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Research Article

Binding of the hop (*Humulus lupulus* L.) chalcone xanthohumol to cytosolic proteins in Caco-2 intestinal epithelial cells

Yan Pang, Dejan Nikolic, Dongwei Zhu, Lucas R. Chadwick, Guido F. Pauli, Norman R. Farnsworth and Richard B. van Breemen

Department of Medicinal Chemistry and Pharmacognosy, UIC/NIH Center for Botanical Dietary Supplements Research, University of Illinois College of Pharmacy, Chicago, IL, USA

Used in the brewing of beer, hops (*Humulus lupulus* L.) contain the prenylated chalcone xanthohumol, which is under investigation as a cancer chemoprevention agent and as a precursor for the estrogenic flavanones isoxanthohumol and 8-prenylnaringenin. The uptake, transport and accumulation of xanthohumol were studied using the human intestinal epithelial cell line Caco-2 to help understand the poor bioavailability of this chalcone. Studies were carried out using Caco-2 cell monolayers 18–21 days after seeding. The apparent K_m and V_{max} values of xanthohumol accumulation in Caco-2 cells were determined, and the protein binding of xanthohumol in sub-cellular fractions of Caco-2 cells was investigated. Approximately 70% of xanthohumol added to the apical side of Caco-2 cells accumulated inside the cells, while 93% of the intracellular xanthohumol was localized in the cytosol. Xanthohumol accumulation was temperature dependent and saturable with an apparent K_m value of 26.5 ± 4.66 μM and an apparent V_{max} of 0.215 ± 0.018 nmol/mg protein/min. Facilitated transport was not responsible for the uptake of xanthohumol, instead, accumulation inside the Caco-2 cells was apparently the result of specific binding to cytosolic proteins. These data suggest that specific binding of xanthohumol to cytosolic proteins in intestinal epithelial cells contributes to the poor oral bioavailability observed previously *in vivo*.

Keywords: Bioavailability / Caco-2 cells / Hops / Intestinal absorption / Xanthohumol

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1 Introduction

The strobiles of *Humulus lupulus* L. (hops) are used as a flavoring agent for beer, and dietary supplements containing hop extracts are under investigation as botanical alternatives to synthetic or equine estrogens for the relief of hot flashes and related symptoms in menopausal women [1]. The most estrogenic constituent of hop preparations is 8-prenylnaringenin, which is formed at low levels during processing by the cyclization of desmethylxanthohumol. Occurring naturally in hops, isoxanthohumol is weakly estrogenic [1]. Although not estrogenic itself, the hops chalcone xanthohumol (see structure in Fig. 1) can cyclize to form the weak estrogen isoxanthohumol under the acidic conditions of the stomach [2]. In addition, isoxanthohumol can be metabolized by human liver enzymes to form the potent estrogen 8-prenylnaringenin [2]. Therefore, xanthohumol might function as a pro-estrogen, if it is absorbed and transported to the liver following oral administration.

Xanthohumol is the most abundant prenylated chalcone in hops. This chalcone (see Fig. 1) has been advocated as a cancer chemoprevention agent [3, 4] that might function by inhibiting the cytochrome P450-catalyzed activation of chemical carcinogens [5], detoxifying carcinogens through the induction of the antioxidant response element and such proteins as quinone reductase [6, 7], inhibiting the production of nitric oxide [8], or by inducing apoptosis in cancer cells [9]. In addition to its potential chemoprevention activity, xanthohumol inhibits diacylglycerol acyltransferase and thereby can reduce the accumulation of triglycerides and potentially lower the risk of atherosclerosis [10, 11].

Correspondence: Dr. Richard B. van Breemen, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, 833 S Wood Street, Chicago, IL 60612-7231, USA
E-mail: breemen@uic.edu
Fax: +1-312-996-7107

Abbreviations: **AP**, apical; **BL**, basolateral; **HBSS**; Hank's balanced salt solution; **TEER**, transepithelial electrical resistance

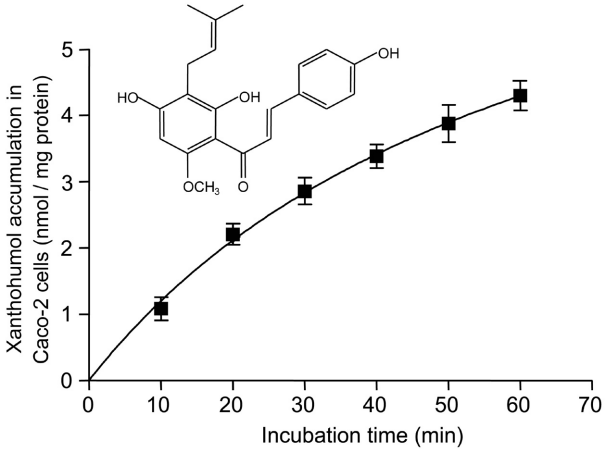

Figure 1. The time course of xanthohumol accumulation by Caco-2 cells. The Caco-2 cell monolayers were incubated for up to 1 h at 37°C with 10 μM xanthohumol in the apical chamber. Each data point represents the mean ± SE of three independent experiments with different Caco-2 cell monolayers.

