Phytochemicals Induce Breast Cancer Resistance Protein in Caco-2 Cells and Enhance the Transport of Benzo[a]pyrene-3-sulfate

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INTRODUCTION

Breast cancer resistance protein (BCRP/ABCG2) is a 655-amino acid–spanning ATP-binding cassette (ABC) half transporter that is expressed in a variety of human tissues, such as colon, small intestine, kidney, liver, and placenta (Maliepaard et al., 2001).

The localization of BCRP on the apical surface of the small intestinal epithelium, for instance, suggests its strategic function as a protective efflux pump by increasing the elimination of ingested xenobiotics and drugs. A large body of evidence exists from recent studies indicating, in general, an important physiological role of BCRP in the body’s defense against dietary toxins. Several (pro)carcinogens and dietary toxic compounds have been identified as substrates for BCRP, among them are heterocyclic amines (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, 2-amino-3-methylimidazo[4,5-f]chinoline, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole), mycotoxins ( aflatoxin B1, ochratoxin), and the photo toxic chlorophyll breakdown product pheophorbide a (Jonker et al., 2002; Pavek et al., 2004; Schrickx et al., 2006; van Herwaarden et al., 2006).

We have recently shown that the phase II metabolites of the food carcinogen benzo[a]pyrene (BP), benzo[a]pyrene-3-sulfate (BP3S) and benzo[a]pyrene-3-glucuronide, are also effectively transported by BCRP in the human colon adenocarcinoma cell line Caco-2 (Ebert et al., 2005). Caco-2 cells, although derived from the colon, represent an established in vitro model for the human small intestinal epithelium. Therefore, this cell line has been extensively used to study biotransformation and transport processes. As expected, immunofluorescence microscopy analysis of Caco-2 cells revealed that the expression of BCRP is localized to the apical plasma membrane (Xia et al., 2005). In the present study, we used Caco-2 cells because this cell line has been shown to be a useful tool to investigate the characteristics of BCRP (Xia et al., 2005).

Epidemiological studies have shown that diets rich in fruit and vegetables are associated with a lower risk of developing cancer.
food-related malignancies such as colon cancer (Greenwald et al., 2001; Terry et al., 2001). Plant-derived food contains numerous structurally diverse chemical compounds (e.g., polyphenolic compounds, flavonoids, dietary indoles, or isothiocyanates), some of which have been identified as natural anticarcinogens (Wattenberg, 1992; Yang et al., 2001). The anticarcinogenic effects of these phytochemicals are believed to be mediated by different biochemical mechanisms, such as antioxidative activities (radical scavenging), altered apoptosis, enhanced DNA repair, and the modulation of detoxifying enzymes (Steinmetz and Potter, 1991; Wargovich, 1997).

Dietary enzyme inducers are formally divided into monofunctional and bifunctional inducers (Nguyen et al., 2003). Monofunctional enzyme inducers (e.g., isothiocyanates) act via binding to Nrf2 (nuclear factor-erythroid 2-related factor 2), a member of the basic leucine zipper NF-E2 family of transcription factors. Nrf2 triggers the induction of phase II enzymes such as glutathione-S-transferases (GST) and UDP glucuronosyltransferases (UGT) by binding to antioxidant responsive element (ARE) enhancers ($5^{'-}GGA/GGATTTNNGC/GG-3'$) in the promoter of these genes (Moskaug et al., 2004; Nguyen et al., 2003; Rushmore et al., 1991). Bifunctional inducers (e.g., flavonoids) induce gene expression through both ARE and xenobiotic response elements (XREs: $5^{'-}T/GCCGTG^{C}G/C-A-3'$), the latter requires the transactivation of the aryl hydrocarbon receptor (AhR) (Hankinson, 1995).

Many food-derived carcinogens such as BP need to be activated to genotoxic metabolites. Therefore, the coexpression of detoxifying enzymes and the corresponding ABC transport proteins seem to play a crucial role in the development of food-related cancers. Both phase I (e.g., cytochrome P450 [CYP] 1A1, CYP1B1) and phase II (e.g., UGT1A6 and GSTs) xenobiotic-metabolizing enzymes (XMEs) involved in the detoxification of BP are known targets within the AhR gene battery. We have recently shown that in Caco-2 cells BCRP is inducible by classical AhR ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polycyclic aromatic hydrocarbons (PAHs) (Ebert et al., 2005).

