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ARTICLE

Transport of Hop Bitter Acids across Intestinal Caco-2 Cell Monolayers

Ko Cattoor,[†] Marc Bracke,[§] Dieter Deforce,[‡] Denis De Keukeleire,[†] and Arne Heyerick*,[†]

[†]Laboratory of Pharmacognosy and Phytochemistry, and [‡]Laboratory of Pharmaceutical Biotechnology, Faculty of Pharmaceutical Sciences, UGent-Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium, and [§]Laboratory of Experimental Cancer Research, Faculty of Medicine, University Hospital Ghent, De Pintelaan 185, B-9000 Ghent, Belgium

Several health-beneficial properties of hop bitter acids have been reported (inhibition of bone resorption and anticarcinogenic and anti-inflammatory activities); however, scientific data on the bioavailability of these compounds are lacking. As a first approach to study the bioavailability, the epithelial transport of hop α- and β-acids across Caco-2 monolayers was investigated. Hop acids were added either to the apical or to the basolateral chamber and, at various time points, amounts transported to the receiving compartment were determined. The monolayer integrity control was performed by using marker compounds (atenolol and propranolol), transepithelial electrical resistance (TEER) measurement, and determination of the fluorescein efflux. The TEER and fluorescein efflux confirmed the preservation of the monolayer integrity. The membrane permeability of the α-acids (apparent permeability coefficients for apical to basolateral transport (P_{appAB}) ranged from 14×10^{-6} to 41×10^{-6} cm/s) was determined to be substantially higher than that of the β-acids (P_{appAB} values ranging from 0.9×10^{-6} to 2.1×10^{-6} cm/s). Notably, the β-acids exhibited significantly different bidirectional P_{app} values with efflux ratios around 10. The involvement of carrier-mediated transport for β-acids (active efflux pathway by P-gp, BCRP, and/or MRP-2 type efflux pumps) could be confirmed by transport experiments with specific inhibitors (verapamil and indomethacin). It appears that α-acids are efficiently absorbed, whereas the permeability of β-acids is low. Limiting factors in the absorption of β-acids could involve P-gp and MRP-2 type efflux transporters and phase II metabolism.

KEYWORDS: Caco-2 transport; hop bitter acids; α-acids; β-acids

INTRODUCTION

Hops (*Humulus lupulus* L.) provide bitterness and flavor to beer and also function as a natural preservative. The increasing numbers of scientific reports on the health-beneficial properties of beer may not only relate to the presence of ethanol (*1*−*5*) but, more importantly, to unique biological activities of hop-derived constituents (*6*−*9*). Previous bioactivity-related research focused mainly on polyphenols (prenylflavonoids) present in hops with phytoestrogenic activities and chemopreventive properties (8-prenylnaringenin and xanthohumol) (*10*, *11*), but recent in vitro and in vivo studies report on various biological activities of hop bitter acids and derivatives, including inhibition of bone resorption and anti-inflammatory and anticarcinogenic properties (*12*, *13*). Humulone (IC_{50} = 5.9 nM) and lupulone were reported to inhibit bone resorption (*14*, *15*). Because of the recently established relationship between osteoporosis and inflammation, it should be mentioned that the direct anti-inflammatory effects of hop acids could also contribute to the prevention of osteoporosis (*16*). From several in vitro studies, individual hop acids proved themselves to be interesting candidates for anti-inflammatory therapy, reporting COX-2 inhibitory activities (IC_{50} = 1.6−55 μM) (*17*−*19*). Both α- and β-acids showed anticarcinogenic properties including dose-dependent induction of apoptosis of cancer cells (*20*−*23*), antiproliferative activity (by arresting cell growth of invasive cancer cells (*23*, *24*)), and antiangiogenic activity. Shimamura and coworkers reported that humulone dose-dependently prevented angiogenesis, inhibited tube formation by endothelial cells from rats, reduced cell growth of endothelial mouse cells, and suppressed the expression of vascular endothelial growth factor (VEGF) (*8*). Lupulone (2.5−50 μg/mL) induced a concentration-dependent inhibition of endothelial cell proliferation and had a strong inhibitory effect on neovascularization (*25*).

Our study attempted to probe the intestinal absorption of hop bitter acids, which comprise so-called α-acids or humulones and β-acids or lupulones (**Figure 1**) (*26*). The mixture of α-acids contains mainly three analogues, cohumulone, *n*-humulone, and adhumulone, which are differentiated by the nature of the acyl side chain. Likewise, the β-acids comprise colupulone, lupulone, and adlupulone (*27*).

*Address correspondence to this author at the Center for Evidence-based Development of Natural Therapeutics, Faculty of Pharmaceutical Sciences, Laboratory of Pharmacognosy and Phytochemistry, UGent-Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium [telephone + 32 (0)9 264 8058; mobile +32 473 562165; fax +32 (0)9 264 8192; e-mail Arne.Heyerick@UGent.be].

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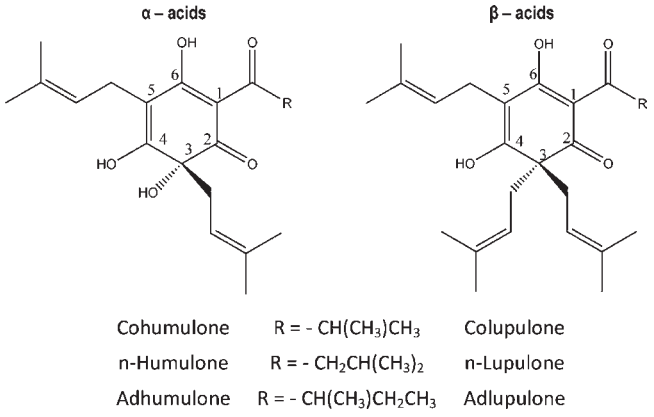

Figure 1. Chemical structures of hop bitter acids.

Although great activity on health-beneficial effects is noted in the literature, knowledge on the absorption, distribution, metabolism, and excretion (ADME) of these compounds is limited. Only one study reports on the human absorption and pharmacokinetics of reduced iso-α-acids. C_{max} (1−3 μg/mL) was reached 4 h after dosing, and the $AUC_{0-8\ h}$ ranged from 7.15 to 18.8 μg h/mL following oral administration of 1000 mg of dihydro-iso-α-acids (*28*). Insights into the bioavailabilities and the effective bioactivities are essential to understand health benefits associated with preparations containing hop-derived compounds such as beer and hop-based food supplements. Recently, induction of the quinone reductase activity by α- and iso-α-acids and activation of CYP3A4, CYP2B6, and some MDR1 levels in human hepatocytes have been reported (*6*, *29*). It appears that hop acids stimulate both phase I and phase II detoxification processes. This may be relevant with respect to the bioavailabilities as enhanced activities stimulate metabolism and excretion, resulting in lower overall bioavailabilities on repeated ingestion.

Because of the wide use of hops as main ingredient for beer brewing and the increasing amount of hop-based food supplements, the in vitro absorption of hop α- and β-acids using Caco-2 cell monolayers was investigated. The α-acids function as precursors for the main bittering principles in beer (iso-α-acids), and typically their concentrations are low, but techniques such as "dry hopping" can introduce levels up to 14 ppm (*30*). The β-acids are not present in beer, but may be present in significant quantities together with the α-acids in hop-derived food supplements that are based on carbon dioxide or ethanolic extracts. Furthermore, given the subtle differences in molecular structure between the compounds under investigation, comparison of the results allows identification of possible structure−activity relationships. The concentrations of hop acid chosen (50 μM) in the presented study can be reasoned by taking into account that an intestinal exposure to 50 μM or ≈18 ppm hop bitter acid is in line with a dry-hopped beer or a single oral dose of a food supplement containing about 10−20 mg of hop acids.