To complement the knowledge of its biological activities, several investigations have addressed the metabolism and bioavailability of xanthohumol. For example, incubations with human liver microsomes [12–14] and recombinant human UDP-glucuronosyltransferases and sulfotransferases [15] have been carried out, and these results predict extensive metabolism of xanthohumol in the liver. However, *in vivo* rodent studies indicate that orally administered xanthohumol is not detected in plasma and that a majority of this dose is excreted unchanged with the feces [16, 17]. These findings suggest that the chemopreventive activity of xanthohumol might be restricted by its poor bioavailability.

The aim of the present study was to investigate intestinal permeability as a limiting factor in the bioavailability of xanthohumol using the Caco-2 cell monolayer model. The Caco-2 cell line is derived from a human colon adenocarcinoma and can undergo spontaneous enterocytic differentiation in culture to resemble epithelial cells of the small intestine. This model has become a standard tool for the prediction of intestinal drug absorption in humans and for mechanistic studies of drug transport [18].

2 Materials and methods

2.1 Materials

Xanthohumol was isolated from a methanolic extract of spent hops and characterized by spectroscopy (in particular, NMR and MS) as described previously [14]. Chrysin, sucrose, propranolol, sulfatase (type VI from *Aerobacter aerogenes*), sodium azide, rotenone, oligomycin, dipyridamole, protease inhibitor cocktail, BSA, and DTT were purchased from Sigma-Aldrich (St. Louis, MO). The Caco-2 cell line, Eagle's minimum essential medium and fetal bovine serum were purchased from the American Type Culture Collection (Rockville, MD). Trypsin-EDTA (0.25%), Hank's balanced salt solution (HBSS), HEPES buffer solution, PBS (610), penicillin, and streptomycin were purchased from Life Technology (Grand Island, NY).

Transwell polycarbonate inserts (24-mm diameter, 4.71-cm^2 surface area and 0.4-μm pore size) were purchased from Corning Costar (Cambridge, MA). Transepithelial electrical resistance (TEER) was measured using a Millicell-ERS instrument from Millipore (Bedford, MA), and ultrafiltration centrifuge tubes with a 10000 molecular weight cut-off were purchased from Millipore.

2.2 Cell culture

Caco-2 cells were cultured at 37°C in Eagle's minimum essential medium with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in an atmosphere of 5% carbon dioxide and 90% relative humidity. When the Caco-2 cells (passage numbers 23–32) had reached approximately 70–80% confluence, they were removed using 0.25% trypsin containing EDTA, seeded onto polycarbonate membranes in Transwell plates at a density of 2×10^5 cells/insert and cultured until late confluence. The cell culture medium was changed every other day after seeding. The integrity of each monolayer of differentiated cells was monitored by measuring the TEER. Only monolayers with TEER values >300 Ωcm^{-2} were utilized.

2.3 Assays of Caco-2 cell permeability and xanthohumol transport

In preparation for the Caco-2 cell monolayer assays, the cell culture medium was removed from both the apical (AP) and basolateral (BL) chambers of the Transwell plates. The cells were washed three times with HBSS containing 20 mM HEPES at pH 7.4 and pre-incubated for 30 min at 37°C on a shaker bath at 50 rpm. A 10-mM stock solution of xanthohumol in DMSO was diluted to different concentrations in HBSS/HEPES buffer such that the final concentration of DMSO was less than 1%. These xanthohumol test solutions were added either to the AP side (for AP → BL measurement) or to the BL side (BL → AP measurement) of the monolayers, and blank HBSS/HEPES buffer was added to the other side. As control experiments, the low permeability standard sucrose (100 μM) and the high permeability standard propranolol (50 μM) were added to the AP chambers of parallel incubations.

Samples from the recipient compartments were collected, replaced with fresh buffer at specific time points (10, 20, 30, 40, and 60 min or 1, 2, 3, and 4 h) and then analyzed by LC-MS as described below for the determination of xanthohumol concentration. After 1 h of incubation, the TEER of each monolayer was measured and found to be >300 Ωcm^{-2}, indicating good monolayer integrity. In addi-

tion, the apparent permeability coefficients (P_{app}) of sucrose and propranolol were measured in parallel incubations using the LC-MS methods of van Breemen *et al.* [18] with the modifications noted in the Section 2.6. The P_{app} value of sucrose was low at $(1.96 \pm 0.50) \times 10^{-7}$ cm/s, indicating that the Caco-2 cell monolayers utilized in this study possessed suitable monolayer integrity. As an additional control, the P_{app} value of propranol was determined to be $(6.75 \pm 0.20) \times 10^{-5}$ cm/s, which is typical of high permeability compounds.