To date, numerous structurally diverse compounds naturally occurring in plant food have been identified as AhR agonists (Denison and Nagy, 2003). This is raising the question whether these nonclassical rather weak AhR agonists present in our daily food may contribute to the barrier function of the small intestine by increasing the expression of BCRP. Therefore, the present study aimed at the investigation whether nonnutritive plant constituents, some of which with reported AhR-agonistic activity (e.g., quercetin curcumin, dibenzoylmethane [DBM]) and/or reported antitumorogenic properties (silymarin, indole-3-carbinol [I3C], (+)-epicatechin, resveratrol), and the synthetic antioxidant tert-butyl hydroquinone (TBHQ) have an effect on the expression and transport function of BCRP. To test the possible involvement of an ARE in the regulation of BCRP, the monofunctional inducers sulforaphane (SUL) and diethylmaleate (DEM) were also included in this study.

Moreover, the transport of BP3S was investigated in Caco-2 cells treated with selected phytochemicals (DBM and quercetin) to test the functional expression of BCRP in those cells. BP3S is the major phase II metabolite formed by Caco-2 cells, and although this metabolite is not derived from the ultimate carcinogen of BP, (+)-anti-BP-7,8-diol-9,10-epoxide ((+)-anti-BPDE), it distinctly contributes to the detoxification of BP. Because the genotoxic metabolite (+)-anti-BPDE is formed in a three-step reaction sequence (oxidation, hydrolysis, and second oxidation), it is likely that an enhanced conjugation to and export of sulfoconjugates of BP decreases the amount of substrate available for the enzyme systems involved in the formation of the ultimate carcinogen (+)-anti-BPDE.

**MATERIALS AND METHODS**

**Chemicals.** Alpha-naphthoflavone (α-NF) (7,8-benzoflavone), chrys (5,7-dihydroxyflavone), curcumin ((1,6,8E)-1,7-bis(4-hydroxy-3-methoxy-phenyl)-1,6-heptadiene-3,5-dione), β-l-sulforaphane, DBM (1,3-diphenyl-1,3-propanedione), DEM (maleic acid diethyl ester), (−)-epicatechin (−)-cis-3,3',4',5,7-pentahydroxyflavone), flavone, genistin (4',5,7-trihydroxyisoflavone), ISC, quercetin (3',4',5,7-tetrahydroxyflavonol), resveratrol (trans-3',4',5-trihydroxystilbene), silymarin (isomeric mixture of silybin, silydianin, silychristin), and TBHQ were purchased from Sigma (Deisenhofen, Germany). The purity of all phytochemicals exceeded 97%. TCDD (99.9% purity) was obtained from Promochem (Wesel, Germany). MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) was obtained from Calbiochem (Darmstadt, Germany). Indeno[1,2,3-cd]-fluoranthene (INF), benzo[a]fluoranthene (Ba[F]), and the phase I metabolite of BP, 3-hydroxybenzo[a]pyrene (3-OH-BP), were synthesized at the Biochemical Institute for Environmental Carcinogens (Grosshansdorf, Germany). The purity of the compounds was greater than 99% as indicated by GC-MSD and HPLC analyses. Indolol[3,2-b]-carbazole (ICZ) was a kind gift from Prof. J. Bergman (Karolinska Institute, Stockholm, Sweden).

Stock solutions of all test compounds were prepared in dimethylsulfoxide (DMSO) and stored at −20°C until used.

When handling PAH and especially TCDD, all safety precautions should be followed. Contaminated waste should be collected separately and eliminated appropriately.

**Cell culture.** The human colon adenocarcinoma cell line Caco-2 was obtained from the European Collection of Cell Cultures (Porton Down, UK) and maintained in Dulbecco’s modified Eagle’s medium (GIBCO–Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO2 in air at 37°C. The cells were used at passages 56–66 for all experiments. MCF-7 wild-type (WT) and MCF-7AHR200 cells were used at passages 8–12, and they were cultured in the same medium as Caco-2 cells. MCF-7AHR200 cells were generously provided by Dr Grace C. Yeh (National Cancer Institute at Frederick, National Institutes of Health, Frederick, MD). MCF-7AHR200 cells have been selected for BP resistance, and they express an extremely low amount of AhR protein (Ciolino et al., 2002).