The human epithelial Caco-2 cell monolayer model using differentiated Caco-2 cells is commonly applied as a screening tool for the prediction of intestinal absorption of drug candidates and phytochemicals and for mechanistic studies of drug transport across epithelial layers (*31*). Although derived from a colon carcinoma, Caco-2 cells can differentiate, after growth, into a monolayer to exhibit the morphological characteristics of small intestinal cells (e.g., intercellular tight junctions and apical microvilli). The adjacent cells adhere through tight junctions formed at the apical side of the monolayer. Caco-2 cells express several active transport systems that resemble those found in brush borders of the human small intestine, including carriers for the transport of amino acids (*32*−*35*), nucleotides (*36*), bile acids (*37*−*39*), vitamins (*40*, *41*), oligopeptides (*42*−*45*), and monocarboxylic acids (*46*, *47*). Phospho-glycoprotein (P-gp), multidrug-resistance associated proteins (MRP), and breast-cancer resistance protein (BCRP) are also expressed in the cell membranes of Caco-2 cells and induce a basolateral-to-apical flux of xenobiotic compounds (*48*, *49*). MRP-1 proteins, expressed on the basolateral side of Caco-2 cells, enhance active transport from the basolateral to the apical compartment. More importantly, in vitro permeability coefficients measured for reference compounds obtained in the Caco-2 cell model have shown good correlation with results based on in vivo studies (*50*, *51*).

Although the intestinal permeability of other hop constituents (8-prenylnarigenin (*52*) and xanthohumol (*53*, *54*)) has been investigated, our study focuses on hop α- and β-acids as pure cohumulone and colupulone, respectively, and a mixture of *n*-humulone + adhumulone and *n*-lupulone + adlupulone, respectively. As the experiments have been performed at short incubation times, saturation kinetics apply, thus minimizing the effects of nonsaturable passive functions (*55*). The results provide first insights into the intestinal absorption of hop acids.

MATERIALS AND METHODS

Materials. Atenolol, propranolol, verapamil hydrochloride, indomethacin, Sulfatase type H1 (from *Helix pomatia*), sodium fluorescein, HEPES sodium salt 99%, D-(+)-glucose, and all HBSS buffer constituents were purchased from Sigma-Aldrich (Bornem, Belgium). Hop CO_2 extract was obtained from Hopsteiner (Mainburg, Germany). HPLC/LC-MS solvents (analytical grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Glutamine, nonessential amino acid solution, penicillin G sodium, fungizone amphotericin B, and fetal bovine serum were purchased from Gibco (Invitrogen Corp., Merelbeke, Belgium).

Cell Culture. Caco-2 cells (American Type Culture Collection (ATCC), Rockville, MD) originating from a human colorectal carcinoma were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing GLUTAMAX, supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were grown in 25 cm^2 culture flasks in an atmosphere of 10% CO_2 and 90% relative humidity at 37 °C (Forma Scientific, Marietta, OH). Cells were passaged every 7 days (90−95% confluence) at a split ratio of 1:10. For transport studies, Caco-2 cells were seeded at a density of 1×10^5 cells/insert on Transwell membrane inserts (0.4 μm pore diameter, 6.5 mm diameter, Corning Costar, Corning, NY) and cultured until late confluence. Cell culture medium was changed every other day. Monolayers were investigated between days 18 and 24 post-seeding. Cells with passage numbers 25−50 were used. The integrity of each monolayer of differentiated cells was monitored by measuring the transepithelial electrical resistance (TEER) with a Millicel-ERS volt-ohmmeter (Millipore, Bedford, MA). TEER is a measure for the presence of tight junctions between adjacent cells. Volumes amounted to 200 μL at the apical side and 750 μL at the basolateral side of the monolayer.

Methods. *Purification of* **α-** *and* **β-***Acids.* Individual hop α- and β-acids were separated on the basis of a pH-dependent fluid−fluid extraction, followed by semipreparative HPLC. Hop CO_2 extract (10 g) was dissolved in diethyl ether (50 mL) in a separation funnel. α-Acids (pK_a values = 4.7−5.6) were extracted with Na_2CO_3 (0.15 M, 3 × 50 mL) and β-acids (pK_a values = 6.7−7.6) with NaOH (0.1 M, 3 × 50 mL) (*56*). The aqueous phases containing either the deprotonated α-acids or the deprotonated β-acids were acidified with HCl (12 M, pH 2) and extracted with diethyl ether (3 × 200 mL). The fractions enriched in either α-acids or β-acids were further purified by preparative HPLC (Gilson, Villiers-le-bel, France). The injection volume (300−400 mg/mL) was 200 μL. A Varian C-18 (21.4 × 250 mm, 10 μm) column was used. Isocratic elution with a flow rate of 15 mL/min was applied with a mobile phase consisting of 30:70 (v/v) H_2O/CH_3CN + 0.025% HCOOH. The residues of the respective fractions (cohumulone, *n*-humulone + adhumulone, colupulone, *n*-lupulone + adlupulone) were extracted with diethyl ether after acidification with HCl (12 M, pH 2). The compounds were stored at −20 °C. Purities were confirmed by LC-MS (Agilent 1200 LC-MS,

Agilent, Waldbronn, Germany). The Agilent Chemstation software package (rev. B.02.01) was used to control the analytical system as well as for data acquisition and processing. As stationary phase, a 3.5 μm Zorbax SB C-18 column (150 × 30 mm; Agilent), was used. The mobile phase consisted of water + 0.025% HCOOH (A) and methanol + 0.025% HCOOH (B). The flow rate was 0.5 mL/min. The initial mobile phase, 60% B, was increased linearly to 100% B over 30 min and maintained during 5 min. Finally, the mobile phase was adjusted to 60% B in 1 min and re-equilibrated at 60% B for 4 min prior to the next injection. The MS parameters in the APCI mode were tuned to maximize formation of the deprotonated analyte. Quantitative analysis was operated in selected ion monitoring (SIM) and in the negative-ion mode using target ions at $[M - H]^-$ m/z 347 and 361 for cohumulone and *n*-humulone + adhumulone, respectively, and m/z 399 and 413 for colupulone and *n*-lupulone + adlupulone, respectively. Purities higher than 95% were confirmed for all compounds.

Bidirectional Transport Studies. The transport medium was Hanks' buffered saline solution (HBSS) containing 10 mM HEPES + 25 mM D-(+)-glucose, adjusted to pH 7.4. The osmolarity was around 350 mOsm/L, verified with an osmometer (Knauer, Berlin, Germany). Prior to the experiments, the cell culture was removed from both AP and BL chambers of the Transwell plate. The cells were washed three times and preincubated (37 °C, 10% CO_2) with transport medium for 30 min. TEER was measured before the experiments. Only cells with initial TEER values > 300 $\Omega \cdot cm^2$ were used. Stock solutions (20 mM in EtOH) of cohumulone, *n*-humulone + adhumulone, colupulone, and *n*-lupulone + adlupulone were diluted in transport medium to a final concentration of 50 μM. Due to coelution of ad- and *n*-analogues in preparative HPLC method, these compounds were applied as a mixture (*n*-humulone + adhumulone and *n*-lupulone + adlupulone, respectively). Because of their closely related molecular structure, in-source ionization efficiencies were expected to be similar in LC-MS analysis. Final EtOH contents were < 0.5%. The transport experiment was initiated by adding 50 μM hop sample to either the AP chamber (for absorptive transport study) or the BL chamber (for secretive transport study). Blank transport medium was added to the other (receiving) chamber. Each experiment was performed in triplicate (three sequential wells with Caco-2 monolayers were tested, in correspondence with several other reports on methodologies of the in vitro transport experiments across Caco-2 monolayers (*57*)). Samples from the receiving compartment were collected after 10, 20, and 30 min, respectively. During the experiments, each sampling volume (600 μL) was replaced by an equal volume of blank transport medium. Samples were stored at −20 °C until LC-MS analysis.

