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Research Article

Flavonoids alter P-gp expression in intestinal epithelial cells *in vitro* and *in vivo*

Katrin Lohner[1], Kerstin Schnäbele[2], Hannelore Daniel[1], Doris Oesterle[3], Gerhard Rechkemmer[2], Martin Göttlicher[3] and Uwe Wenzel[4]

[1] Molecular Nutrition Unit, Department of Food and Nutrition, Technical University of Munich, Freising, Germany
[2] Biofunctionality of Food, Department of Food and Nutrition, Technical University of Munich, Freising, Germany
[3] GSF Institute of Toxicology, Neuherberg, Germany
[4] Molecular Nutrition Research, Interdisciplinary Research Center, Justus-Liebig-University of Giessen, Giessen, Germany

Flavonoids are secondary plant metabolites included in our diet but are also provided in a growing number of supplements. They are suggested to interact with intestinal transport systems including phospho-glycoprotein (P-gp) which mediates the efflux of a variety of xenobiotics back into the gut lumen. In human intestinal Caco-2 cells, we tested the effects of 14 different flavonoids on P-gp expression *in vitro*. Protein expression levels were quantified by Western blotting, flow cytometry, and real-time PCR. Except apigenin, all flavonoids at concentrations of 10 μM increased P-gp expression in Western blotting experiments when cells were exposed to the compounds over 4 wk. Flavone was one of the most effective P-gp inducers in Caco-2 cells and its effects were, therefore, also assessed for changes in P-gp *in vivo* in the gastrointestinal tract of C57BL/6 mice. P-gp expression was significantly increased by flavone (400 mg/kg body weight × day over 4 wk) in the small intestine but not in the colon which displayed intrinsically the highest expression level. In conclusion, the increase in P-gp expression caused by flavonoids in intestinal epithelial cells *in vitro* and also *in vivo* may serve as an adaptation and defense mechanism limiting the entry of lipophilic xenobiotics into the organism.

Keywords: C57BL / Caco-2 human intestinal epithelial cells / Flavonoids / Flow cytometry / P-glycoprotein expression

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1 Introduction

Phospho-glycoprotein (P-gp) is a high M_r membrane glycoprotein (1280 amino acids, 170 kDa) encoded by the human *MDR1* gene, localized to chromosome 7q21 [1]. As a member of the large ATP-binding cassette (ABC) transporter superfamily [2], P-gp consists of two similar halves, each half including a hydrophobic transmembrane region and a cytosolic nucleotide-binding domain responsible for ATP-hydrolysis [3]. P-gp is constitutively expressed at the apical surface of epithelial cells in several organs including liver, kidney, and especially intestine [4]. It acts as an energy-dependent efflux pump with a broad specificity for chemically apparently unrelated hydrophobic compounds [5]. In its physiological function to export endogenous substrates such as the steroids cortisol, aldosterone, corticosterone, or 17-beta-estradiol, it is thought to protect cells from exogenous xenobiotics like environmental toxins or drugs [6]. As an epithelial drug-efflux pump P-gp plays a crucial role also in drug absorption, distribution, and excretion [7]. Moreover, its overexpression is a major cause for the failure of cancer chemotherapy in humans, as it is responsible for decreased drug accumulation in multidrug-resistant cells and consequently for reduced cytotoxicity of the administered compound [8].

Correspondence: Professor Uwe Wenzel, Interdisciplinary Research Center, Molecular Nutrition Research, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany
E-mail: uwe.wenzel@ernaehrung.uni-giessen.de
Fax: +49-641-99-39229

Abbreviations: ECL, enhanced chemiluminescence; **FACS,** fluorescence-assisted cell sorting; **FITC,** fluorescein-isothiocyanate; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **MEM,** Minimal Essential Medium; **P-gp,** phospho-glycoprotein

Flavonoids are a subclass of dietary polyphenolic compounds present in fruits, vegetables, and herbal plants and are proposed to promote human health by their antioxidant, antiviral, or anticarcinogenic properties [9]. However, flavonoids may also be regarded as xenobiotics and most of the flavonoids like other polyphenols are treated as xenobiotics in the body by undergoing extensive metabolism by phases I and II enzyme systems. Moreover, efflux transporters, such as P-gp [10] are also involved in flavonoid handling. Flavonoids are abundant in food, and the total dietary intake of polyphenols is estimated to be around 1 g/day; with 100–150 mg/day provided by flavonoids in Western diets [11]. Intake may be considerably higher in people consuming over-the-counter botanical and dietary supplements [12]. In view of the role of P-gp in not only limiting the oral availability of xenobiotics but also in view of possible interactions of drugs and dietary constituents, it seems important to understand the actions of flavonoids on P-gp-functions and expression level, respectively. Whereas some flavonoids were shown to inhibit P-gp-mediated transport processes by directly interacting with the vicinal ATP-binding sites, the steroid-binding site, or the substrate-binding domains [12, 13], others, like epicatechin from green tea, were shown to activate P-gp by a heterotropic allosteric mechanism [14]. Information on flavonoid effects on P-gp expression, however, appears scarce. Some herbal constituents such as hyperforin were demonstrated to activate the pregnane X receptor, an orphan nuclear receptor acting as a key regulator of *MDR1* [15] gene expression causing in turn increased intestinal P-gp levels [16].