To measure the kinetics of xanthohumol accumulation by Caco-2 cells, a 10-mM stock solution of xanthohumol in DMSO was diluted with HBSS/HEPES buffer to obtain concentrations of 2.5, 5, 10, 20, 30, 40, and 50 μM. The xanthohumol solutions were added to the AP side of Caco-2 cell monolayers, blank HBSS/HEPES buffer was added to the BL side, and the cells were incubated for 10 min at 37°C. After incubation, each cell monolayer was washed twice with fresh HBSS/HEPES buffer and then extracted with 1 mL of methanol for 30 min. The methanol extracts were collected for quantitative analysis of xanthohumol by LC-MS. All experiments were carried out three times.

The role of transporter proteins in xanthohumol accumulation in Caco-2 cells was investigated by comparing the cellular uptake of xanthohumol in the presence or absence of various transport inhibitors. Caco-2 cell monolayers were rinsed three times with PBS (pH 7.4) and then preincubated for 30 min with or without (control) 10 mM sodium azide and 50 mM 2-deoxyglucose. Alternatively, the monolayers were preincubated for 10 min with or without (control) 10 μM rotenone and 10 mM 2-deoxyglucose, 20 μM oligomycin, or 10 μM dipyridamole. After preincubation, 10 μM xanthohumol was added to the AP side of each monolayer, and the Caco-2 cells were incubated for another 10 min. After the incubation, the monolayers were washed three times using blank PBS and were cut out from the inserts using a tissue culture insert cutter Nalge Nunc International (Rochester, NY). Methanol (1 mL) was added to each monolayer, and the amount of xanthohumol in the cells was determined using LC-MS.

2.4 Subcellular distribution of xanthohumol in Caco-2 cells

For the investigation of the subcellular distribution of xanthohumol in Caco-2 cells, the monolayers were incubated as described above for the kinetics experiments except that only 10 μM xanthohumol was used, and the incubation time was extended to 1 h. The cell monolayers were rinsed three times with ice-cold PBS and then scraped off with a plastic scraper. The cells were pelleted by centrifugation (10 min at $420 \times g$) and then lysed by incubation for 1 h at 4°C in 1 mL hypotonic buffer (20 mM Tris at pH 7.4, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM DTT, 1 mM EDTA, and 1% protease inhibitor cocktail). Next, the cells were homogenized and centrifuged at $940 \times g$ for 15 min at 4°C to obtain a crude nuclear pellet. The supernatant was centrifuged at $16\,000 \times g$ for 3 h at 4°C to collect the membrane pellet and a supernatant containing cytosolic proteins. Aliquots of 0.15, 0.3 or 2 mL methanol were added to each nuclear pellet, membrane pellet and cytosolic protein fraction, which were then incubated for 30 min at room temperature before being centrifuged at $940 \times g$ for 106 min. Next, the supernatants were collected and analyzed for xanthohumol using LC-MS.

The protein content of the Caco-2 cells and their sub-cellular preparations were determined by the method of Bradford [19] with a Bio-Rad protein assay reagent. BSA was used as a standard. The protein levels were used for normalization and comparison of xanthohumol levels in different samples.

2.5 Binding of xanthohumol to cytosolic proteins

After the Caco-2 monolayers reached late confluence, the cytosolic protein fraction was isolated for binding studies as described above for the subcellular distribution experiment. After extraction, the cytosolic protein was diluted to 0.15 mg/mL using stabilizing buffer consisting of 75 mM Tris pH 7.4, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 1% protease inhibitor cocktail. A 40-μL aliquot of 10 μM xanthohumol was incubated for 1 h at room temperature with 200 μL of the 0.15 mg/mL cytosolic protein fraction. After the incubation, the unbound xanthohumol and other low mass compounds were removed by ultrafiltration through a 10 000 molecular weight cut-off membrane using a procedure similar to that described by Nikolic *et al.* [20] except that a mixture of cytosolic proteins was substituted for purified protein. Briefly, the cytosolic protein mixture containing xanthohumol-protein complexes in the ultrafiltration chamber was washed with stabilizing buffer to remove unbound compounds, and xanthohumol was released from protein by adding methanol/water (90:10; v/v). The ultrafiltrate was collected and then analyzed using LC-MS. To control for nonspecific binding to proteins and/or to the ultrafiltration apparatus, a control experiment was carried out, which was identical except that denatured cytosolic proteins were substituted for the active cytosolic proteins. The cytosolic proteins (200 μL of a 0.15-mg/mL solution) were denatured in the ultrafiltration chamber using methanol/water (90:10; v/v) so that the amount of protein in the chamber for the control measurements was identical to that used for the experiments.

2.6 LC-MS

All LC-MS analyses were carried out using an Agilent (Palo Alto, CA) G1946A single quadrupole mass spectrometer equipped with an 1100 HPLC system. The dwell time for SIM during quantitative analysis was 440 ms per ion.