Cellular Uptake Experiments. The same preincubation protocol was followed as described under Bidirectional Transport Studies. In cellular uptake studies, the hop acids (final concentration of 50 μM) were added to the AP chambers. Blank transport medium was added to the BL chamber. Different monolayers were incubated with hop acids for 15, 30, and 60 min, respectively. Each experiment was performed in triplicate. Samples were withdrawn from the AP and the BL chambers, followed by EtOH extraction of the cell monolayers. Then, 200 μL of EtOH was added to the AP chamber, 750 μL of EtOH was added to the BL chamber, and the monolayers were extracted for 30 min. Afterward, samples from the AP and BL chambers were collected and stored at −20 °C until LC-MS analysis.

Enzymatic Hydrolysis of Cellular Uptake Samples. Cell fraction samples (300 μL) were diluted with 1.5 mL of sodium acetate buffer (0.1 M, pH 5), and a preparation containing both β-glucuronidase and sulfatase from *H. pomatia* (30 μL of a 330 units/mL solution in sodium acetate buffer (0.1 M, pH 5)) was added. Samples were incubated for 2 h at 37 °C. Afterward, samples were acidified with 200 μL of H_3PO_4 (1.0 M, pH 2) and extracted with ethyl acetate (extraction was repeated once). The collected organic phases were dried under nitrogen and redissolved in methanol (100 μL) prior to analysis. Replicate control samples were included with no enzyme treatment to determine the extent of glucuronidation and/or sulfation. Cellular levels of conjugated hop acids were calculated by subtracting the amount of free hop acid (no enzyme treatment) from the amount of total hop acids (+ β-glucuronidase/sulfatase). Similarly, aliquots of medium from the basolateral (300 μL) and apical (100 μL) compartment were combined with the mixture of β-glucuronidase/sulfatase (30 μL of a 330 units/mL solution in sodium acetate buffer (0.1 M, pH 5)), incubated, and extracted as above to determine total amount of hop acids. Replicate aliquots also were extracted without enzyme treatment to determine the amounts of conjugated hop acid.

Control Measurements. The low-permeability standard atenolol (50 μM) and the high-permeability standard propranolol (20 μM) were added to the monolayers simultaneously with the test compounds. TEER was measured before and after the experiments. Monolayers with low TEER values assumed to exhibit extensive leakage through imperfect occluding junctions or holes in the monolayer were discarded. After the transport studies, sodium fluorescein was used as a paracellular leakage marker. Two hundred microliters of HBBS containing 1 mg/mL sodium fluorescein was added to the AP chamber of each monolayer. After 1 h of incubation, the amount of fluorescein transported to the BL chamber was measured by fluorescence spectrophotometry (λ_{exc} = 480 nm, λ_{em} = 530 nm). The results were compared with values reported in the literature. Only cells with TEER exceeding 250−300 $\Omega \cdot cm^2$ were used (*18*). Values for the atenolol flux are usually $< 1.0 \times 10^{-6}$ and $> 10 \times 10^{-6}$ cm/s for the propranolol flux (*19*). A transport of sodium fluorescein not exceeding 1%/h indicates preservation of the monolayer (*58*).

Stability of Hop Bitter Acids. The stability of hop α-and β-acids in the experimental conditions was evaluated. Stock solutions of 20 mM (EtOH) were diluted to a final concentration of 50 μM of cohumulone, *n*- + adhumulone, colupulone, and *n*- + adlupulone and incubated at 37 °C for 1 h. Afterward, samples (200 μL) were transferred into vials and analyzed using LC-MS.

Inhibitor Studies. In transporter inhibition studies, either verapamil (100 μM) or indomethacin (100 μM) was added into both AP and BL chambers. After a preincubation period in the presence of specific inhibitors, the same experimental protocol was followed as described under Bidirectional Transport Studies. Furthermore, the transport of probe substrates rhodamine 123 (as substrate for P-gp) and 5-chloromethylfluorescein diacetate (CMFDA) (as substrate for MRP-2) was monitored as positive control.

LC-MS Analysis of Samples. Samples from the transepithelial permeability study were directly applied to liquid chromatography−mass spectrometry (LC-MS) analysis (Agilent 1200 LC-MS, see Purification of α-Acids and β-Acids for a detailed description). As stationary phase, a 3.5 μm Zorbax SB C-18 column (150 × 3.0 mm; Agilent) was used. The mobile phase consisted of 10 mM ammonium acetate (pH 5.0) + 2% CH_3CN (A) and methanol (B). The flow rate was 0.6 mL/min. The initial mobile phase, 10% B, was increased linearly to 90% B over 8 min, maintained for 4 min, and further increased to 100% B in 1 min and maintained during 4 min. Finally, the mobile phase was adjusted to 10% B in 1 min and re-equilibrated at 10% B for 4 min prior to the next injection. Quantitative analysis of the hop acids was carried out as described under Purification of α-Acids and β-Acids. Quantitative analysis of atenolol and propranolol was operated in the SIM positive-ion mode using target ions at $[M + H]^+$ m/z 260.3 and 267.3 for propranolol and atenolol, respectively.

Data Presentation of Transport Experiments. The results of the transport experiments are expressed as an apparent permeability coefficient (P_{app} (in cm/s)), which was calculated as described previously (*51*). $P_{app} = \delta Q/\delta t \times A^{-1} \times C^{1-}$with $\delta Q/\delta t$ (nmol/s) = the slope of the cumulative concentration of the compound in the receiving chamber over time, A (cm^2) = the membrane surface area of the cell monolayer, and C_0 ($nmol/cm^3$): = the initial compound concentration in the donor compartment. Other important properties are the percent amount transported to the BL chamber compared with the amount added to the donor compartment (%T) and the efflux ratio (ER), which is used to represent the extent of efflux and is calculated according the equationefflux ratio = P_{appBA} (mean)/P_{appAB} (mean)where P_{appAB} and P_{appBA} are, respectively, the apparent permeability coefficients for transport from the apical to the basolateral compartment and from the basolateral to the apical compartment.

Statistical Analysis. SPSS release 17.0 for Windows (SPSS, Inc., Chicago, IL) was used for all statistical analyses. All experiments had a minimum of three independent observations (three sequential wells with Caco-2 monolayers were tested) for each test group. Each experiment was replicated at least once such that $N \leq 6$ unless indicated otherwise. Data were expressed as means ± SEM when applicable. Normality of distribution

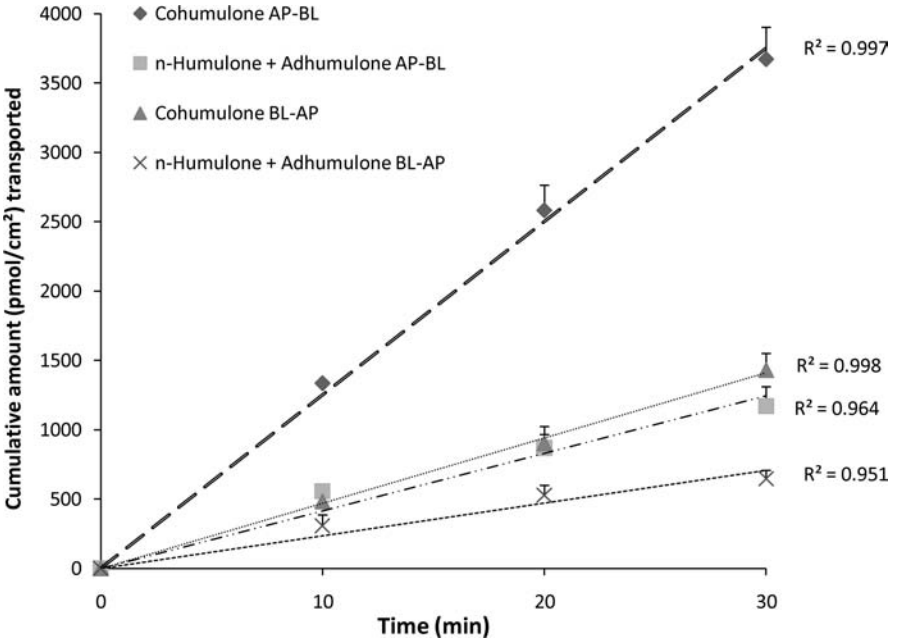

Figure 2. Apical (AP)-to-basolateral (BL) and basolateral (BL)-to-apical (AP) transport of α-acids (cohumulone and *n*-humulone + adhumulone) across Caco-2 monolayers. Hop α-acids (50 μM) were added to either the apical (AP-to-BL) or the basolateral (BL-to-AP) compartment of monolayers at 0 min. Samples from the receiving compartment were collected at 10, 20, and 30 min and analyzed by LC-MS as described under Materials and Methods. Data are means ± SEM for three replicates. Trendlines fitted through zero show good linearity ($R^2 > 0.95$).

was investigated using the Shapiro−Wilk test. Comparison of means between more than two groups was performed using one-way analysis of variance (ANOVA) followed by Bonferroni posthoc comparison of statistical significance. Linear regression was performed when correlation analysis was warranted. Results were considered to be statistically significant when $P < 0.05$.