**Gene expression experiments.** Caco-2 cells were cultured in small glass petri dishes (28 cm2, coated with 1% [wt/vol] gelatine) or six-well plates (9.3 cm2) for 14 days after reaching confluence and subsequently treated with test compounds freshly dissolved in culture medium. After 24, 48, or 72 h of incubation, the medium was removed, the cell monolayer was washed with ice-cold phosphate-buffered saline (PBS), and cells were harvested for RNA isolation.

“Superinduction” of BCRP mRNA by coinubcation with ICZ and 26S proteasome inhibitor MG-132. Caco-2 cells were cultured in six-well plates...
for 17 days and treated with either 2.5 μM of ICZ or with a mixture of 2.5 μM ICZ and 20 μM of MG-132. Controls were treated with 0.1% DMSO (vol/vol). After 8 and 24 h of incubation, the medium was replaced, the cell monolayer was washed with ice-cold PBS, and cells were harvested for RNA isolation.

**Preparation of mRNA and real-time quantitative PCR analysis.** Total RNA was isolated using TRIzol reagent (Invitrogen, Eggenstein, Germany) according to the manufacturer’s protocol with an additional 70% ethanol wash. One hundred nanograms of RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega, Heidelberg, Germany) with 100 pmol of oligo (dT)18 as primer for primer for 1 h at 37°C. Real-time quantitative PCR (qPCR) were run on a Mx3000P cycler (Stratagene, Amsterdam, The Netherlands). PCRs were run in triplicates containing SYBR Green I (Molecular Probes, Leiden, The Netherlands) as described previously (Ebert et al., 2005). Primers used were as follows—for human BCRP (accession no. AF098951): 5′-CAGGTCTTGTTGTCAATCTCA-3′ (sense) and 5′-TCCATATCGTGAATGCTGAAG-3′ (antisense), and for human β-actin (accession no. NM 01101): 5′-CGTCCACCCGAAATGCTT-3′ (sense) and 5′-GTTTCGCGCCAAGTAGGTATTG-3′ (antisense). The thermal cycling comprised an initial denaturation step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 30 s. By employing Mx3000P software, the mRNA amount of BCRP in each sample was normalized to its β-actin content and then normalized to DMSO control to obtain fold inductions as described before (Ebert et al., 2005).

**Transient transfection.** In order to test whether the expression of BCRP mRNA is AhR dependent, AhR-deficient MCF-7AHR200 cells were seeded at a density of 2 × 10⁵ cells per well in six-well plates and transfected with different amounts (0.2–1.6 μg DNA/cm² growth area) of the human AhR expression plasmid pSp30AhR2 (generously provided by Dr. C. Bradfield, University of Wisconsin, Madison, WI; Delwick et al., 1993) using the calcium phosphate coprecipitation technique. Twenty-four hours after transfection, medium was removed and the cells were harvested for total RNA isolation as described above, and the expression of BCRP and AhR mRNA was determined by real-time qPCR.

**Western blotting.** Caco-2 cells were cultured in large culture flasks (75 cm²) for 9 days and treated for 3 days with different test compounds or DMSO (0.1% [vol/vol]) as indicated in the figures. MCF-7 cells were grown in culture dishes (145 cm²) until reaching 80% confluence and subsequently treated for 24 h with test compounds as designated in the figures. To obtain whole-cell lysates, cells were harvested by scraping into a small volume of ice-cold lysis buffer (2 mM Tris, 50 mM mannitol, pH 7.0) supplemented with 10 μM phenylmethylsulfonyl fluoride and homogenized by passing through a 27-gauge hypodermic needle for several times. Soluble fractions were obtained by centrifugation. Membrane-enriched cell fractions from Caco-2 cells were prepared as follows: the cells were collected as described above and pelleted by brief centrifugation. After an initial homogenization by passing through a 27-gauge hypodermic needle, an additional homogenization followed by ultrasonication on ice using a microtip. Cell debris were removed by centrifugation (5 min/3000 × g at 4°C). The supernatant was subjected to centrifugation for 50 min at 4°C and 50,000 × g. The resulting pellet was resuspended in PBS. Protein contents of samples were determined by the bicinchoninic acid method (Smith et al., 1985) with bovine serum albumin as a standard. Aliquots of membrane-enriched fractions (45 μg) or cell lysates (100–120 μg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (8 or 10% polyacrylamide resolving gel) and electrotransferred onto nitrocellulose membranes as described previously (Ebert et al., 2005). Probing was performed overnight at 4°C with anti-BCRP antibody BXP-21 (Calbiochem, dilution 1:2000). Blots were then incubated with secondary horse-radish peroxidase–conjugated antibody (1:10,000) for 1.5 h at room temperature. Bands were visualized by enhanced chemiluminescence detection kit (ECL advance) following the manufacturer’s instructions (Amersham Biosciences, Braunschweig, Germany).