To assess whether the exposure of intestinal cells to flavonoids at a concentration of 10 μM over a time period of 4 wk affects P-gp expression, we used Caco-2 cells as an *in vitro* model and determined P-gp-mRNA levels by real-time PCR and the protein density by Western blotting and flow cytometry, respectively. For flavone, that proved to markedly increase P-gp expression in Caco-2 cells, we also tested the activity *in vivo* in C57BL/6 mice receiving the flavonoid by gavage.

2 Materials and methods

2.1 Materials

Media and supplements for cell culture were from Invitrogen (Karlsruhe, Germany). Cell culture plates were purchased from Renner (Dannstadt, Germany). Flavonoids were obtained from the following sources: eriodictyol and genistein were from Extrasynthese (Lyon, France); isoxanthohumol was generously provided by Professor Dr. Hans Becker (Pharmacognosy and Analytical Phytochemistry, University of Saarland, Germany); Cyanidin-3-glucoside was a kind gift from Professor Peter Winterhalter (Food Chemistry, Technical University of Braunschweig, Germany); (+/−)-catechin was obtained from BASF (Ludwigshafen, Germany); and all other compounds were from Sigma (Deisenhofen, Germany). Purity of the flavonoids used was generally higher than 98%. Protease Inhibitor Cocktail was purchased from Roche (Mannheim, Germany), protein determination assay from BioRad (Munich, Germany), and polyvinylidene difluoride (PVDF) membranes from Millipore (Bedford, USA). Fat-free powdered milk was from Heirler (Radolfzell, Germany), all antibodies used for Western blotting were from Santa Cruz Biotechnology (Heidelberg, Germany), and the enhanced chemiluminescence (ECL) detection system from Amersham Biosciences (Freiburg, Germany). MDR1 antibody Clone 17F9 was obtained from BD Biosciences (Pharmingen, USA), RNAwiz from Ambion (Austin, USA), M-MLV Reverse Transcriptase and Reaction Buffer from Promega (Mannheim, Germany), and dNTPs and RNase inhibitor from Fermentas (St. Leon-Rot, Germany). The standard diet used in animal experiments was V1534 from Ssniff (Soest, Germany). All the other materials were from Sigma.

2.2 Cell culture

The human colon carcinoma cell line Caco-2 was obtained from the German National Resource Centre for Biological Material (Braunschweig, Germany) and was used between passages 10 and 40. The cells were grown in a humidified atmosphere at 37°C with 5% CO_2 in culture medium consisting of Minimal Essential Medium (MEM) with Earle's salts and 2 mM L-glutamine, supplemented with 10% v/v FBS, 50 μg/mL gentamycin and 0.1 mM MEM nonessential amino acids. Cells were subcultured at preconfluent densities by the use of a solution containing 0.05% trypsin and 0.5 mM EDTA. Caco-2 cells were cultured in T-75 flasks for Western blotting and in six-well cell culture plates for real-time and flow cytometry experiments, respectively. Starting on day 3 of postconfluency, cells were exposed to 10 μM flavonoid in the culture medium for 4 wk with medium changes every second day. Viability, as assessed by trypan blue exclusion, and morphology of the cells remained unaltered by the treatments. Flavonoids were dissolved in DMSO with final concentrations of the solvent not exceeding 1% v/v. Control cells were treated with the same amount of solvent.