Table 1. Caco-2 cell monolayer permeability to xanthohumol measured using LC-MS

Initial concentration (μM)	Concentration of xanthohumol in the receiving chamber at different time points (nM)							
	10 min	20 min	30 min	40 min	60 min	2 h	3 h	4 h
5 (AP[a)]-BL[b)])	0	0	0	0	0			
10 (AP-BL)	0	0	0	0	1.9	11.9	18.9	28.1
50 (AP-BL)	0	0	0	0	4.0			
10 (BL-AP)					14.7	51.9	85.0	132.0

a) AP = apical chamber.
b) BL = basolateral chamber.

For the quantitative analysis of xanthohumol, chrysin (0.1 μM final concentration) was added to each sample as an internal standard. RP HPLC separations were carried out using a Waters (Milford, MA) Xterra C_{18} column (2.1 × 20 mm, 3.5-μm id) at a flow rate of 0.3 mL/min. The solvent system consisted of a 30 min gradient from 20–90% ACN in water. After holding at 90% ACN for 3.5 min, the column was re-equilibrated at 20% ACN for 4 min before the next analysis.

During LC-MS, the deprotonated molecule of xanthohumol was monitored at *m/z* 353, and the deprotonated molecule of the internal standard chrysin was measured at *m/z* 253 using SIM. The standard curve for xanthohumol quantitation was linear (R^2 = 0.998, N = 3) over the range 32 fmol (11.3 pg) to 20 pmol (7.08 pg) injected on-column. The LOD (defined as an S/N of 3) was 9.6 fmol (3.4 pg), and the LOQ (S/N of 10) was 32 fmol (11.3 pg).

Sucrose and propranolol were measured using LC-MS as described previously [18], except that sucrose was substituted for mannitol. The standard curve for the quantitative analysis of sucrose was linear (R^2 = 0.995) from 58 fmol (19.8 pg) to 57 pmol (19.5 ng) injected on-column. The LOD and LOQ for sucrose were 17.4 fmol (5.95 pg) and 58 fmol (19.8 pg), respectively.

2.7 Statistical analysis

Data were analyzed statistically using the Student's *t*-test, and non-linear regression analysis was carried out using GraphPad (San Diego, CA) Prism 4. Differences between means of measurements of $p < 0.05$ were considered significant.

3 Results

3.1 Permeability of Caco-2 cell monolayers to xanthohumol

The apparent permeability coefficients of xanthohumol across Caco-2 cell monolayers were determined in the AP to the BL direction and in the BL to the AP direction. Using typical incubation time points for Caco-2 cell monolayer experiments (10, 20, 30, 40, and 60 min) and initial donor side concentrations of xanthohumol of 5, 10 and 50 μM, xanthohumol could be detected using LC-MS on the receiving side of the Caco-2 cell monolayer only for the 10 and 50 μM initial concentrations at the 60-min time point. After 60-min, xanthohumol was detected on the receiving side of the Caco-2 cell monolayer at 2 and 4 nM for the 10 and 50 μM initial donor concentrations, respectively. Therefore, the Caco-2 permeability experiment was repeated using the longer time points of 1, 2, 3, and 4 h.

As indicated by the data shown in Table 1, the rate at which xanthohumol crossed the Caco-2 cell monolayer was approximately linear over the 4-h incubation period. Based on these data, the apparent permeability coefficient of xanthohumol (at an initial concentration of 10 μM) in the AP to BL direction was calculated to be $(1.33 \pm 0.03) \times 10^{-7}$ and was slightly higher in the BL to AP direction at $(2.37 \pm 0.58) \times 10^{-7}$ cm/s. Since the apparent permeability coefficient of the low permeability marker compound sucrose was similar at $(1.96 \pm 0.5) \times 10^{-7}$ cm/s, the intestinal absorption of xanthohumol is predicted to be poor. For comparison, the P_{app} value of the high permeability standard, propranol, was $(6.75 \pm 0.20) \times 10^{-5}$ cm/s in this system.

3.2 Accumulation of xanthohumol in Caco-2 cells

The amount of xanthohumol that crossed from the AP to the BL side or from the BL to the AP side of the Caco-2 cell monolayer was very low. For example, only 0.8 ± 0.1% of the 10 μM xanthohumol added to the AP side of the Caco-2 cell monolayer was detected in the BL compartment after 1 h of incubation at 37°C. However, LC-MS analysis indicated that a majority of the xanthohumol that was introduced to one side of the monolayer or the other (*i.e.* 72.0 ± 2.3% when xanthohumol was added to the AP side) became concentrated in the Caco-2 cells. The remainder of the xanthohumol (27.2 ± 2.5%, when xanthohumol was added to the AP side of the monolayer) was still in the donor compartment after 1 h as measured using LC-MS.