**Transport of BP3S endogenously formed from 3-OH-BP in Caco-2 cells.** Caco-2 cells were grown on Transwell inserts (4.71 cm² growth area, 0.4-μm pore size, polycarbonate membranes; Corning Costar Co., Cambridge, MA) and cultured for 16 days after seeding and subsequently pretreated with quercetin (25 μM), DBM (50 μM), or DMSO (0.1%). The medium was replaced every second day. The integrity of the cell monolayer was routinely checked before and after the transport experiment by measuring the transepithelial electrical resistance as described previously (Ebert et al., 2005).

Because AhR agonists quercetin and DBM are capable to induce phase I enzymes (CYP1A1 and CYP1B1) and the pretreatment consequently would lead to a decrease in the amount of substrate available for the conjugating sulfotransferases (SULT) in comparison to untreated cells, we therefore blocked phase I enzymes by incubation with α-NF, a well-established CYP1A1 and CYP1B1 inhibitor (Shimada et al., 1998). Cells were preincubated with α-NF (150 μM) for 1 h. The incubation experiment was subsequently started by replacing the medium with medium containing 5 μM of the substrate 3-OH-BP and α-NF (50 μM final concentration). All treatment compounds were added to both chambers of the Transwell plate. The plates were placed on an orbital shaker during the experiment. At the indicated times, medium samples from both chambers of the Transwell plate were collected and stored at −20°C until analyzed for BP3S by HPLC.

**HPLC analysis.** The sample cleanup and HPLC analysis of the medium samples were performed as described in detail previously (Buesen et al., 2002). The separation of the BP metabolites was performed employing HPLC (HP 1100 series, Agilent Technologies, Waldbronn, Germany) on a reversed-phase C18 column (PAH 16 plus, 5-μm particle size, J. T. Baker, Griesheim, Germany). The detection was carried out using a diode-array detector (HP 1100 series). The amounts of BP metabolites were quantified by means of an internal standard, INF.

**Statistical analysis.** Statistical analyses were performed with SigmaStat software. Differences between mean values were determined by one-way ANOVA followed by a Tukey-Kramer posttest. Statistically significant differences were set at p ≤ 0.05.

**RESULTS**

**Effect on BCRP mRNA Expression in Caco-2 Cells**

Flavonoids are the most abundant nonnutritive compounds in our daily diet. Due to their polynuclear and (partly) aromatic structure, they are able to interact with the AhR and thereby induce, for instance, phase I enzymes (Zhang et al., 2003). Based on our previous observation that AhR agonists are able to induce BCRP expression in colon cancer cells (Ebert et al., 2005), we therefore investigated the effects of several flavonoids (chrysirin, flavone, quercetin, and genistein) and other phytochemicals with reported anticarcinogenic activities (resveratrol and I3C) on the expression of BCRP mRNA in Caco-2 cells. As shown in Figure 2A, the stilbene resveratrol and the flavone chrysin were most effective in inducing the expression of BCRP mRNA, followed by the flavonol quercetin and flavone, the unsubstituted parent compound of flavonones. Interestingly, resveratrol is a reported AhR antagonist (Ciollino et al., 1998). Treatment with I3C, a breakdown product of glucobrassicin, a constituent of cruciferous vegetables, showed a clear but lower induction of BCRP mRNA compared with the other treatments. The phytoestrogen genistein had the lowest effect on the induction of BCRP mRNA, consistent with the relatively low induction of the AhR target gene CYP1A1 in MCF-7 cells reported by Zhang et al. (2003). I3C showed
Effect of Flavonoids on BCRP Expression in Caco-2 Cells