RESULTS

Bidirectional Transport of Marker Compounds across Caco-2 Cell Monolayers and Monolayer Integrity. The Caco-2 cell monolayers were assessed with respect to their barrier properties using model compounds known for passive diffusion. Propranolol and atenolol showed P_{app} values of $(53.1 \pm 5.0) \times 10^{-6}$ and $(0.45 \pm 0.18) \times 10^{-6}$ cm/s, respectively, which is in correspondence with reported data. The results indicate that the monolayers can be used to discriminate between compounds with low and high permeabilities. Final concentrations did not affect TEER or transepithelial transport. Because the transport of compounds did not influence TEER, the preservation of integrity of the monolayers was confirmed.

Stability of Hop Bitter Acids. To investigate the stability of the studied compounds, control experiments with α- and β-acids were carried out. After 1 h of incubation in blank transport medium without Caco-2 cells, recoveries were all >98%, indicating no significant degradation for the α- or β-acids during the experimental conditions applied.

Bidirectional Transport of α- and β-Acids across Caco-2 Cell Monolayers. The present study was undertaken to investigate the in vitro transport of hop bitter acids as cohumulone and *n*-humulone + adhumulone (α-acids) and colupulone and *n*-lupulone + adlupulone (β-acids) using Caco-2 cell monolayers (**Figure 1**). The transport characteristics were determined for two directions, apical-to-basolateral (AP-to-BL) and basolateral-to-apical (BL-to-AP). The cumulative amounts transported into the receiving chamber as a function of time are shown in **Figures 2** and **3**. Significantly higher amounts of α-acids were transported to the basolateral chamber compared with the β-acids. For the α-acids, the fractions transported into the receiver chambers increased linearly within 30 min in both absorptive and secretive directions. For the β-acids, the transport in the absorptive direction also showed a linear relationship, but, in efflux transport, a different relationship was observed. **Table 1** presents two transport parameters P_{app} and efflux ratios (ratio of efflux versus influx) for the hop acids in the AP-to-BL as well as in the BL-to-AP direction. The P_{appAB} values ranged from 0.9×10^{-6} to 40.9×10^{-6} cm/s. The highest membrane permeability was determined for cohumulone (40.9×10^{-6} cm/s), being about 3-fold higher than the flux rate of *n*-humulone + adhumulone and 20−40 times higher than the transport rates of the β-acids. In secretive transport, the P_{appBA} of the α-acids in the BL-to-AP direction was 15.7×10^{-6} cm/s for cohumulone and 7.2×10^{-6} cm/s for *n*-humulone + adhumulone. Cohumulone and *n*-humulone + adhumulone showed higher absorption than secretion with efflux ratios of 0.4 for cohumulone and 0.5 for *n*-humulone + adhumulone. Notably, colupulone and *n*-lupulone + adlupulone exhibited substantially different bidirectional P_{app} values with efflux ratios of 10.3 and 9.7, respectively.

Effect of Inhibitors on the Transport of α- and β-Acids. The transport of rhodamine 123 showed a substantial efflux with an efflux ratio of 3.0, which was significantly reduced to 0.3 in the presence of verapamil as a P-gp inhibitor. For CMFDA, the presence of indomethacin as a MRP inhibitor caused reduction of the efflux ratio from 6.4 to 0.16. The results confirmed that P-gp and MRP-2 were both present in the Caco-2 monolayers

To identify the efflux transporters involved, bidirectional transport of colupulone and *n*-lupulone + adlupulone across Caco-2 monolayers was examined in the presence and absence of specific transport inhibitors. As a control experiment, bidirectional transport of α-acids in the presence of verapamil and indomethacin was also examined. As expected, the transport of cohumulone and *n*-humulone + adhumulone was not influenced. The efflux ratios in the presence of selective inhibitors verapamil (P-gp influence) and indomethacin (MRP-2 influence) are listed in **Table 1**. In the presence of verapamil (100 μM), a significant

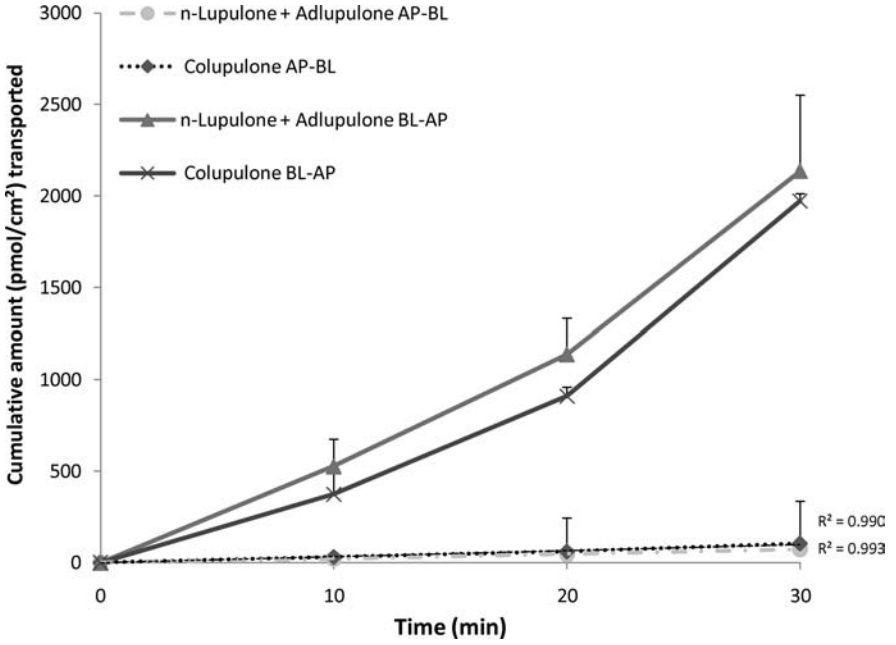

Figure 3. Apical (AP)-to-basolateral (BL) and basolateral (BL)-to-apical (AP) transport of β-acids (colupulone and *n*-lupulone + adlupulone) across Caco-2 monolayers. Hop β-acids (50 μM) were added to either the apical (AP-to-BL) or the basolateral (BL-to-AP) compartment of monolayers at 0 min. Samples from the receiving compartment were collected at 10, 20, and 30 min and analyzed by LC-MS as described under Materials and Methods. Data are means ± SEM for three replicates. Trendlines from linear regression analysis fitted through zero show good linearity for AP-to-BL transport ($R^2 > 0.95$). BL-to-AP transport showed enhanced transport upon longer treatment periods.