2.3 Membrane preparation and Western blot analysis

For Western blot analysis, cells were washed with ice-cold PBS, pH 7.4, without Ca^{2+} and Mg^{2+} but containing 1 mM EDTA and harvested by scraping with a rubber policeman. Homogenization in lysis-buffer consisting of 10 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, was performed by 20 strokes using a motor-driven homogenizer. For protease inhibition the lysis buffer contained 0.8 mM Pefabloc SC and 0.2% v/v Protease Inhibitor Cocktail (1 mM PMSF,

10 μg/mL pepstatin A, 20 μg/mL leupeptin, 10 μg/mL aprotinin). The cell homogenates were centrifuged at 400 × *g* for 20 min at 4°C to remove nuclei and cell debris. The supernatant containing the cell membrane proteins was subsequently transferred to a new 1.5 mL Eppendorf tube and centrifuged at 40 000 × *g* for 20 min at 4°C. The resulting pellet was resuspended in membrane buffer containing 10 mM HEPES, pH 7.5, 1 mM EDTA, 10% w/v glycerine, 1 mM DTT, and 0.2% v/v Protease Inhibitor Cocktail. The protein concentration was determined according to the OD at 600 nm using the BioRad protein assay (BioRad). Protein aliquots were mixed with Laemmli buffer containing 50 mM Tris, 100 mM DTT, 10% glycerin, 2% SDS, and 0.1% bromphenol blue [17] and stored at −20°C. Identical amounts of membrane protein (30 μg/lane) were loaded onto 7.5% v/v polyacrylamide minigels, separated by SDS-PAGE prior to semidry electrotransfer (23 V for 3 h) onto PVDF membranes [18]. The membranes were blocked for nonspecific binding by incubation in Tris-buffered saline (TBS) (25 mM Tris, 140 mM NaCl, 3 mM KCl, pH 7.4) containing 0.05% v/v Tween-20 and 5% w/v fat-free powdered milk for 60 min at room temperature. P-gp was immunodetected using the rabbit polyclonal primary IgG antibody Mdr H-241 by incubation for 2 h at room temperature in a dilution of 1:500 with 0.1% w/v fat-free powdered milk. After incubation with horseradish peroxidase-conjugated goat antirabbit IgG secondary antibody for 60 min at room temperature in a dilution of 1:1000 and 0.1% w/v fat-free powdered milk, blots were detected using an ECL system. The ECL signals were visualized using a Hyperfilm, scanned and quantified with the ProteomWeaver software (Definiens, Munich, Germany). Following development, blots were stripped with Western blot stripping buffer (62.5 mM Tris-HCl, 2% SDS, and 100 mM β-mercaptoethanol) for 30 min at 50°C. Subsequent to being blocked again, equal protein loading and transfer to the membrane were assessed by immunodetection of β-actin with the mouse antihuman primary IgG antibody Actin C4 (MP Biomedicals, Irvine, CA) followed by the horseradish peroxidase-conjugated goat antimouse IgG secondary antibody, both in a dilution of 1:1000 with 0.1% w/v fat-free powdered milk by the method described above.

2.4 Flow cytometry analysis

To allow gentle detachment of the cell monolayer and to obtain single cells with intact cell surfaces for efficient antibody-binding, cell monolayers were trypsinized for 3–5 min, as described above. Cells were resuspended and homogenized gently with 1 mL fresh medium. One million cells were subsequently centrifuged for 3 min at 250 × *g* at room temperature and resuspended in 50 μL of PBS. Fluorescein-isothiocyanate (FITC)-conjugated mouse antihuman monoclonal MDR1 antibody Clone 17F9 was added at 0.5 μg antibody/10^6 cells and incubated for 1 h at room temperature in the dark. Subsequently, cells were washed by adding 900 μL of PBS and pelleted by centrifugation (3 min at 250 × *g* at room temperature). Cells were resuspended in 1 mL PBS and stored on ice in the dark until use. To measure the expression of P-gp a Beckton Dickinson FACSCalibur flow cytometer (BD Biosciences, San Jose, USA) equipped with an argon-ion laser (emission at 488 nm) was used. The forward scatter (FSC) detector was set at E-1 with a threshold value of 80. Green fluorescence from FITC was collected through a 530 ± 30 nm band pass filter. 2×10^4 cells of each sample were measured for statistical analyses using logarithmic amplification. CellQuest software from BD was used for acquisition and analysis of the data. Results are shown in histogram form, with the ordinate representing the cell number and the abscissa representing the relative fluorescence intensity on a logarithmic scale. Comparison of the different levels of P-gp expression in the cell membranes of the estimated cell populations was carried out by using the median of relative fluorescence intensity after subtraction of the corresponding median value of unstained cells.