Different flavonoids (flavone, chrysin, quercetin, and silymarin), resveratrol, and DBM were tested for their ability to induce BCRP in Caco-2 cells. To allow comparisons between the observed effects, the phytochemicals were used at the same concentration of 25 μM. Silymarin, a chemoprotective agent from the seeds of milk thistle (Silybum marianum), is a mixture of three isomeric substituted flavonoids (silibin, silydianin, and silychristin [Kohno et al., 2002]), and its molar concentration was calculated based on the major component silibin (for chemical structures, see Fig. 1). Of the tested flavonoids, flavone and quercetin showed a strong induction (Fig. 2B), consistent with the results from the mRNA analysis. Silymarin could increase the expression of BCRP also, whereas chrysin had no effect on BCRP expression. Resveratrol had a moderate effect, and DBM had a strong inducing effect on BCRP expression.

Effect of Monofunctional Inducers (ARE Activators) on BCRP mRNA and Protein Expression in Caco-2 Cells

Quercetin and TBHQ are two established antioxidants and inducers of ARE (Nrf2)-mediated gene expression (Nguyen et al., 2003). In preliminary experiments, both compounds exhibited a strong effect on the induction of BCRP; we next tested whether further ARE activators would be able to increase the expression of BCRP, too. The synthetic compound DEM and the isothiocyanate SUL are prototypical ARE activators without any AhR-agonistic activity (Nguyen et al., 2003). Both compounds had no effect on the expression of BCRP on mRNA (Fig. 3A) and on protein level; only DEM showed a very low induction, while SUL-treated cells showed no response (Fig. 3B). Similarly, the constituent of green tea and known antioxidant (-)-epicatechin (25 and 50 μM) had no inducing effect on BCRP expression on mRNA level, and a slight decrease of BCRP expression could be observed at a concentration of 50 μM (Fig. 3B). In contrast, treatment with both TBHQ and quercetin showed a pronounced increase in BCRP mRNA. As shown in Figure 3A, the highest increase could be detected for TBHQ and quercetin at a concentration of 50 μM (5.9- and 5.3-fold). On protein level, TBHQ at a concentration of 100 μM could induce BCRP to the greatest extent, followed by TBHQ at 50 μM and quercetin at 50 μM (Fig. 3B). As TBHQ and quercetin have been reported to be AhR agonists, the results clearly indicate that AhR-dependent signaling pathways are involved in the regulation of BCRP, whereas Nrf2 and its cognate response element ARE seem not to be involved.
Effect of Phytochemicals on BCRP Expression and MCF-7 WT Cells and AhR-Deficient MCF-7AHR200 Cells

To further substantiate our hypothesis that the regulation of BCRP is mediated by AhR-dependent signaling pathways, MCF-7 WT and AhR-deficient MCF-7AHR200 cells were incubated with the PAH and AhR agonist B[k]F (see Fig. 4A) as positive control and with different phytochemicals (Figs. 4B and 4C). The most pronounced induction of BCRP could be observed in MCF-7 WT cells treated with TBHQ and DBM (Fig. 4C) followed by quercetin (Fig. 4C), curcumin, and resveratrol (Fig. 4B). Silymarin had only a moderate effect on the induction of BCRP in this cell line. Most interesting, in MCF-7AHR200 cells, BCRP was almost not detectable on a basal level and none of the test compounds could markedly enhance the expression of BCRP. This strongly supports our hypothesis of the AhR being involved in the regulation of both basal and inducible protein expression of BCRP. Consistently, the PAH B[k]F at a concentration of 5μM, which is 10-fold lower than that of DBM or TBHQ, could increase the expression of BCRP to a similar amount as DBM or TBHQ.

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Transient Transfection

As AhR-deficient MCF-7AHR200 cells showed a nearly nondetectable level of basal expression of BCRP, it was interesting to test whether the introduction of AhR cDNA could restore the expression of the BCRP gene. As shown in Figure 5, transient transfection with AhR expression plasmid pSportAHR2 (Dolwick et al., 1993) could substantially increase the expression of BCRP mRNA and of AhR mRNA (not shown) as determined by real-time qPCR 24 h posttransfection. This result is providing further evidence of the role the AhR obviously plays in BCRP regulation.