Table 1. Apparent Permeability Coefficients (P_{app}) and Efflux Ratios in the Absence and Presence of the Specific Inhibitors Verapamil and Indomethacin[a]

	P_{app} ($\times 10^{-6}$ cm/s)			P_{app} + verapamil ($\times 10^{-6}$ cm/s)		P_{app} + indomethacin ($\times 10^{-6}$ cm/s)		efflux ratio +	efflux ratio +
hop acid	AP-BL	BL-AP	efflux ratio	AP-BL	BL-AP	AP-BL	BL-AP	verapamil	indomethacin
COH (50 μM)	41 ± 2	16 ± 2	0.4 ± 0.1	37 ± 3	16 ± 1	41 ± 3	15 ± 1	0.4 ± 0.1	0.4 ± 0.1
N-HUM + ADHUM(50 μM)	14 ± 2	7.2 ± 0.6	0.5 ± 0.1	18. ± 3	9.6 ± 1.4	17 ± 2	9.8 ± 1.0	0.5 ± 0.2	0.5 ± 0.1
COL (50 μM)	2.1 ± 0.9	21 ± 3	10.3 ± 4.3	2.5 ± 0.6	10 ± 2	27 ± 1	5.6 ± 0.4	4.2 ± 2.0*	0.2 ± 0.1**
N-LUP + ADLUP (50 μM)	0.9 ± 0.1	8.9 ± 1.0	9.7 ± 3.6	1.4 ± 0.3	10 ± 2	14 ± 5	2.7 ± 0.5	7.0 ± 1.7	0.2 ± 0.1**

[a] Hop α-acids (50 μM) (cohumulone = COH and *n*-humulone + adhumulone = N-HUM + ADHUM) and β-acids (colupulone = COL and *n*-lupulone + adlupulone = N-LUP + ADLUP) were added to the apical (AP-BL) or basolateral (BL-AP) compartment of monolayers at 0 min. After 10, 20, and 30 min, samples were withdrawn from the receiving compartment and analyzed by LC-MS as described under Materials and Methods. Data are means ± SEM for three replicates. Comparison of efflux ratios (in the absence and presence of inhibitors) between more than two groups was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc comparison of statistical significance. Statistically significant differences versus the efflux ratios in the absence of inhibitors are indicated: *$p < 0.05$; **$p < 0.005$.

decrease of the efflux ratio was observed compared to the data in the absence of the P-gp-inhibitor (from 10.3 and 9.7 to 4.2 and 7.0, respectively). The presence of indomethacin (MRP-2 inhibitor) (50 μM) caused almost complete inhibition of the efflux of colupulone and *n*-lupulone + adlupulone (**Table 1**). A significant decrease in the efflux ratio from 10.3 and 9.7 to 0.2 for colupulone and *n*-lupulone + adlupulone, respectively, was observed. The results suggest that both MRP and P-gp are involved in the efflux of hop β-acids.

Cellular Uptake of α- and *β*-Acids. The amounts of α- and β-acids recovered in the AP chamber, BL chamber, and cell monolayer are shown in **Figure 4**. After 60 min of incubation of the α-acids with the Caco-2 cells, the amount absorbed in the cell fraction was 60%, whereas 30% was transported to the BL chamber. A residual amount of 10% was still present at the AP chamber. After 60 min of incubation of the β-acids with the Caco-2 cells, the amount recovered in the cell fraction was only 8%, whereas 2.1% was present unchanged at the BL chamber. A residual 4.0% was recovered in the AP chamber. More than 95% of the administered amount of α-acids was recovered unchanged. The formation of phase II metabolites of α-acids seems unlikely within the duration of the experiments. The recovery of the β-acids was <15%. These observations suggest formation of phase II metabolites and/or degradation products of β-acids in the Caco-2 cells within the duration of the experiment (60 min).

Enzymatic Hydrolysis of Cellular Uptake Samples. As a consequence of the low recovery of the β-acids (<15%), enzymatic hydrolysis of fractions from apical and basolateral compartments and cell monolayers was carried out with a mixture of sulfatase and glucuronidase activity to determine the amount of sulfate and/or glucuronide conjugates (**Figure 4**). The amounts, quantified after deconjugation, rendered a recovery of 97.5 ± 13.6%. About 81−90% of the total amount of β-acids present in the apical compartment, basolateral compartment, and cell fraction was present as a conjugated sulfate or glucuronidated product. Following apical supplementation, 42 ± 9% of the total amount of the β-acid conjugates were present in the apical compartment and 11 ± 3% in the basolateral compartment, whreas 47 ± 18% remained in the cell fraction.

DISCUSSION

Oral bioavailability is one of the most important pharmacokinetic properties of drugs. Various intestinal processes such as permeation, efflux, intestinal metabolism, and microbiotic

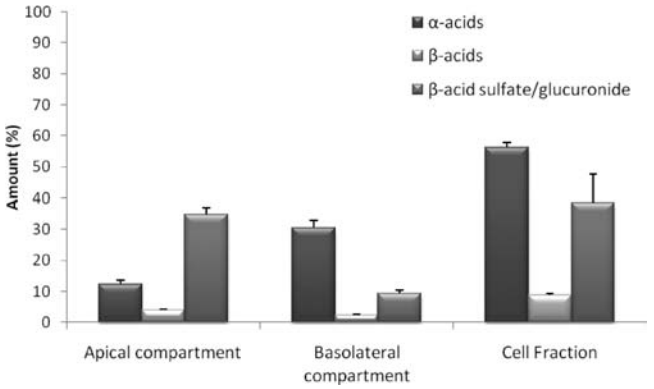

Figure 4. Distribution of α- and β-acids in the apical compartment, basolateral compartment, and cell fraction. Hop α- and β-acids (50 μM) were added to the apical compartment of monolayers at 0 min. After 60 min, samples were withdrawn from the apical compartment, basolateral compartment, and cell fraction and analyzed by LC-MS as described under Materials and Methods. Data are means ± SEM for three replicates.

metabolism in the colon may affect the bioavailabilities of hop acids. As a first aspect of the study of the bioavailability of hop acids, permeability experiments of the target compounds in both absorptive (AP-to-BL) and secretive (BL-to-AP) modes were carried out to determine if a carrier-mediated transport or a carrier-mediated efflux mechanism is involved. The P_{app} values determined for the α-acids approximate the P_{app} value of propranolol. Propranolol is a lipophilic compound, which is known for its high permeability across epithelial membranes. Rapidly and completely absorbed compounds are generally lipophilic and distribute readily into the cell membranes. Because the surface area of the brush border membranes is >1000-fold larger than the paracellular surface area (*59*), it is suggested that the hop α-acids are quickly and efficiently transported exclusively by the transcellular route (*60*) in the absorptive direction. On the other hand, absorption via a paracellular pathway cannot be neglected, but it is generally low, because intercellular tight junctions restrict free paracellular movement between epithelial cells (*61*).

The absorptive transport of both α- and β-acids shows a linear increase during 30 min, indicating the absence of saturation characteristics under the experimental conditions applied, which may suggest passive transport, although AP-to-BL and BL-to-AP permeabilities with a wider concentration range (for example, 5−200 μM) on the initial side are necessary to confirm the transport mechanism. The vast majority of well-absorbed drugs are transported passively across cell membranes (*31*). Active transport of dissociated forms with anionic transporters or facilitated diffusion would rather lead to a sigmoid-like relationship, albeit only at sufficiently high concentrations, because carrier-mediated transport is saturable. If a compound has a low passive permeability, saturation of the carrier will result in a decreased absorbed fraction (*60*, *62*).

There was no significant difference between transport in the absorptive direction and transport in the secretive direction for the α-acids, because P_{appAB} and P_{appBA} are comparable. Influx exceeded efflux, albeit with small differences. It may be derived that α-acids show a bidirectional transport by passive diffusion (i.e., nontransporter mediated). This suggests that passive diffusion of α-acids could play a role in permeation across intestinal cells, not only in the Caco-2 monolayers but possibly also in vivo. Factors influencing diffusion include structural and chemical properties of molecules, that is, charge, molecular weight, hydrogen bonding potential, and hydrophobicity. Blanco et al. demonstrated that diffusion of hop acids across lipophilic bilayers is dependent on log *P*, pK_a, and molecular size. Hop acids with smaller molecular sizes possessed good lipophilicity (log *P*), indicating a more efficient transport across the cell membrane (*63*).

