ther.* 2008, *10*, R8.

[6] Prehm, P., Inhibitors of hyaluronan export prevent proteoglycan loss from osteoarthritic cartilage. *J. Rheumatol.* 2005, *32*, 690–696.

[7] Le Graverand-Gastineau, M. P., Disease modifying osteoarthritis drugs: facing development challenges and choosing molecular targets. *Curr. Drug Targets* 2010.

[8] Prehm, P., Schumacher, U., Inhibition of hyaluronan export from human fibroblasts by inhibitors of multidrug resistance transporters. *Biochem. Pharmacol.* 68, *2004*, 1401–1410.

[9] Monz, K., Maas-Kuck, K., Schumacher, U., Schulz, T. *et al.*, Inhibition of hyaluronan export attenuates cell migration and metastasis of human melanoma. *J. Cell. Biochem.* 2008, *105*, 1260–1266.

[10] Schulz, T., Schumacher, U., Prehm, P., Hyaluronan export by the ABC-transporter MRP5 and its modulation by intracellular cGMP. *J. Biol. Chem.* 2007, *282*, 20999–21004.

[11] Xin, Z. C., Kim, E. K., Lin, C. S., Liu, W. J. *et al.*, Effects of icariin on cGMP-specific PDE5 and cAMP-specific PDE4 activities. *Asian J. Androl.* 2003, *5*, 15–18.

[12] O'Brien, J., Wilson, I., Orton, T., Pognan, F., Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* 2000, *267*, 5421–5426.

[13] Bjornsson, S., Simultaneous preparation and quantitation of proteoglycans by precipitation with Alcian Blue. *Anal. Biochem.* 1993, *210*, 282–291.

[14] Nakayama, G. R., Caton, M. C., Nova, M. P., Parandoosh, Z., Assessment of the Alamar Blue assay for cellular growth and viability *in vitro*. *J. Immunol. Methods* 1997, *204*, 205–208.

[15] Klempner, M. S., Mikkelsen, R. B., Corfman, D. H., Andre, S. J., Neutrophil plasma membranes. I. High-yield purification of human neutrophil plasma membrane vesicles by nitrogen cavitation and differential centrifugation. *J. Cell Biol.* 1980, *86*, 21–28.

[16] Stern, M., Stern, R., An ELISA-like assay for hyaluronidase and hyaluronidaseinhibitors. *Matrix* 1992, *12*, 397–403.

[17] Terry, D. E., Chopra, R. K., Ovenden, J., Anastassiades, T. P., Differential use of Alcian blue and toluidine blue dyes for the quantification and isolation of anionic glycoconjugates from cell cultures: application to proteoglycans and a high-molecular-weight glycoprotein synthesized by articular chondrocytes. *Anal. Biochem.* 2000, *285*, 211–219.

[18] van Gieson, I., Laboratory notes of technical methods for the nervous system. *N. Y. Med. J.* 1889, *50*, 57–60.

[19] Lowry, O. H., Rosebrough, A. L., Farr, A. L., Randall, R. S., Protein measurement with folin phenol reagent. *Anal. Biochem.* 1951, *87*, 265–275.

[20] Benderdour, M., Tardif, G., Pelletier, J. P., Di Battista, J. A. *et al.*, Interleukin 17 (IL-17) induces collagenase-3 production in human osteoarthritic chondrocytes *via* AP-1 dependent activation: differential activation of AP-1 members by IL-17 and IL-1beta. *J. Rheumatol.* 2002, *29*, 1262–1272.

[21] Kozaci, L. D., Buttle, D. J., Hollander, A. P., Degradation of type II collagen, but not proteoglycan, correlates with matrix metalloproteinase activity in cartilage explant cultures. *Arthritis Rheum.* 1997, *40*, 164–174.

[22] Sasaki, K., Hattori, T., Fujisawa, T., Takahashi, K. *et al.*, Nitric oxide mediates interleukin-1-induced gene expression of matrix metalloproteinases and basic fibroblast growth factor in cultured rabbit articular chondrocytes. *J. Biochem. (Tokyo)* 1998, *123*, 431–439.

[23] Billinghurst, R. C., Wu, W., Ionescu, M., Reiner, A. *et al.*, Comparison of the degradation of type II collagen and proteoglycan in nasal and articular cartilages induced by interleukin-1 and the selective inhibition of type II collagen cleavage by collagenase. *Arthritis Rheum.* 2000, *43*, 664–672.

[24] Bayliss, M. T., Howat, S., Davidson, C., Dudhia, J., The organization of aggrecan in human articular cartilage. Evidence for age-related changes in the rate of aggregation of newly synthesized molecules. *J. Biol. Chem.* 2000, *275*, 6321–6327.

[25] Cho, Y. C., You, S. K., Kim, H. J., Cho, C. W. *et al.*, Xanthohumol inhibits IL-12 production and reduces chronic allergic contact dermatitis. *Int. Immunopharmacol.* 2010, *10*, 556–561.

[26] Dorn, C., Weiss, T. S., Heilmann, J., Hellerbrand, C., Xanthohumol, a prenylated chalcone derived from hops, inhibits proliferation, migration and interleukin-8 expression of hepatocellular carcinoma cells. *Int. J. Oncol.* 2010, *36*, 435–441.