2.5 RNA extraction, reverse transcription PCR and quantitative real-time PCR

Total RNA was isolated with RNAwiz. RNA concentration was determined by UV spectroscopy at 260 nm. One microgram of total RNA was used for cDNA synthesis in a final volume of 40 μL. RNA was heated to 65°C for 5 min with 1 × M-MLV Reverse Transcriptase Reaction Buffer and 300 μM each of dATP, dGTP, dCTP, and dTTP, and subsequently cooled on ice for 5 min. First strand DNA synthesis was accomplished with 0.08 μg random hexamer primers, 12.5 U of RNase inhibitor, and 200 U of M-MLV Reverse Transcriptase. The completed reverse transcription mixture was preincubated for 10 min at room temperature. PCR was carried out for 50 min at 42°C and a final step of 15 min at 70°C was performed to inactivate the reaction. Samples were cooled and stored at −20°C until use.

Primer design for LightCycler (Roche Applied Sciences)-based quantitative real-time PCR was done with regard to primer dimer formation, selfpriming formation, and primer melting temperature using the LigtCycler Probe Design Software (Roche). Blast search in the published sequence database GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) revealed that primers are gene-specific and if possible, those primers which span at least one intron were chosen. Primers of housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S were used as internal controls. Based on these criteria, the following primers (MWG, Ebersberg, Germany) were used; the primer sequences used for MDR1 amplification were: forward primer 5′-GGCCTAATGCCGAACAC-3′ and reverse primer 5′-CTGCCAAGGGGTCGTA-3′, corresponding to residues 3086–3070 and residues 3271–3256, respectively, of the

published cDNA sequence (Acc. No.: AF016535). The primers were designed to generate a 202-bp product spanning exons 25 and 26. The primer sequences used for GAPDH amplification were: forward primer 5′-GGCTCTCCAGAACATCATCCCTGC-3′ and reverse primer 5′-GGGTGTCGCTGTTGATGAAGTCAGAGG-3′, corresponding to residues 603–626 and residues 871–848 (Acc. No.: NM 002046), which yield a 269-bp product (exon 8). The primer sequences used for 18S ribosomal RNA amplification were: forward primer 5′-CCAAAGTCTTTGGGTTCCGG-3′ and reverse primer 5′-ACCAACTAAGAACGGCCATG-3′, corresponding to residues 1146–1165 and residues 1350–1331 (Acc. No.: X03205), generating a 205-bp product.

Each PCR reaction was conducted with 25 ng reverse-transcribed total RNA (cDNA-RNA hybrid) in a final volume of 10 μL consisting, furthermore, of 4 mM $MgCl_2$, 0.4 μM forward and reverse primers, and 1 × FastStart DNA Master SYBR Green I (containing "Hot Start" Taq DNA polymerase, reaction buffer, dNTP mix, $MgCl_2$, and SYBR Green I dye). After initial denaturation at 95°C for 10 min, amplification was performed by 50 cycles using the following parameters: 95°C for 15 s (denaturation), 62°C for 10 s (annealing), 72°C for 20 s (elongation), and 82°C (18S) or 86°C (MDR1 and GAPDH), respectively, for 5 s (melting primer dimers prior to quantification). A negative control without cDNA was also performed for 50 cycles to uncover possible contamination.

Normalization to the housekeeping genes GAPDH and 18S and calculation of the relative amount of target mRNA were performed by the Excel-based tools BestKeeper [19] and Q-Gene [20].

2.6 Animals and treatment

Female mice (C57BL/6J) at the age of 6 wk, weighing 17.0–18.4 g, were obtained from Harlan Winkelmann (Borchen, Germany). Four animals were kept *per* cage in a specific pathogen-free animal facility under controlled conditions of a 14 h light/10 h dark cycle. All the mice had free access to tap water and a standard diet, and were weighed weekly. Animal handling and experimentation were performed in accordance with the Germans Animal protection law and approved by the Animal Care und Use Committee of the state of Bavaria (AZ 211-2531-37/00). For detection and quantification of P-gp in the small and large intestine, eight mice were randomly divided into two groups (four animals *per* group): one group was treated with 400 mg/kg body weight *per* day of flavone, control mice received vehicle alone. Due to its low solubility in water, flavone was suspended in a saline/Myrj solution (0.1% Myrj 53, 0.9% NaCl) and given orally using a pharynx tube five times a week for 4 wk from the age of 7 wk onwards. Three days after the last administration, mice (weighing 19.5–22.7 g) were sacrificed by cervical dislocation and the complete intestine was harvested. The excised intestine was perfused with ice-cold saline to remove food contents and separated into duodenum (approximately 3–4 cm), jejunum (approximately 16–18 cm), ileum (approximately 10–15 cm), and colon (approximately 6–8 cm). The individual segments were placed on an ice-cold glass plate, cut open lengthwise and the intestinal mucosa was collected by scraping with an ice-cold microscope slide. With ice-cold lysis-buffer, tissues were transferred to Eppendorf tubes, frozen in liquid nitrogen immediately, and stored at −80°C until use. For Western blot analysis tissue samples were prepared as described above.