To investigate the uptake of xanthohumol by Caco-2 cells, the time course of xanthohumol accumulation was measured. As shown in Fig. 1 for 10 μM xanthohumol

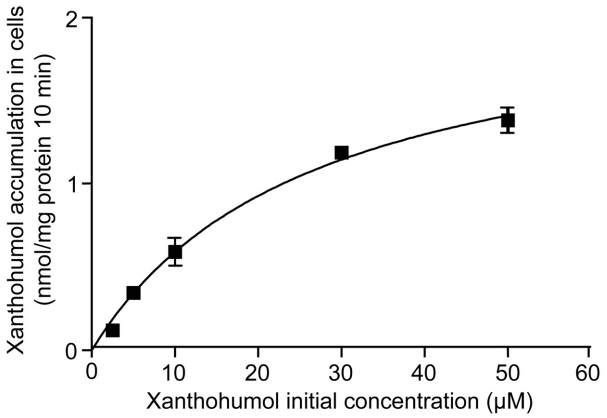

Figure 2. Concentration dependence of xanthohumol accumulation by Caco-2 cells. Caco-2 cells were incubated for 10 min at 37°C with initial concentrations of xanthohumol on the apical side of the monolayer ranging from 2.5–50 μM. Each data point represents the mean of three determinations with different Caco-2 cell monolayers.

added to the AP side of the monolayer, the rate of uptake by the Caco-2 cells was initially rapid and decreased as the concentration outside the cells decreased. Although xanthohumol readily entered Caco-2 cells in just a few minutes, almost none exited the cells on the BL side of the cells. Therefore, the low apparent permeability of xanthohumol in the Caco-2 cell model was not caused by low membrane permeability, but instead, was due to accumulation of xanthohumol inside the Caco-2 cells.

To investigate the mechanism of xanthohumol accumulation in Caco-2 cells, the effects of temperature and initial concentration of xanthohumol in the AP compartment on cellular accumulation were measured. Incubation of xanthohumol with Caco-2 monolayers at 4°C instead of 37°C (2-min incubation with 10 μM xanthohumol) decreased the amount of xanthohumol entering the cells by 47%. When the initial concentration of xanthohumol on the AP side of the Caco-2 cell monolayer was increased incrementally from 2.5 to 50 μM, the amount of xanthohumol taken up by the cells over 10 min increased but not proportionally (see Fig. 2). This indicated that the accumulation of xanthohumol by Caco-2 cells was concentration dependent and saturable. The kinetic parameters of xanthohumol uptake from the AP side of Caco-2 cells were evaluated by using nonlinear regression analysis and the Michaelis-Menten equation. During the first 10 min of incubation, the apparent K_m value was 26.5 ± 4.7 μM, and the V_{max} was 0.215 ± 0.018 nmol/mg protein/min.

To determine whether transporter proteins were involved in the uptake of xanthohumol by Caco-2 cells, the effects on uptake of several types of inhibitors were investigated. Dipyridamole was used as a nonspecific inhibitor of transporters whereas sodium azide, oligomycin and rotenone were evaluated as metabolic inhibitors that could prevent the energetic process of active transport. The results of these uptake inhibitor studies are shown in Fig. 3. Instead of inhibiting xanthohumol uptake, treatment with each inhibitor enhanced accumulation of xanthohumol by Caco-2 cells. Dipyridamole and rotenone each increased xanthohumol accumulation more than threefold compared to control incubations without inhibitors. Treatment with sodium azide or oligomycin increased intracellular levels of xanthohumol more than twofold. These results suggest that xanthohumol is not taken into Caco-2 cells by transporters. On the contrary, an active efflux process appears to reduce the rate of accumulation.

3.3 Subcellular localization of xanthohumol within Caco-2 cells

To investigate the localization of xanthohumol on a subcellular level, Caco-2 cell monolayers were incubated for 1 h with 10 μM AP xanthohumol. The cells were harvested, lysed and separated into cytosolic, membrane and nuclear fractions by centrifugation. The cytosol contained 94 ± 1.0% of the intracellular xanthohumol with the remainder being divided equally between the membrane (3 ± 0.9%) and nuclear (3 ± 0.9%) fractions.

The binding of xanthohumol to cytosolic proteins was investigated using ultrafiltration LC-MS, a technique developed in our laboratory for the evaluation of binding between proteins and ligands [21]. In this affinity-binding assay, xanthohumol was incubated with cytosolic proteins from Caco-2 cells that were either denatured (control) or in their native form. After removal of unbound xanthohumol using ultrafiltration, any xanthohumol that remained bound to the cytosolic proteins was eluted and analyzed using LC-MS. As shown in Fig. 4, the xanthohumol peak corresponding to the incubation with native cytosolic proteins was enhanced more than 20-fold compared to the control incubation. Therefore, xanthohumol bound specifically to one or more cytosolic proteins in their native conformation but not after they were denatured by treatment with methanol/water (90:10; v/v).