Weak acids can indeed cross cytoplasmatic lipophilic membranes in undissociated form (*64*,*65*). Knowledge of the pH value of the transport medium and the pK_a value of the hop acid under consideration allows one to calculate the concentration of undissociated molecules at any pH value because [undissociated acid] = [total acid administered]/[$10^{(pH\text{-}pK_a)} + 1$] by rearrangement of the Henderson−Hasselbalch equation. Under the experimental conditions of pH 7.4, the ratio of dissociated/undissociated molecules was approximately 100. The presence of a high percentage of the dissociated forms seems contradictory with the high absorption of α-acids. Presumably, fast transport of the undissociated species from the AP chamber into the cell compartment, followed by a fast transport further to the BL chamber, is maintained by significant protonation of ionized α-acids due to rebalancing of the shifted acid/conjugated base equilibrium at the AP chamber. Therefore, it would be interesting to study the effects of the pH of the transport medium on the epithelial transport of hop acids.

The P_{app} values of the β-acids ($\leq 1 \times 10^{-6}$ cm/s) were comparable to the P_{app} of atenolol, which is a marker for low permeability. This observation suggested that intestinal absorption of hop β-acids is predicted to be poor, which might be associated with a low bioavailability. The extra isoprenyl moiety present in the β-acids compared to the α-acids contributes to a higher lipophilicity and increased steric hindrance, resulting in lower absorption.

Often, transport of drugs across the intestinal epithelial is accompanied by phase II conjugation reactions, which may affect drug absorption. Enzymatic hydrolysis of samples following a 60 min incubation of β-acids with Caco-2 cells indicated that the largest fraction of the β-acid amount is present as a sulfate and/or glucuronide conjugate, suggesting that intestinal absorption of the lipophilic β-acids is limited by substantial glucuronidation and/or sulfation by the enterocytes. For Caco-2 cells derived from human colon carcinoma, expression of human phase II metabolizing enzymes has been confirmed (*66*). Therefore, the β-acid phase II metabolites found in the present study indicated that similar types of metabolites of β-acids might also be found in the human small intestine. It is also interesting that the intracellularly formed sulfated and glucuronidated β-acids were highly abundant in the apical compartment, whereas only low levels of those metabolites were found in the basolateral compartment. This suggests that a certain type of active transporter is involved in the selective efflux of the metabolites formed in the Caco-2 cells. Membrane transporters, especially the efflux transporters P-gp and MRP-2, are known to affect absorption and oral bioavailability of drugs. Previous studies demonstrated that P-gp and MRP-1−3 are capable of transporting phase II metabolites such as glutathione, glucuronide, and sulfate conjugates (*67*).

Verapamil and indomethacin are representative inhibitors for in vitro screening and appropriate standards for such screenings. In drug discovery, the P-gp substrate specificity, the so-called "Rule of Fours", can be applied as an aid in predicting whether or not a test compound is likely to be a candidate for P-gp involvement. The rule can be summarized as follows: a compound is more likely to be a P-gp substrate when it has (N + O) ≥ 8 and a molecular weight (MW) > 400 with weak acidic character (p*Ka* > 4). Most P-gp substrates can be defined as lipophilic and neutral species. MRP-2 is directly responsible for the intestinal elimination of organic anions, complementing the substrate

specificity of P-gp. The bidirectional transport of colupulone and *n*-lupulone + adlupulone exhibited efflux ratios of 10.3 and 9.7, respectively, suggesting the involvement of carrier-mediated transport for β-acids (active efflux pathway by P-gp, BCRP, and/or MRP-2 type efflux pumps). Co-administration of verapamil or indomethacin did not modify the transport of hop α-acids. In the current study, both P-gp and, to a larger extent, MRP-2 decreased the efflux of β-acids to the AP compartment and increased the disposition of the β-acids to the BL compartment.

For the α-acids as well as for the β-acids, the different side chain at C-1 (isopentanoyl in *n*- and ad-, isobutyroyl in coanalogues) resulted in different absorption efficiencies. The coanalogues seemed to be more efficiently absorbed than the *n*- + ad-analogues. The different nature of the side chain is reflected by differences in diffusion and transport rates. The above observations suggest that the dissimilarity in the molecular structure and corresponding lipophilicity between the investigated α- and β-acids is sufficiently high to result in substantial differentiated absorption efficiencies, substrate affinity for efflux transporters, and metabolizing enzymes. Differences in lipophilicity are also demonstrated by the corresponding log *P* values (calculated using "Molinspiration MiTools", based on ZINC database) (*68*) of α-acids ranging from 3.2 to 3.7 for cohumulone and *n*-humulone, respectively, in contrast with 5.8−6.3 for, respectively, colupulone and *n*-lupulone.

Although no substantial degradation in the experimental setup was observed, experiments including the stability of these acids during gastrointestinal digestion by ex vivo studies in the stomach and intestinal fluid could be a great contribution to the discussion of the findings of this study in a broader context.

In conclusion, the present study demonstrates fast and efficient absorption of hop α-acids. Cohumulone showed higher absorption than *n*-humulone + adhumulone. On the basis of the low apparent permeability coefficient of β-acids, the in vivo absorption of orally administered β-acids is predicted to be low. The limiting factors in the absorption of β-acids could be the involvement of P-gp and MRP-2 type efflux transporters and/or substantial phase II metabolism reactions. The results from the Caco-2 cell culture model must be interpreted with caution because clearance and toxicity have not been measured, whereas they should have an impact in vivo. It must be of great interest to compare the tissue culture data obtained in this work with future pharmacokinetic data generated in vivo.

ABBREVIATIONS USED

P-gp, phospho-glycoprotein; MRP, multidrug resistance associated protein; BCRP, breast cancer resistance protein; TEER, transepithelial electrical resistance; P_{app}, apparent permeability coefficient; LC-MS, liquid chromatography−mass spectrometry; HBSS, Hank's balanced salt solution; AP → BL, apical-to-basolateral transport; BL → AP, basolateral-to-apical transport; CMFDA, 5-chloromethylfluorescein diacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

LITERATURE CITED

(1) Doll, R.; Peto, R.; Hall, E.; Wheatley, K.; Gray, R. Mortality in relation to consumption of alcohol − 13 years observations on male Britisch doctors. *Br. Med. J.* **1994**, *309* (6959), 911–918.

(2) Thun, M. J.; Peto, R.; Lopez, A. D.; Monaco, J. H.; Henley, S. J.; Heath, C. W.; Doll, R. Alcohol consumption and mortality among middle-aged and elderly US adults. *N. Engl. J. Med.* **1997**, *337* (24), 1705–1714.

(3) Hamilton, S. R.; Hyland, J.; McAvinchey, D.; Chaudhry, Y.; Hartka, L.; Kim, H. T.; Cichon, P.; Floyd, J.; Turjman, N.; Kessie, G.; Nair, P. P.; Dick, J. Effects of chronic dietary beer and ethanol consumption on experimental colonic carcinogenesis by azoxymethane in rats. *Cancer Res.* **1987**, *47* (6), 1551–1559.

(4) Fuchs, F. D.; Chambless, L. E. Is the cardioprotective effect of alcohol real? *Alcohol* **2007**, *41*, 399–402.

(5) Romeo, J.; Warnberg, J.; Nova, E.; Diaz, L. E.; Gomez-Martinez, S.; Marcos, A. Moderate alcohol consumption and the immune system: a review. *Br. J. Nutr.* **2007**, *98*, S111–S115.

(6) Bohr, G.; Klimo, K.; Zapp, J.; Becker, H.; Gerhauser, C. Cancer chemopreventive potential of humulones and isohumulones (hops α- and iso-α-acids): induction of NAD(P)H:quinone reductase as a novel mechanism. *Nat. Prod. Commun.* **2008**, *3* (12), 1971–1976.

(7) Gerhauser, C. Beer constituents as potential cancer chemopreventive agents. *Eur. J. Cancer* **2005**, *41* (13), 1941–1954.