Superinduction of BCRP mRNA

Inhibition of the proteasomal degradation of the ligand-activated AhR complex caused a superinduction of BCRP mRNA. The phenomenon of superinduction by inhibition of
the 26S proteasomes has been described for the AhR target gene CYP1A1 by Ma and Baldwin (1999). The ligand-activated AhR protein complex is released from the nucleus into the cytosol, ubiquitinated, and subsequently degraded by 26S proteasomes. This mechanism controls the activity of the ligand-activated AhR and was used in this study to investigate the possible involvement of the AhR in the regulation of the BCRP gene.

As shown in Figure 6, the incubation with the natural AhR agonist ICZ (2.5 µM) could elevate the levels of both BCRP mRNA after 8 h (fourfold) and 24 h (twofold). Cotreatment with MG-132 resulted in a slight further increase after 4 h (sevenfold) and to a pronounced induction of BCRP mRNA after 24 h of incubation (23-fold increase). The results of this experiment clearly demonstrate that the expression of the BCRP gene is most likely regulated via AhR-dependent pathways.

FIG. 4. Role of aryl hydrocarbon receptor (AhR) in breast cancer resistance protein (BCRP) expression. Effect of phytochemicals (B, C) and the polycyclic aromatic hydrocarbon benzo[k]fluoranthene (B[k]F, 5µM) (A) on BCRP expression in human breast cancer cells MCF-7 and AhR-deficient MCF-7AHR200 cells. While all test compounds could substantially increase the expression of BCRP in MCF-7 WT cells, the AhR-deficient cell line MCF-7AHR200 showed a nearly nondetectable basal level of BCRP and no response to the treatment. MCF-7 cells were grown until 80% confluence and treated with resveratrol (Resv), curcumin (Curc), silymarin (Sily), r-butyl hydroquinone (TBHQ), quercetin (Querc), dibenzoyl methane (DBM), or dimethylsulfoxide (0.1%, Ctrl) for 24 h. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out on membrane-enriched cell fractions (80 µg) (A, B) or whole-cell lysates (B) (120 µg) on a 8% resolving gel with BXP-21 primary antibody as described in the “Materials and Methods” section. One representative blot is shown.

FIG. 6. Superinduction of breast cancer resistance protein (BCRP) mRNA expression in Caco-2 cells after treatment with indolo[3,2-b]-carbazole (ICZ) and carbobenzoxy-l-leucyl-l-leucyl-l-leucinal (MG-132) (inhibitor of 26S proteasomes) for 8 and 24 h. BCRP mRNA expression was quantified relative to untreated control (0.1% dimethylsulfoxide) by real-time quantitative PCR with β-actin as the housekeeping gene.

Effect of a Pretreatment with Quercetin or DBM on the Transport of BP3S

As presented in Figure 7A, Western blot analysis of Caco-2 cells showed that several plant constituents and reported AhR agonists (Denison and Nagy, 2003) had an inducing effect on the expression of BCRP. The most striking increase in BCRP expression could be observed after treatment of Caco-2 cells with TCDD (50nM), which served as a positive control, followed by the flavonol quercetin (25 µM) and DBM (50 µM), a constituent of liquorice.

In order to test whether BCRP induced by quercetin and DBM was functionally active, we incubated pretreated Caco-2...
cells with the precursor of BP3S, 3-OH-BP, at 5μM and analyzed medium contents for the endogenously formed sulfate conjugate after 2, 4, and 6 h.

As shown in Figure 7B, only DBM pretreatment of Caco-2 cells led to a significant increase in apically transported BP3S after 2, 4, and 6 h of incubation \((p < 0.05, \text{one-way ANOVA, Tukey-Kramer posttest})\). The greatest increase could be observed after 6 h for DBM. The pretreatment with quercetin led only to a low enhancement of apically transported BP3S compared with control cells. This stands in contrast to the relatively strong induction of BCRP by quercetin in Caco-2 and MCF-7 WT cells. A possible explanation for this phenomenon may be that quercetin has not been completely metabolized by the beginning of the experiment and competes with 3-OH-BP for the conjugating enzymes (SULTs). This is supported by the observation that the total amounts of BP3S that were transported out of the cells (to the apical and basal chamber of the Transwell) were even lower than that of the control cells. Moreover, sulfoconjugates of quercetin are also a substrate for BCRP; therefore, remaining quercetin may compete for both interacting mechanisms (conjugation and apically transport).