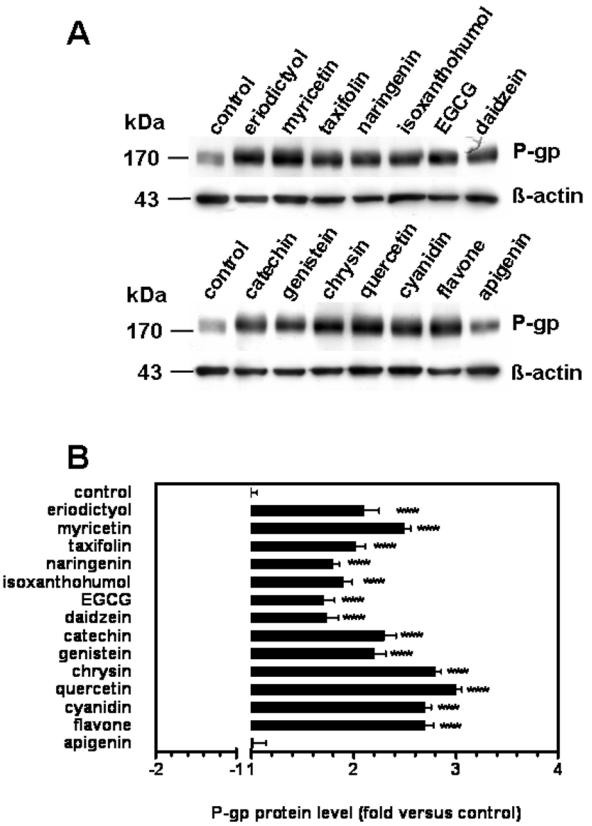

Figure 1. P-gp and β-actin levels in plasma membranes of Caco-2 cells treated for 4 wk with 10 μM of the flavonoids or exposed only to medium (control) as assessed by Western blotting. (A) Representative experiment. Proteins were extracted from Caco-2 as described in the Section 2.3 and separated by SDS-PAGE. P-gp protein levels were determined by Western blotting. Shown is a representative gel. (B) P-gp levels quantified by densitometry in relation to β-actin. Data are the mean values ± SD from three independent experiments. ***p <0.001 *vs.* control.

2.7 Statistical analysis

Differences between groups were tested using One-way ANOVA and Tukey's Multiple Comparison test (Graph Pad