4 Discussion

The low apparent permeability coefficient of xanthohumol across the Caco-2 cell monolayer, which was similar to the low permeability standard sucrose, explains the poor bioavailability of xanthohumol observed by Avula *et al*. [17]. However, it should be noted that, in case only the donor and the receiver compartments of the Caco-2 cell system had been analyzed, it might have been inferred that Caco-2 cells are impermeable to xanthohumol. Instead, LC-MS analysis of the cells from these incubations indicated that xanthohumol enters Caco-2 cells quickly with a V_{max} of 0.215 ± 0.018 nmol/mg protein/min and a K_m value of 26.5 ±

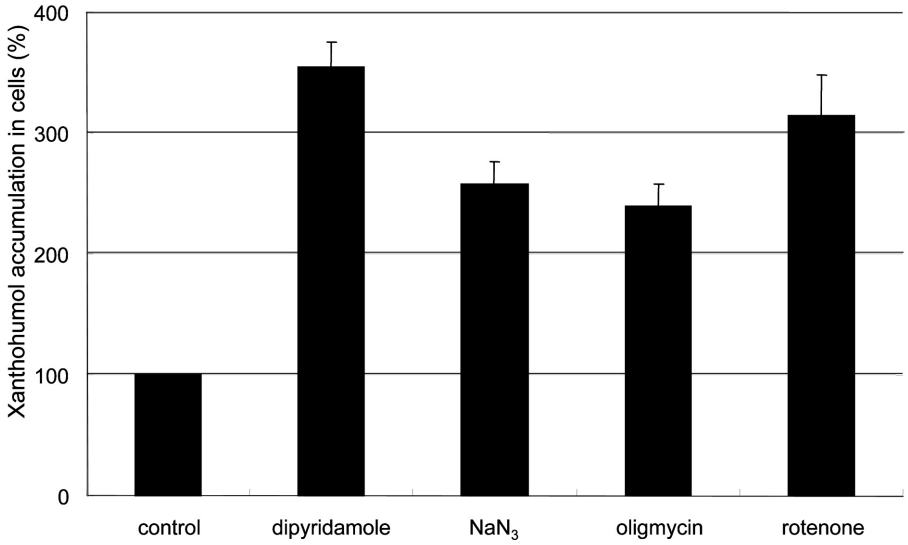

Figure 3. Effects of the nonspecific transporter inhibitor dipyridamole and various metabolic inhibitors on xanthohumol accumulation by Caco-2 cells. Incubations were carried out with 10 μM xanthohumol for 10 min in the absence (control) or presence of each inhibitor. The Caco-2 cell monolayers were preincubated for 10 min with each inhibitor prior to the addition of xanthohumol to the apical chamber. Each column represents the mean ± SE of three independent monolayer experiments.

4.7 μM. Therefore, the permeability of intestinal epithelial cells to xanthohumol is high but unidirectional. Although xanthohumol readily enters Caco-2 cells, it becomes trapped and does not exit quickly.

In contrast to our LC-MS measurements of xanthohumol permeability, Rodriguez-Proteau *et al.* [22] reported that the P_{app} of xanthohumol was high at $\sim 1.5 \times 10^{-5}$ cm/s in both the AP to BL and BL to AP directions. Although P_{app} values of reference compounds were not reported by Rodriguez-Proteau and coworkers, propranolol was used as a high permeability standard in our study and produced a P_{app} of 6.75×10^{-5} cm/s. This value for propranolol was approximately 100-fold higher than the P_{app} values of xanthohumol measured in the present study, which were 1.33×10^{-7} in the AP to BL direction and 2.37×10^{-7} cm/s in the BL to AP direction.

An important difference between our method and that of Rodriguez-Proteau was the use of LC-MS instead of scintillation counting for the quantitative analysis of xanthohumol in the Caco-2 cell preparations. Whereas LC-MS responds selectively to compounds of a particular mass eluting at a particular time, scintillation counting responds only to radioactivity and has no chromatography step to enhance selectivity. If radio-labeled xanthohumol metabolites or degradation products crossed the Caco-2 cell monolayer, they could be detected using scintillation counting and mistaken for xanthohumol. However, the LC-MS assay used here can distinguish between xanthohumol and its metabolites or degradation products. Since xanthohumol is known to form phase II metabolites such as sulfate and glucuronic acid conjugates [13] and Caco-2 cells are capable of forming phase II metabolites from botanical compounds such as resveratrol [23], it is possible that the detection of radioactive metabolites contributed to the difference between our results and those of Rodriguez-Proteau *et al.* [22]. Because the Caco-2 cell uptake of xanthohumol was concentration dependent, temperature dependent and a saturable process, transporter proteins or the involvement of binding proteins had to be taken into account. To investigate whether or not facilitated transport is involved in the accumulation of xanthohumol, Caco-2 cells were treated with various transport inhibitors, and the uptake of xanthohumol was measured. Instead of interfering with accumulation, inhibition of transport by Caco-2 cells actually enhanced the uptake of xanthohumol. Therefore, an active efflux pump system for xanthohumol must exist in Caco-2 cells. Additional evidence for the existence of an efflux pathway was provided by the ratio of the apparent permeability coefficients for xanthohumol across the Caco-2 cell monolayer, which favored the movement of xanthohumol in the BL to AP direction (2.37/1.33 for BL to AP/AP to BL). Independent confirmation of the efflux of xanthohumol by Caco-2 cells was also reported by Rodriguez-Proteau *et al.* [22], who found that xanthohumol is a substrate for the multridrug resistance protein MDR-1.