**DISCUSSION**

The anticarcinogenic effects of minor components of plant-derived food have been intensively studied in the past years (Havsteen, 2002). The majority of these studies focused on the modulation of detoxifying enzymes such as UGT and GST, since the conjugation of potentially harmful compounds and procarcinogens prevents the formation of reactive (genotoxic) intermediates and accelerates their clearance from the body. ABC transport proteins such as BCRP are located in the plasma membrane, and they play a key role in this detoxification process by mediating the active transport of water-soluble conjugates across the cell membrane and thereby preventing adverse effects that would result from an accumulation of conjugated xenobiotics (Haimerl et al., 2004). Although the interaction of several flavonoids with ABC transporters such as P-glycoprotein (Di Pietro et al., 2002), multidrug resistance-associated protein 1 (Leslie et al., 2001), and BCRP (Zhang and Morris, 2003) on a functional level have been reported; this study for the first time provides evidence that flavonoids and other nonnutritive food compounds are able to modulate BCRP on a transcriptional level in Caco-2 and MCF-7 cells.

The average intake of flavonoids that are naturally present in the human diet has been estimated to be ~1 g/day (Formica and Regelson, 1995); although based on their beneficial health effects, specific flavonoids (e.g., quercetin and genistein) are marketed as nutritional supplements; therefore, the intake of these compounds is likely to be higher. Flavonoids are able to interact with the AhR and exert agonistic or antagonistic effects, dependent on the test system and concentration used (Ashida et al., 2000; Ciolino et al., 1998; Gehm et al., 1996; Zhang et al., 2003).

Resveratrol (trans-3, 4′,5-trihydroxystilbene) is a phytoalexin found in grape skin and wine and has gained intense investigation due to its reported cancer chemopreventive activity (Signorelli and Ghidoni, 2005). Resveratrol is an agonist for the estrogen receptor (ER) but an antagonist for the AhR (Ciolino et al., 1998; Gehm et al., 1997). Our results show a clear induction of BCRP in MCF-7 WT cells after treatment with resveratrol (25μM) but no response in MCF-7AHRE300 cells, which lack functional AhR protein. Very recently, ER response elements have been discovered in the promoter region of the BCRP gene (Ee et al., 2004). Given that...
the induction of BCRP in MCF-7 WT cells by resveratrol is based on its ER-agonistic effect, an increase in BCRP should have been detected in AhR-deficient MCF-7AHR200 cells also. However, an explanation of the mechanism involved in the observed induction of BCRP in MCF-7 remains speculative and would require further investigation.

Results from Western blot analysis have shown a striking increase in BCRP expression mediated by quercetin and TBHQ. Both the natural flavonone quercetin and TBHQ, a metabolite of the synthetic food preservative butylated hydroxyanisole, are well-known antioxidants and therefore are able to induce gene expression via interaction with AREs in the promoter of target genes (Nguyen et al., 2003). This prompted us to investigate the effects of other well-known ARE activators on BCRP expression in Caco-2 cells. The functional expression of Nrf2 in this cell line has been reported before (Banning et al., 2005; Bonnesen et al., 2001). The natural isothiocyanate and monofunctional inducer SUL is a breakdown product of glucosinolates present in cruciferous vegetables. SUL does not interact with the AhR, and it has been shown to induce Nrf2 target genes such as phase II enzyme GST in Caco-2 cells (Bonnesen et al., 2001). Our results show that SUL had no effect on BCRP expression on both mRNA and protein level in Caco-2 cells. As second prototypical ARE activator, the synthetic compound DEM had no effect on BCRP mRNA expression too, and the protein level was affected only marginally. These results suggest that most likely no ARE enhancer may be involved in the expression of BCRP, although this needs more detailed investigations. The strong inducing effect of quercetin and TBHQ are therefore most likely based on their AhR-agonistic activity, since both compounds have been reported to bind to the AhR (Ciolino et al., 1999; Gharavi and El-Kadi, 2005). Another natural antioxidant, (−)-epicatechin, a constituent of green tea, has been tested in this study for its ability to modulate BCRP expression. Interestingly, this flavonoid did not induce BCRP on mRNA level but rather repressed the expression of BCRP on protein level at a concentration of 50μM, suggesting an AhR-antagonistic effect exerted by this compound. Although quercetin and (−)-epicatechin share a similar pattern of hydroxyl group substitution, the C-ring of (−)-epicatechin lacks planarity, a feature considered essential for binding to the AhR. A downregulation of BCRP expression below basal level could be observed in previous experiments, when Caco-2 cells have been treated with 3′-methoxy-4′-nitroflavone, a potent AhR antagonist (Ebert et al., 2005).