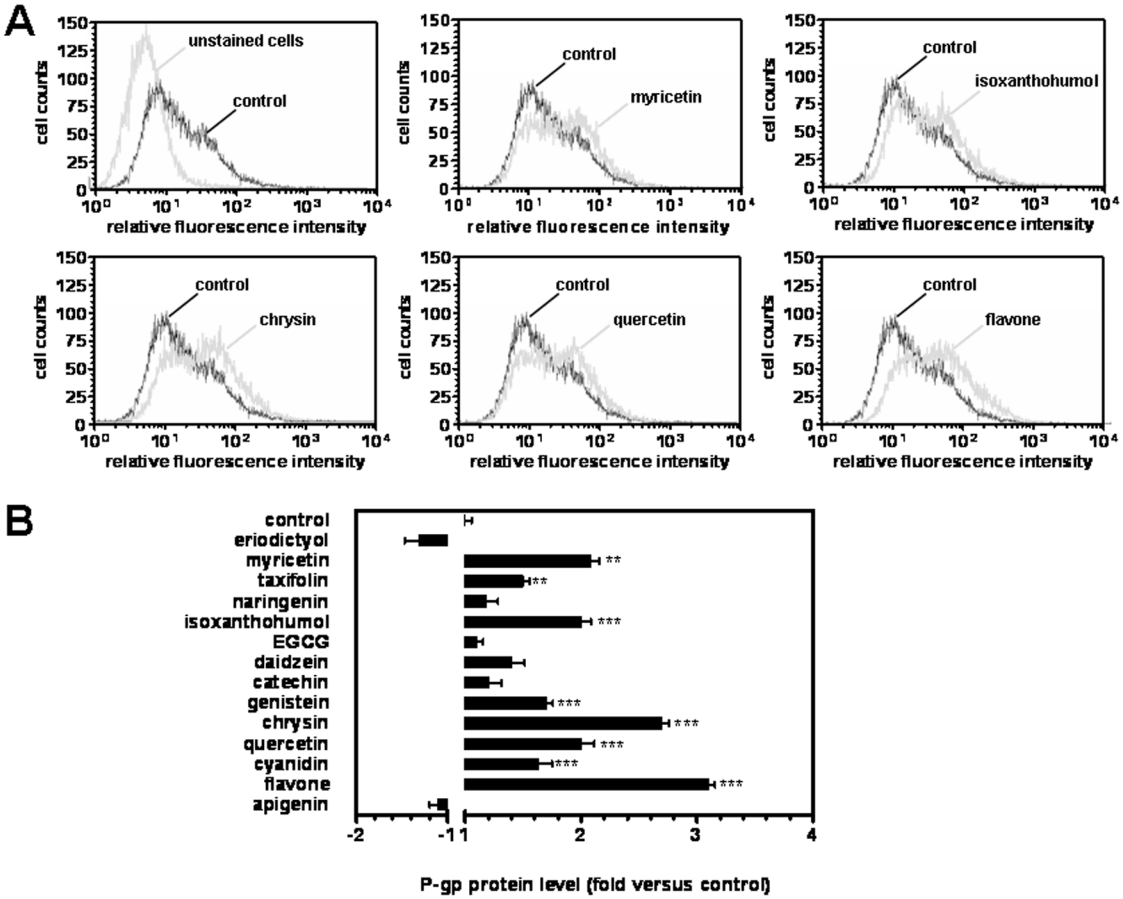

Figure 2. Flow cytometric analysis of P-gp protein expression in Caco-2 cells after flavonoid exposure for 4 wk. (A) Caco-2 cells cultured in medium alone (control) or media containing 10 μM flavonoid were stained with an FITC-labeled MDR1 antibody and relative fluorescence intensities of single cells were determined by the use of a Beckton Dickinson FACSCalibur flow cytometer. Representative results are shown and panel (B) shows the medians of fluorescence intensities of flavonoid-treated cells *vs.* controls. Data are the mean values ± SD from three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control.

Prism, San Diego, CA). For each variable, at least three independent experiments were carried out. Data are given as mean ± SD. Significance was set at $p < 0.05$.

3 Results

3.1 Almost all flavonoids tested increase P-gp protein levels in Caco-2 cells

Fourteen different flavonoids were tested at a concentration of 10 μM for their ability to affect P-gp expression in crude membranes of Caco-2 cells. The test compounds flavone, chrysin, and apigenin represented the flavone subclass, quercetin and myricetin the flavonols, catechin, epigallocatechin-3-gallate (EGCG) and taxifolin the flavanols, naringenin and eriodictyol the flavanones, genistein and daidzein the isoflavone group. In addition, we used the anthocyan cyanidin-3-glucosid and the chalcon isoxanthohumol. Exposure of cells to thirteen flavonoids for 4 wk resulted in a significant increase in P-gp protein levels in the cell membrane when assessed by Western blotting (Fig. 1). Only apigenin failed to affect P-gp protein levels while nine of the 14 flavonoids caused at least a more than two-fold increase in P-gp protein (Fig. 1B). We also used flow cytometry analysis to confirm the Western-blot findings. A significant increase in relative fluorescence intensity of P-gp-stained cells by the FITC-conjugated MDR1 antibody was obtained in case of eight of the tested compounds (Fig. 2).

3.2 Flavonoids appear to change P-gp protein levels *via* an increase in transcriptional activity

Real-time PCR was used to investigate the effects of flavonoids on the P-gp mRNA levels in order to assess whether the effects occur *via* transcriptional activation or through an impaired protein turnover. Twelve of the test compounds led also to a significant increase in P-gp mRNA levels (Fig. 3). Naringenin and eriodictyol that failed to enhance

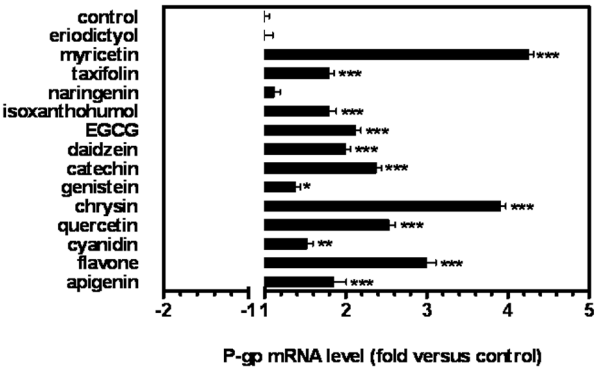

Figure 3. P-gp mRNA levels in Caco-2 cells exposed for 4 wk to 10 μM flavonoids dissolved in culture medium or to medium alone (control), as determined by real-time PCR. The mRNA levels were determined by the use of SYBR Green I with Roche's LightCycler. Results were normalized to the housekeeping genes GAPDH and 18S (BestKeeper). Data are the mean values ± SD of three independent experiments. *p <0.05, **p <0.01, ***p <0.001 *vs.* control.