The uptake and accumulation of xanthohumol by Caco-2 cells should not be considered typical of chemoprevention agents. For comparison, the chemoprevention agent resveratrol, occurring in grapes, peanuts and some other food sources, is not known to accumulate in Caco-2 cells. Unlike xanthohumol, the intestinal permeability and absorption of resveratrol is high with a P_{app} value in the AP to BL direc-

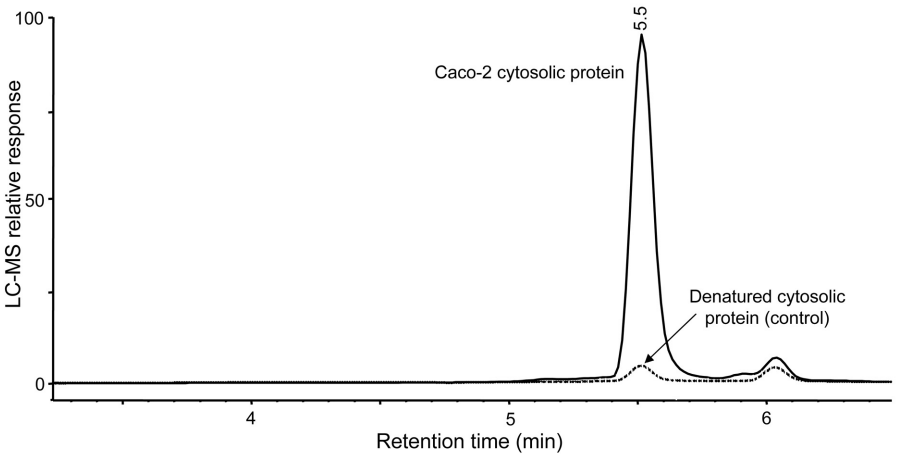

Figure 4. Ultrafiltration LC-MS investigation of the binding of xanthohumol to Caco-2 cell cytosolic proteins. Enhancement of the area of the HPLC peak corresponding to xanthohumol at 5.5 min in the experimental incubation (solid line) indicates specific binding of xanthohumol to cytosolic proteins. The control incubation (dashed line) containing denatured cytosolic protein showed no specific binding. The small peak corresponding to xanthohumol in the control was due to nonspecific binding and adsorption of xanthohumol to the ultrafiltration apparatus.

tion of $2.0 \times 10^{-}5$ cm/s [23]. Furthermore, unlike xanthohumol, resveratrol is not a substrate for P-glycoprotein or MDR-1. However, like xanthohumol, resveratrol forms phase II conjugates in Caco-2 cells.

Since facilitated transport was not responsible for the accumulation of xanthohumol by Caco-2 cells, the subcellular localization of xanthohumol was investigated using differential centrifugation and LC-MS. Instead of binding to cellular membranes or being sequestered in cellular organelles, xanthohumol was localized almost exclusively (94%) in the cytosol. Subsequent ultrafiltration LC-MS experiments indicated that xanthohumol was bound to cytosolic proteins with molecular weights of more than 10 000 Da (the molecular weight cut-off of the ultrafiltration membrane). Furthermore, xanthohumol did not bind to these proteins after they were denatured (Fig. 4).

Studies are in progress to identify the primary proteins responsible for the accumulation of xanthohumol by Caco-2 cells. It is already known that xanthohumol binds covalently to the cytosolic protein Keap1 through a Michael-type addition reaction between Keap1 cysteine sulfydryl groups and the α-β unsaturated ketone of xanthohumol [7]. In fact, Keap1 alkylation by xanthohumol has been reported to be a mechanism [7] for the possible cancer chemoprevention activity of xanthohumol [4]. Although Michael-type addition results in covalent binding, these reactions are reversible. Therefore, even if the majority of xanthohumol in the cytosol had bound covalently to cysteine-rich cytosolic proteins such as Keap1, considerable amounts of xanthohumol might still be recovered during treatment of the protein with 90% methanol and ultrafiltration.

Based on the low apparent permeability coefficient of xanthohumol determined in this investigation, the systemic bioavailability of orally administered xanthohumol can be predicted to be very low. These *in vitro* findings are consistent with *in vivo* observations of low bioavailability of xanthohumol in rats [17]. However, since xanthohumol is accumulated by intestinal epithelial cells, from which most forms of colon cancer originate, xanthohumol might be an effective colon cancer chemoprevention agent.