[27] Dorn, C., Kraus, B., Motyl, M., Weiss, T. S. *et al.*, Xanthohumol, a chalcon derived from hops, inhibits hepatic inflammation and fibrosis. *Mol. Nutr. Food Res.* 2010, *54*, S205–S213.

[28] Lust, S., Vanhoecke, B., Van, G. M., Boelens, J. *et al.*, Xanthohumol activates the proapoptotic arm of the unfolded protein response in chronic lymphocytic leukemia. *Anticancer Res.* 2009, *29*, 3797–3805.

[29] Cho, Y. C., Kim, H. J., Kim, Y. J., Lee, K. Y. *et al.*, Differential anti-inflammatory pathway by xanthohumol in IFN-gamma and LPS-activated macrophages. *Int. Immunopharmacol.* 2008, *8*, 567–573.

[30] Kac, J., Plazar, J., Mlinaric, A., Zegura, B. *et al.*, Antimutagenicity of hops (*Humulus lupulus* L.): bioassay-directed fractionation and isolation of xanthohumol. *Phytomedicine* 2008, *15*, 216–220.

[31] Vanhoecke, B., Derycke, L., Van, M. V., Depypere, H. *et al.*, Antiinvasive effect of xanthohumol, a prenylated chalcone present in hops (*Humulus lupulus* L.) and beer. *Int. J. Cancer* 2005, *117*, 889–895.

[32] Plazar, J., Filipic, M., Groothuis, G. M., Antigenotoxic effect of xanthohumol in rat liver slices. *Toxicol. In Vitro* 2008, *22*, 318–327.

[33] Lupinacci, E., Meijerink, J., Vincken, J. P., Gabriele, B. *et al.*, Xanthohumol from hop (*Humulus lupulus* L.) is an efficient inhibitor of monocyte chemoattractant protein-1 and tumor necrosis factor-alpha release in LPS-stimulated RAW 264.7 mouse macrophages and U937 human monocytes. *J. Agric. Food Chem.* 2009.

[34] Harikumar, K. B., Kunnumakkara, A. B., Ahn, K. S., Anand, P. *et al.*, Modification of the cysteine residues in IkappaBalpha kinase and NF-kappaB (p65) by xanthohumol leads to suppression of NF-kappaB-regulated gene products and potentiation of apoptosis in leukemia cells. *Blood* 2009, *113*, 2003–2013.

[35] Lee, S. H., Kim, H. J., Lee, J. S., Lee, I. S., Kang, B. Y., Inhibition of topoisomerase I activity and efflux drug transporters' expression by xanthohumol from hops. *Arch. Pharm. Res.* 2007, *30*, 1435–1439.

[36] Guerreiro, S., Monteiro, R., Martins, M. J., Calhau, C. *et al.*, Distinct modulation of alkaline phosphatase isoenzymes by 17beta-estradiol and xanthohumol in breast cancer MCF-7 cells. *Clin. Biochem.* 2007, *40*, 268–273.

[37] Nikolic, D., Li, Y., Chadwick, L. R., Pauli, G. F., van Breemen, R. B., Metabolism of xanthohumol and isoxanthohumol, prenylated flavonoids from hops (*Humulus lupulus* L.), by human liver microsomes. *J. Mass Spectrom.* 2005, *40*, 289–299.

[38] Coldham, N. G., Sauer, M. J., Identification, quantitation and biological activity of phytoestrogens in a dietary supplement for breast enhancement. *Food Chem. Toxicol.* 2001, *39*, 1211–1224.

[39] Possemiers, S., Bolca, S., Grootaert, C., Heyerick, A. *et al.*, The prenylflavonoid isoxanthohumol from hops (*Humulus lupulus* L.) is activated into the potent phytoestrogen 8-prenylnaringenin *in vitro* and in the human intestine. *J. Nutr.* 2010, *136*, 1862–1867.

[40] Stevens, J. F., Page, J. E., Xanthohumol and related prenylflavonoids from hops and beer: to your good health. *Phytochemistry* 2004, *65*, 1317–1330.

[41] Russell, D. L., Salustri, A., Extracellular matrix of the cumulus–oocyte complex. *Semin. Reprod. Med.* 2006, *24*, 217–227.

[42] Scarchilli, L., Camaioni, A., Bottazzi, B., Negri, V. *et al.*, PTX3 interacts with inter-alpha-trypsin inhibitor: implications for

hyaluronan organization and cumulus oophorus expansion. *J. Biol. Chem.* 2007, *282*, 30161–30170.

[43] Ko, W. C., Shih, C. M., Lai, Y. H., Chen, J. H., Huang, H. L., Inhibitory effects of flavonoids on phosphodiesterase isozymes from guinea pig and their structure–activity relationships. *Biochem. Pharmacol.* 2004, *68*, 2087–2094.

[44] Dell'Agli, M., Maschi, O., Galli, G. V., Fagnani, R. *et al.*, Inhibition of platelet aggregation by olive oil phenols *via* cAMP-phosphodiesterase. *Br. J. Nutr.* 2008, *99*, 945–951.