P-gp transcript levels (Fig. 3) also did not lead to increased protein levels when assessed by flow cytometry (Fig. 2B), whereas Western blotting suggested an enhanced P-gp expression (Fig. 1B). Flavone and chrysin appeared to be the strongest P-gp inducers with similar activation factors in the Western blotting, flow cytometry, and real-time PCR experiments (Figs. 1–3). Most of the flavonoids seemed to mediate their effects *via* an increased transcription of the *MDR1*-gene or *via* stabilization of the mRNA. However, the significant increase in P-gp mRNA levels caused by apigenin (Fig. 3) that failed to translate into increased P-gp protein levels (Figs. 1 and 2) may indicate that some flavonoids may also change the protein turnover as a second mechanism of regulation.

3.3 Flavone increases P-gp expression in intestinal epithelial cells also *in vivo*

Since flavone-induced P-gp expression increased most efficiently in Caco-2 cells with similar increases at the mRNA and protein level, we selected this compound for our *in vivo* study. C57BL/6 mice received over 4 wk flavone by gavage and P-gp expression was determined by Western-blotting. P-gp levels in general showed a pronounced longitudinal distribution substantially increasing from proximal to distal intestine with highest densities in the colon (Fig. 4). Jejunal levels were 2.5-fold higher than in duodenum and colonic levels were 7.5-fold higher than in the most proximal segment (Fig. 4B). Treating animals with flavone led to an increase in P-gp in all gut segments when compared to corresponding regions in control animals (Fig. 4) with the most pronounced effects in jejunum (56% increase) followed by duodenum (36%), and ileum (28%) whereas colonic levels increased only by 10% (Fig. 4).

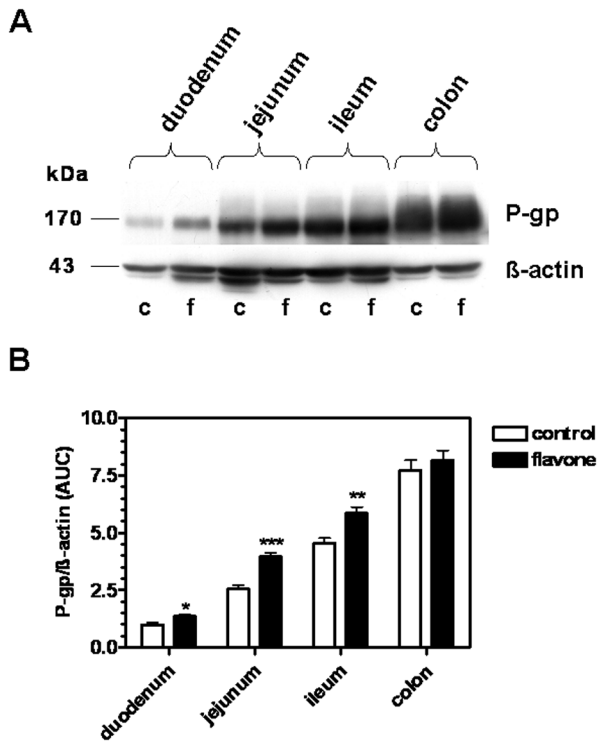

Figure 4. P-gp protein levels in various segments of the intestine of C57BL/6 mice in control animals (n = 4) or those receiving flavone by gavage (n = 4). (A) P-gp and β-actin levels in the gut segments from animals treated over 4 wk with vehicle alone (c, control) or 400 mg flavone (f)/kg body weight × day using Western blotting. (B) Densitometric analysis of the Western blot data was accomplished by the use of Proteome Weaver software. Data are given as the mean values ± SD. *p <0.05, **p <0.01, ***p <0.001 *versus* the control from the same intestinal segment.

