Effect of (mixtures of) flavonoids on the *in vitro* and *in vivo* bioavailability of PhIP: a biologically based modelling approach Maaike E. Schutte

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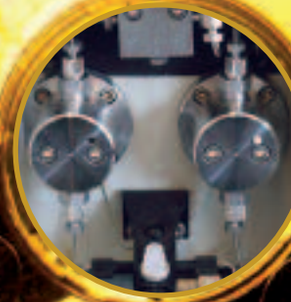

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Promotoren:	Prof. Dr. Ir. I.M.C.M. Rietjens Hoogleraar in de Toxicologie Wageningen Universiteit
	Prof. Dr. Ir. J.P. Groten Hoogleraar in de Combinatietoxicologie Wageningen Universiteit
Co-promotoren:	Dr. G.M. Alink Universitair Hoofddocent, Sectie Toxicologie Wageningen Universiteit
	Dr. Ir. J.J.M. van de Sandt Manager research chemical safety TNO Kwaliteit van Leven, Zeist
Promotiecommissie:	Prof. Dr. V.J. Feron Universiteit Utrecht
	Prof. Dr. F.G.M. Russel Radboud Universiteit Nijmegen
	Prof. Dr. Th. Walle Medical University of South Carolina, Charleston, USA
	Prof. Dr. R.F. Witkamp Wageningen Universiteit

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Author Maaike Evelien Schutte

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Abstract

The transport of food ingredients across the intestinal epithelium is an important factor determining the absorption upon oral intake. Uptake of compounds in the intestine may be influenced by transport proteins such as the ATP binding cassette transporters (ABC transporters). ABC transporters have been shown to be involved in the efflux of several food related compounds and in the efflux of xenobiotics. The pro-carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) was found to be transported by efflux transporters from the intestinal cells back into the intestinal lumen and in this way its uptake into the body could be prevented. Also it has been shown that flavonoids, present in fruits, vegetables, nuts, wine and tea are capable of inhibiting the efflux of substrates by ABC transporters. The aim of this thesis was to investigate the possible effect of flavonoids and mixtures thereof on the transport of PhIP across the intestinal barrier and to predict the absorption of PhIP through kinetically modelling. The results presented in this thesis show that different flavonoids are capable of increasing the apical to basolateral PhIP transport through Caco-2 monolayers, an *in vitro* model for the intestinal epithelium. It is also shown that the flavonoids can do so by acting as inhibitors of the apical ABC transporters in the intestinal cells. Using typical inhibitors for the ABC transporters experiments in Caco-2 monolayers revealed that especially MRP2 and BCRP are involved in the apical excretion of PhIP and these transporters can be inhibited by the flavonoids.

Furthermore, an *in silico* model describing this process taking into account passive diffusion and active transport of PhIP was developed. Using the *in silico* model it could be demonstrated that for several flavonoids, including flavone, kaempferol chrysoeriol, myricetin, luteolin, naringenin, quercetin and apigenin, their apparent Ki values for inhibition of the active transport to the apical side are in the 5 to 50 μM range and thus within the physiological concentration range that may be achieved within the intestine upon supplement intake.

Additional experiments revealed that several binary flavonoid mixtures and one mixture containing five model flavonoids increased the apical to basolateral PhIP transport through the Caco-2 monolayer. Assuming competitive inhibition of the apparent active transporter by the flavonoids and concentration-additivity for their inhibiting effect, the kinetic model could be extended and thus adequately described the experimental values obtained for the flavonoid mixtures. This illustrates that the effect of different flavonoids present in the diet is additive and

from this it can be concluded that the effects of flavonoids on PhIP bioavailability can even be expected at levels achieved upon normal dietary intake.

Finally, it was demonstrated that the observation that quercetin increases the transport of PhIP through Caco-2 monolayers *in vitro* could be confirmed in an *in vivo* rat model. Co-administration of PhIP and quercetin significantly increased the blood $AUC_{(0\text{-}8hr)}$ of PhIP in rats to 131±14% of the $AUC_{(0\text{-}8hr)}$ for rats dosed with PhIP alone.