(8) Shimamura, M.; Hazato, T.; Ashino, H.; Yamamoto, Y.; Iwasaki, E.; Tobe, H.; Yamamoto, K.; Yamamoto, S. Inhibition of angiogenesis by humulone, a bitter acid from beer hop. *Biochem. Biophys. Res. Commun.* **2001**, *289* (1), 220–224.

(9) Tagashira, M.; Watanabe, M.; Uemitsu, N. Antioxidative activity of hop bitter acids and their analogs. *Biosci., Biotechnol., Biochem.* **1995**, *59* (4), 740–742.

(10) Possemiers, S.; Bolca, S.; Eeckhaut, E.; Depypere, H.; Verstraete, W. Metabolism of isoflavones, lignans and prenylflavonoids by intestinal bacteria: producer phenotyping and relation with intestinal community. *FEMS Microbiol. Ecol.* **2007**, *61* (2), 372–383.

(11) Colgate, E. C.; Miranda, C. L.; Stevens, J. F.; Bray, T. M.; Ho, E. Xanthohumol, a prenylflavonoid derived from hops induces apoptosis and inhibits NF-κB activation in prostate epithelial cells. *Cancer Lett.* **2007**, *246* (1−2), 201–209.

(12) Zanoli, P.; Zavatti, M. Pharmacognostic and pharmacological profile of *Humulus lupulus* L. *J. Ethnopharmacol.* **2008**, *116* (3), 383–396.

(13) Van Cleemput, M.; Cattoor, K.; De Bosscher, K.; Haegeman, G.; De Keukeleire, D.; Heyerick, A. Hop (*Humulus lupulus*)-derived bitter acids as multipotent bioactive compounds. *J. Nat. Prod.* **2009**, *72* (6), 1220–1230.

(14) Tobe, H.; Muraki, Y.; Kitamura, K.; Komiyama, O.; Sato, Y.; Sugioka, T.; Maruyama, H. B.; Matsuda, E.; Nagai, M. Bone resorption inhibitors from hop extract. *Biosci., Biotechnol., Biochem.* **1997**, *61* (1), 158–159.

(15) Kondo, K. Beer and health: preventive effects of beer components on lifestyle-related diseases. *Biofactors* **2004**, *22* (1−4), 303–310.

(16) Mundy, G. R. Osteoporosis and inflammation. *Nutr. Rev.* **2007**, *65* (12), S147–S151.

(17) Yamamoto, K.; Wang, J. N.; Yamamoto, S.; Tobe, H. Suppression of cyclooxygenase-2 gene transcription by humulon of beer hop extract studied with reference to glucocorticoid. *FEBS Lett.* **2000**, *465* (2−3), 103–106.

(18) Tripp, M.; Darland, G.; Lerman, R.; Lukaczer, D.; Bland, J.; Babish, J. Hop and modified hop extracts have potent in vitro anti-inflammatory properties. *Proc. 1st Int. Humulus Symp.* **2005**, *668*, 217–227.

(19) Hougee, S.; Faber, J.; Sanders, A.; van den Berg, W. B.; Garssen, J.; Smit, H. F.; Hoijer, M. A. Selective inhibition of COX-2 by a standardized CO_2 extract of *Humulus lupulus* in vitro and its activity in a mouse model of zymosan-induced arthritis. *Planta Med.* **2006**, *72* (3), 228–233.

(20) Lamy, V.; Roussi, S.; Chaabi, M.; Gosse, F.; Lobstein, A.; Raul, F. Lupulone, a hop bitter acid, activates different death pathways involving apoptotic TRAIL-receptors, in human colon tumor cells and in their derived metastatic cells. *Apoptosis* **2008**, *13* (10), 1232–1242.

(21) Chen, W. J.; Lin, J. K. Mechanisms of cancer chemoprevention by hop bitter acids (beer aroma) through induction of apoptosis mediated by fas and caspase cascades. *J. Agric. Food Chem.* **2004**, *52* (1), 55–64.

(22) Tobe, H.; Kubota, M.; Yamaguchi, M.; Kocha, T.; Aoyagi, T. Apoptosis to HL-60 by humulone. *Biosci., Biotechnol., Biochem.* **1997**, *61* (6), 1027–1029.

(23) Lamy, V.; Roussi, S.; Chaabi, M.; Gosse, F.; Schall, N.; Lobstein, A.; Raul, F. Chemopreventive effects of lupulone, a hop β-acid, on

human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. *Carcinogenesis* **2007**, *28* (7), 1575–1581.

(24) Stephan, T. E.; Ngo, E. O.; Nutter, L. M. Hexahydrocolupulone and its antitumor cell proliferation activity in vitro. *Biochem. Pharmacol.* **1998**, *55* (4), 505–514.

(25) Siegel, L.; Miternique-Grosse, A.; Griffon, C.; Klein-Soyer, C.; Lobstein, A.; Raul, F.; Stephan, D. Antiangiogenic properties of lupulone, a bitter acid of hop cones. *Anticancer Res.* **2008**, *28* (1A), 289–294.

(26) Stevens, R. Chemistry of hop constituents. *Chem. Rev.* **1967**, *67* (1), 19–71.

(27) Verzele, M. 100 years of hop chemistry and its relevance to brewing. *J. Inst. Brew.* **1986**, *92* (1), 32–48.

(28) Hall, A. J.; Babish, J. G.; Darland, G. K.; Carroll, B. J.; Konda, V. R.; Lerman, R. H.; Bland, J. S.; Tripp, M. L. Safety, efficacy and anti-inflammatory activity of ρ iso-α-acids from hops. *Phytochemistry* **2008**, *69* (7), 1534–1547.

(29) Teotico, D. G.; Bischof, J. J.; Peng, L.; Kliewer, S. A.; Redinbo, M. R. Structural basis of human pregnane X receptor activation by the hops constituent colupulone. *Mol. Pharmacol.* **2008**, *74* (6), 1512–1520.

(30) Fritsch, A.; Shellhammer, T. H. α-Acids do not contribute bitterness to lager beer. *J. Am. Soc. Brew. Chem.* **2007**, *65* (1), 26–28.

(31) Stenberg, P.; Norinder, U.; Luthman, K.; Artursson, P. Experimental and computational screening models for the prediction of intestinal drug absorption. *J. Med. Chem.* **2001**, *44* (12), 1927–1937.

(32) Hu, M.; Borchardt, R. T. Transport of large neutral amino acid in a human intestinal epithelial cell line (Caco-2) − uptake and efflux of phenylalanine. *Biochim. Biophys. Acta* **1992**, *1135* (3), 233–244.

(33) Osiecka, I.; Cortese, M.; Porter, P. A.; Borchardt, R. T.; Fix, J. A.; Gardner, C. R. Intestinal absorption of α-methyldopa − in vitro mechanistic studies in rat small intestinal segments. *J. Pharmacol. Exp. Ther.* **1987**, *242* (2), 443–449.

(34) Thwaites, D. T.; McEwan, G. T. A.; Brown, C. D. A.; Hirst, B. H.; Simmons, N. L. Na^+ independent, H^+ coupled transepithelial β-alanine absorption by human intestinal Caco-2 cell monolayers. *J. Biol. Chem.* **1993**, *268* (25), 18438–18441.

(35) Ranaldi, G.; Islam, K.; Sambuy, Y. D-Cycloserine uses an active transport mechanism in the human intestinal cell line Caco-2. *Antimicrob. Agents Chemother.* **1994**, *38* (6), 1239–1245.

(36) He, Y. P.; Sanderson, I. R.; Walker, W. A. Uptake, transport and metabolism of exogenous nucleosides in intestinal epithelial cell cultures. *J. Nutr.* **1994**, *124* (10), 1942–1949.

(37) Hidalgo, I. J.; Borchardt, R. T. Transport of bile acids in a human intestinal epithelial cell line, Caco-2. *Biochim. Biophys. Acta* **1990**, *1035* (1), 97–103.

(38) Ho, N. F. H. Utilizing bile acid carrier mechanisms to enhance liver and small intestine absorption. *Ann. N.Y. Acad. Sci.* **1987**, *507*, 315–329.