During the incubation of xanthohumol with Caco-2 cells, xanthohumol was found to enter the cells rapidly without the assistance of transporters. Although energy-dependent efflux of xanthohumol was detected toward the AP side of the Caco-2 cell monolayer, this process did not prevent the accumulation inside the cells of more than 70% of the xanthohumol added to the culture. When xanthohumol was added to either the AP or the BL sides of the Caco-2 cell monolayer, the rate of appearance of xanthohumol on the receiving side of the monolayer was similar to compounds such as sucrose, which do not penetrate the cells. Therefore, xanthohumol is predicted to exhibit extremely low bioavailability even though it rapidly enters intestinal epithelial cells.

Once inside the Caco-2 cells, more than 90% of the xanthohumol were accumulated in the cytosol in a process that was temperature dependent and saturable. Furthermore, xanthohumol was observed to bind to cytosolic proteins. Although the systemic bioavailability of orally administered xanthohumol is predicted to be extremely low, high concentrations of xanthohumol could be reached inside intestinal epithelial cells. As a result, orally administered

xanthohumol might be useful as a colon cancer chemoprevention agent.

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5 References

[1] Overk, C. R., Yao, P., Chadwick, L. R., Nikolic, D., *et al.*, *J. Agric. Food Chem.* 2005, *53*, 6246 – 6253.

[2] Nikolic, D., Li, Y., Chadwick, L. R., Pauli, G. F., van Breemen, R. B., *J. Mass Spectrom.* 2005, *40*, 289 – 299.

[3] Miranda, C. L., Stevens, J. F., Helmrich, A., Henderson, M. C., *et al.*, *Food Chem. Toxicol.* 1999, *37*, 271 – 285.

[4] Gerhäuser, C., Alt, A., Heiss, E., Gamal-Eldeen, A., *et al.*, *Mol. Cancer Ther.* 2002, *1*, 959 – 969.

[5] Henderson, M. C., Miranda, C. L., Stevens, J. F., Deinzer, M. L., Buhler, D. R., *Xenobiotica* 2000, *30*, 235 – 251.

[6] Miranda, C. L., Aponso, G. L., Stevens, J. F., Deinzer, M. L., Buhler, D. R., *Cancer Lett.* 2000, *149*, 21 – 29.

[7] Dietz, B. M., Kang, Y. H., Liu, G., Eggler, A. L., *et al., Chem. Res. Toxicol.* 2005, *18*, 1296 – 1305.

[8] Zhao, F., Nozawa, H., Daikonnya, A., Kondo, K., Kitanaka, S., *Biol. Pharm. Bull.* 2003, *26*, 61 – 65.

[9] Pan, L., Becker, H., Gerhäuser, C., *Mol. Nutr. Food Res.* 2005, *49*, 837 – 843.

[10] Tabata, N., Ito, M., Tomoda, H., Omura, S., *Phytochemistry* 1997, *46*, 683 – 687.

[11] Nozawa, H., *Biochem. Biophys. Res. Commun.* 2005, *336*, 754 – 761.

[12] Yilmazer, M., Stevens, J. F., Deinzer, M. L., Buhler, D. R., *Drug Metab. Dispos.* 2001, *29*, 223 – 231.

[13] Yilmazer, M., Stevens, J. F., Buhler, D. R., *FEBS Lett.* 2001, *491*, 252 – 256.

[14] Chadwick, L. R., Nikolic, D., Burdette, J. E., Overk, C. R., *et al., J. Nat. Prod.* 2004, *67*, 2024 – 2032.

[15] Ruefer, C. E., Gerhäuser, C., Frank, N., Becker, H., Kulling, S. E., *Mol. Nutr. Food Res.* 2005, *49*, 851 – 856.

[16] Nookandeh, A., Frank, N., Steiner, F., Ellinger, R., *et al., Phytochemistry* 2004, *65*, 561 – 570.

[17] Avula, B., Ganzera, M., Warnick, J. E., Feltenstein, M. W., *et al., J. Chromatogr. Sci.* 2004, *42*, 378 – 382.

[18] van Breemen, R. B., Li, Y., *Expert Opin. Drug Metab. Toxicol.* 2005, *1*,175 – 185.

[19] Bradford, M. M., *Anal. Biochem.* 1976, *72*, 248 – 254.

[20] Nikolic, D., van Breemen, R. B., *Comb. Chem. High Throughput Screen.* 1998, *1*, 47 – 55.

[21] Johnson, B. M., Nikolic, D., van Breemen, R. B., *Mass Spectrom. Rev.* 2002, *21*, 76 – 86.

[22] Rodriguez-Proteau, R., Mata, J. E., Miranda, C. L., Fan, Y., *et al*., *Xenobiotica* 2006, *36*, 41 – 58.

[23] Yu, C., Shin, Y. G., Chow, A., Li, Y., Kosmeder, J. W., *et al*., *Pharm. Res.* 2002, *19*, 1907 – 1914.