One of the most interesting observations comes from the Western blot analysis of AhR-deficient MCF-7AHR200 cells. These cells expressed an extremely low basal level of BCRP and showed no response to treatment with AhR agonists. Moreover, transient transfection with an expression plasmid for human AhR could restore the expression of BCRP mRNA. Inhibition of the proteasome-mediated degradation of the AhR:ARNT (AhR nuclear translocator) complex by MG-132 led to a further induction of BCRP mRNA by ICZ, a phenomenon that has been described as superinduction (Ma and Baldwin, 1999). These results strongly support our hypothesis that BCRP is under transcriptional control of the AhR.

Homologs to the human BCRP have been discovered in other species, and protein sequence analysis of murine, rat, and porcine Bcrp/abcg2 have shown that they share a relatively high degree of amino acid identity with human BCRP, which is 81, 81, and 85%, respectively (Mao and Unadkat, 2005). Murine Bcrp1 is one of the best-characterized BCRP homologs, and substrate specificity seems to be similar to BCRP (Krishnamurthy and Schuetz, 2005). In contrast to our results obtained with human cells, murine Bcrp1 mRNA is not inducible by 3-methylcholanthrene, a prototypical AhR agonist and procarcinogen (Han and Sugiyama, 2006). The AhR is highly polymorphic, and the inducibility of AhR-regulated genes differs significantly between inbred mouse strains; however, since the mouse strain used by Han and Sugiyama (2006) (BALB/c) carries the high-affinity allele, Ahrab2 (Poland et al., 1987), this provides no explanation. Although human BCRP and murine Bcrp1 seem to share an overlap of substrate specificity, differences in tissue distribution have been reported for both. While the highest expression of BCRP mRNA is found in human placenta, mice show only a moderate level in placenta and have high levels of Bcrp1 in kidney (Allen et al., 1999). Therefore, a very important question for future investigations would be whether species-specific tissue distribution is accompanied by different regulatory mechanisms governing the expression of BCRP and whether PAHs would fail to induce Bcrp1 on the protein level, too.

In conclusion, the data presented in this study show for the first time that several anticarcinogenic phytochemicals, namely, quercetin, DBM, curcumin, and the synthetic antioxidant TBHQ, are able to induce BCRP mRNA and/or protein expression in human colon carcinoma cells, most likely mediated by AhR-dependent mechanisms as supported by the strong induction of BCRP protein by TCDD (50nM). Furthermore, the pretreatment with phytochemicals (quercetin and DBM) enhances the transport of BP3S, a sulfoconjugate of the food carcinogen BP, providing evidence that the induced protein is functionally active. However, an in vitro transport assay with cultured cells is a rather artificial system, though it helps to identify mechanisms in the complex system of procarcinogen detoxification. To further elucidate the role that BCRP plays in the toxicokinetic of PAHs in the intact organism, we are currently investigating the detoxification of BP in Bcrp1 knockout mice.

Nrf2-mediated mechanisms are most likely not involved in the regulation of BCRP, since monofunctional inducers (SUL and DEM) have no effect on BCRP expression. Moreover, a downregulation of BCRP could be observed after treatment of Caco-2 cells with (−)-epicatechin, probably based on an AhR-antagonistic effect. Since the intestinal mucosa is in direct contact to ingested food constituents including procarcinogens and phytochemicals, it therefore acts as a first-line barrier.
against potential harmful compounds. Because food-related cancers such as colon cancer are strongly associated with lifestyle factors including dietary habits (Martinez, 2005), the data presented in this study add to our understanding of how nonnutritive food constituents and additives such as TBHQ may act as chemopreventives/anticarcinogens.

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