(39) Kramer, W.; Wess, G.; Neckermann, G.; Schubert, G.; Fink, J.; Girbig, F.; Gutjahr, U.; Kowalewski, S.; Baringhaus, K. H.; Boger, G.; Enhsen, A.; Falk, E.; Friedrich, M.; Glombik, H.; Hoffmann, A.; Pittius, C.; Urmann, M. Intestinal absorption of peptides by coupling to bile acids. *J. Biol. Chem.* **1994**, *269* (14), 10621–10627.

(40) Dix, C. J.; Hassan, I. F.; Obray, H. Y.; Shah, R.; Wilson, G. The transport of vitamin B12 through polarized monolayers of Caco-2 cells. *Gastroenterology* **1990**, *98* (5), 1272–1279.

(41) Ng, K. Y.; Borchardt, R. T. Biotin transport in a human intestinal epithelial cell line (Caco-2). *Life Sci.* **1993**, *53* (14), 1121–1127.

(42) Dantzig, A. H.; Tabas, L. B.; Bergin, L. Cefaclor uptake by the proton-dependent dipeptide carrier of human intestinal Caco-2 cells and comparison to cephalexin uptake. *Biochim. Biophys. Acta* **1992**, *1112* (2), 167–173.

(43) Saito, H.; Inui, K. Dipeptide transporters in apical and basolateral membranes of the intestinal cell line Caco-2. *Am. J. Physiol.* **1993**, *265* (2), G289–G294.

(44) Thwaites, D. T.; Brown, C. D. A.; Hirst, B. H.; Simmons, N. L. Transepithelial glycylsarcosine transport in intestinal Caco-2 cells mediated by expression of H^+-coupled carriers at both apical and basal membranes. *J. Biol. Chem.* **1993**, *268* (11), 7640–7642.

(45) Ganapathy, M. E.; Brandsch, M.; Prasad, P. D.; Ganapathy, V.; Leibach, F. H. Differential recognition of β-lactam antibiotics by intestinal and renal peptide transporters, PEPT-1 and PEPT-2. *J. Biol. Chem.* **1995**, *270* (43), 25672–25677.

(46) Fisher, R. B. Active transport of salicylate by rat jejunum. *Q. J. Exp. Physiol. Cognate Med. Sci.* **1981**, *66* (2), 91–98.

(47) Takanaga, H.; Tamai, I.; Tsuji, A. pH-dependent and carrier-mediated transport of salicylic acid across Caco-2 cells. *J. Pharm. Pharmacol.* **1994**, *46* (7), 567–570.

(48) Hunter, J.; Hirst, B. H. Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Adv. Drug Delivery Rev.* **1997**, *25* (2−3), 129–157.

(49) Walle, U. K.; Galijatovic, A.; Walle, T. Transport of the flavonoid chrysin and its conjugated metabolites by the human intestinal cell line Caco-2. *Biochem. Pharmacol.* **1999**, *58* (3), 431–438.

(50) Gres, M. C.; Julian, B.; Bourrie, M.; Meunier, V.; Roques, C.; Berger, M.; Boulenc, X.; Berger, Y.; Fabre, G. Correlation between oral drug absorption in humans, and apparent drug permeability in TC-7 cells, a human epithelial intestinal cell line: comparison with the parental Caco-2 cell line. *Pharm. Res.* **1998**, *15* (5), 726–733.

(51) Artursson, P.; Karlsson, J. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochem. Biophys. Res. Commun.* **1991**, *175* (3), 880–885.

(52) Nikolic, D.; Li, Y. M.; Chadwick, L. R.; van Breemen, R. B. In vitro studies of intestinal permeability and hepatic and intestinal metabolism of 8-prenylnaringenin, a potent phytoestrogen from hops (*Humulus lupulus* L.). *Pharm. Res.* **2006**, *23* (5), 864–872.

(53) Rodriguez-Proteau, R.; Mata, J. E.; Miranda, C. L.; Fan, Y.; Brown, J. J.; Buhler, D. R. Plant polyphenols and multidrug resistance: Effects of dietary flavonoids on drug transporters in Caco-2 and MDCKII-MDR1 cell transport models. *Xenobiotica* **2006**, *36* (1), 41–58.

(54) Pang, Y.; Nikolic, D.; Zhu, D. W.; Chadwick, L. R.; Pauli, G. F.; Farnsworth, N. R.; van Breemen, R. B. Binding of the hop (*Humulus lupulus* L.) challcone xanthohumol to cytosolic proteins in Caco-2 intestinal epithelliall cells. *Mol. Nutr. Food Res.* **2007**, *51* (7), 872–879.

(55) Bailey, C. A.; Bryla, P.; Malick, A. W. The use of the intestinal epithelial cell culture model, Caco-2, in pharmaceutical development. *Adv. Drug Delivery Rev.* **1996**, *22* (1−2), 85–103.

(56) HermansLokkerbol, A. C. J.; Hoek, A. C.; Verpoorte, R. Preparative separation of bitter acids from hop extracts by centrifugal partition chromatography. *J. Chromatogr., A* **1997**, *771* (1−2), 71–79.

(57) Press, B.; Di Grandi, D. Permeability for intestinal absorption: Caco-2 assay and related issues. *Curr. Drug Metab.* **2008**, *9* (9), 893–900.

(58) Kamuhabwa, A. R.; Augustijns, P.; de Witte, P. A. In vitro transport and uptake of protohypericin and hypericin in the Caco-2 model. *Int. J. Pharm.* **1999**, *188* (1), 81–86.

(59) Pappenheimer, J. R.; Reiss, K. Z. Contribution of solvent drag through intercellular junctions to absorption of nutrients by the small intestine of the rat. *J. Membr. Biol.* **1987**, *100* (2), 123–136.

(60) Artursson, P.; Palm, K.; Luthman, K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Delivery Rev.* **2001**, *46* (1−3), 27–43.

(61) Furuse, M.; Hirase, T.; Itoh, M.; Nagafuchi, A.; Yonemura, S.; Tsukita, S. Occludin: a novel integral membrane protein localizing at tight junctions. *J. Cell Biol.* **1993**, *123* (6), 1777–1788.

(62) Li, Y. M.; Shin, Y. G.; Yu, C. W.; Kosmeder, J. W.; Hirschelman, W. H.; Pezzuto, J. M.; van Breemen, R. B. Increasing the throughput and productivity of Caco-2 cell permeability assays using liquid chromatography−mass spectrometry: application to resveratrol absorption and metabolism. *Comb. Chem. High Throughput Screen* **2003**, *6* (8), 757–767.

(63) Blanco, C. A.; Rojas, A.; Nimubona, D. Effects of acidity and molecular size on properties of beer bacteriostatic hop derivates. *Trends Food Sci. Technol.* **2007**, *18* (3), 144–149.

(64) Simpson, W. J.; Smith, A. R. W. Factors affecting antibacterial activity of hop compounds and their derivatives. *J. Appl. Bacteriol.* **1992**, *72* (4), 327–334.

(65) Sakamoto, K.; van Veen, H. W.; Saito, H.; Kobayashi, H.; Konings, W. N. Membrane-bound ATPase contributes to hop resistance of *Lactobacillus brevis*. *Appl. Environ. Microbiol.* **2002**, *68* (11), 5374–5378.

(66) Sun, D. X.; Lennernas, H.; Welage, L. S.; Barnett, J. L.; Landowski, C. P.; Foster, D.; Fleisher, D.; Lee, K. D.; Amidon, G. L. Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequences tags and correlation with permeability of 26 drugs. *Pharm. Res.* **2002**, *19* (10), 1400–1416.

(67) Suzuki, H.; Sugiyama, Y. Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. *Eur. J. Pharm. Sci.* **2000**, *12* (1), 3–12.

(68) Irwin, J. J.; Shoichet, B. K. ZINC − a free database of commercially available compounds for virtual screening. *J. Chem Inf. Model.* **2005**, *45* (1), 177–182.

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