4 Discussion

Flavonoid intake arises not only from the consumption of fruits and vegetables but also *via* an increased use of supplements containing extracts derived from different sources including herbs. Although flavonoids are considered to be health-promoting ingredients of the diet, they may also be seen as xenobiotics for which a variety of mechanisms exist to limit uptake into the body and to increase clearance and excretion from the body. Two processes are critical in this regard and for several flavonoids it has been shown that they interfere with such processes. One mechanism is the transformation of the xenobiotics into more water-soluble structures by the enzymes of phase-I and phase-II metabolism for enhanced excretion. Flavonoids were shown to alter the activites and/or expression levels of both phase-I [21, 22] as well as phase-II enzymes [23, 24] in the intestinal tract, thereby affecting the plasma levels of xenobiotics [25, 26]. The other mechanism relates to the secretion of lipophilic xenobiotics back into the intestinal lumen by ATP-

driven export pumps located in the apical plasma membrane, such as P-gp or multidrug resistance-associated protein 2 (MRP2). Many flavonoids appear to serve as substrates for MRP2, especially those in the form of glucosides or glucuronides [27, 28] but some are also P-gp substrates [29, 30]. By this competitive action the flavonoids may increase the bioavailability of other xenobiotics. Other flavonoids, however, were shown to stimulate P-gp transport rates in intestinal cells [14, 31] via allosteric mechanisms that enhance P-gp overall function or efficiency [14] and thereby reduce xenobiotic uptake into the body. Besides such direct interactions with the P-gp protein, they could also alter the number of transporters available especially when provided by the diet over extended time periods and for most of the flavonoids this has not been studied yet.

We therefore assessed in the present study whether a long-term exposure of intestinal epithelial cells to flavonoids alters P-gp expression. With the exception of apigenin, all the tested compounds, when provided at a concentration of 10 μM over 4 wk led to a significant increase in P-gp protein in intestinal epithelial Caco-2 cells based on Western blotting. Although findings from flow cytometry analysis were in line with Western blotting results, a lower number of flavonoids were found to affect P-gp levels, suggesting that Western blotting provided a higher sensitivity.

With the exception of naringenin and eriodictyol all the flavonoids tested also increased transcriptional rate of the *MDR1* gene or P-gp mRNA stability as assessed by quantitative real-time PCR. One possible mechanism by which this could be achieved is *via* activation of the pregnane X receptor, as this orphan nuclear receptor acts as a key regulator of *MDR1* [15] and various flavonoids have been demonstrated to activate pregnane X receptor response elements [32]. In the case of apigenin, the enhanced mRNA level does not translate into increased P-gp protein levels indicating that P-gp degradation may be increased by this compound. In contrast, P-gp protein levels were enhanced after exposure of cells to naringenin and eriodictyol (at least according to Western blot analysis) in spite of a lack of an effect on the mRNA level. This also suggests that protein turnover is affected. In this case, a reduced proteasomal degradation of P-gp may occur as shown for EGCG [33].

Since flavone proved as a strong P-gp inducer in Caco-2 cells at the mRNA as well as at the protein level, we selected this flavonoid to investigate whether similar effects can be observed also *in vivo*. C57BL/6 mice were treated over 4 wk with 400 mg/kg body weight daily by gavage. This relates to a dose of about 10% of those applied in most of the chemoprevention studies in mice [34, 35]. P-gp expression increased along the gastrointestinal tract with highest levels found in the colon, similar to findings for P-gp mRNA levels in the human gut [36]. The higher expression of P-gp in the more distal parts of the gut is suggested to provide a higher protection from insults by chemicals as this region of the gut has a low expression of CYP3A4 [36]. Moreover, an increased transcellular permeability for lipophilic compounds in the terminal regions of the intestine might be compensated by enhanced P-gp levels [37]. Exposure of the colonic epithelium to carcinogenic compounds could also be higher due to bacterial metabolism [38] and lower transit rates demanding higher P-gp activity. In our mice study, it became evident that flavone also *in vivo* altered P-gp levels in all the intestinal segments but the impact was lower *in vivo* than *in vitro* with a maximal increase of about 1.6-fold in jejunum of mice whereas an almost three-fold increase in P-gp levels in Caco-2 cells was observed, respectively. The lower efficiency of flavone *in vivo* could be due to lower concentrations at the site of action as compared to the cells in culture. Additionally, metabolic conversion in the mouse gut could result in the formation of ineffective metabolites when flavone reaches more distal sides [11, 39].

In conclusion, we show that most of the flavonoids used here increase P-gp levels in intestinal epithelial cells *in vitro* but – at least as demonstrated for flavone – also *in vivo*. Their effects appear to be mainly exerted through an activation of *MDR1* gene transcription. This adaptation to flavonoid exposure may be regarded as a mechanism that protects the organism from uptake of potentially hazardous compounds by exporting them back into the lumen and by increasing their excretion, respectively. But our findings also address a very important area of drug–food interactions as flavonoids coming from the diet but more importantly when provided in high doses *via* supplements alter P-gp levels thereby potentially also affecting the oral availability of drugs that are transported by P-gp.

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