Therefore, all together the studies presented in this thesis point to a flavonoid-mediated increase of the bioavailability of PhIP and, thus, a possible adverse effect of these supposed beneficial food ingredients when present in combination with the pro-carcinogen PhIP.

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General introduction, objectives and outline

Based on:
Flavonoid-mediated inhibition of intestinal ABC transporters may affect the oral bioavailability of drugs, food-borne toxic compounds and bioactive ingredients.

Walter Brand, Maaike E. Schutte, Gary Williamson, Jelmer J. van Zanden, Nicole H.P. Cnubben, John P. Groten, Peter J. van Bladeren and Ivonne M.C.M. Rietjens.

Biomedicine and Pharmacotherapy (2006) 60: 508-519

Introduction

The transport of ingested food ingredients across the intestinal barrier is an important factor determining bioavailability upon oral intake. This not only holds for biofunctional food ingredients but also for drugs and for toxic compounds. For small and generally lipophilic compounds the predominant route for intestinal absorption is through transcellular transport. Several mechanisms contribute to the ultimate efficiency of this transcellular transport (*1*). These include passive diffusion, facilitated diffusion, and active transport. Passive and facilitated diffusion occur along a concentration gradient. Both processes result in a net flux from the intestinal lumen at the apical side of the intestinal epithelial cells, through the cytoplasm of the intestinal cells, to the blood stream at the basolateral side of the intestinal cells. The role of active transport in the ultimate outcome of the transcellular transport is more complex. This is because i) the transporters involved are able to transport compounds against a concentration gradient, ii) they are located in either the apical or the basolateral membrane of the epithelial cells, and iii) some of these active transporters transport compounds into the intestinal cell, such as the sodium dependent glucose transporter (SGLT1) involved in active cellular uptake of for example certain flavonoid glycosides (*2-4*), but others including the ATP binding cassette (ABC) transporters, preferentially result in efflux of compounds and/or their metabolites from the intestinal epithelial cell. The overall absorption of a chemical, food ingredient, drug or toxic compound across the intestinal epithelium by transcellular transport can be largely dependent on the activity of these membrane bound transport proteins.

Objective of this thesis

ABC transporters are known to efficiently excrete physiological substrates and xenobiotics out of the cell. In the intestinal epithelium the ABC transporters P-glycoprotein, multidrug resistance protein 2 (MRP2; ABCC2), and breast cancer resistance protein (BCRP; ABCG2) (*5-9*) are localized in the apical membrane of the enterocyte, catalyzing the efflux of compounds to the intestinal luminal side opposing bioavailability. Localized in the basolateral membrane are the ABC transporters MRP1 (ABCC1), MRP3 (ABCC3) and MRP5 (ABCC5) catalyzing the efflux of compounds to the serosal side facilitating bioavailability (*10-14*).
One class of compounds for which it is shown that ABC transporters are involved in their intestinal transport are the heterocyclic amines such as 2-amino-1-methyl-

6-phenylimidazo[4,5-*b*]pyridine (PhIP). Studies using Caco-2 monolayers and typical transport inhibitors have shown that P-gp and MRP2 are likely to be involved in the excretion of PhIP from enterocytes back to the intestinal lumen (*15*). Studies in rodents showed that the bioavailability of PhIP is decreased in the presence of the ABC transporters BCRP (*16*) and MRP2 (*17, 18*).
Research regarding multidrug resistance showed that flavonoids are potent inhibitors of the ABC transporters P-gp, MRP1, MRP2, MRP4, MRP5 and BCRP (*19-24*).
The general objective of this thesis was to obtain a better understanding of the cellular mechanisms underlying the changes in intestinal absorption of toxic xenobiotics due to the presence of (mixtures of) food components. Since ABC transporters are involved in the efflux of xenobiotics like PhIP and flavonoids are capable of modulating the ABC transporter activity, the specific aim of this thesis was to investigate the possible interaction of flavonoids and mixtures thereof on the absorption of PhIP. Since the human diet consists of multiple food constituents and humans are exposed to mixtures of compounds, rather than to single compounds also the effect of mixtures of flavonoids on ABC transporter mediated PhIP transport was studied.
Flavonoids are bioactive food ingredients and are present in fruits and vegetables and may protect against cancer and cardiovascular diseases (reviewed in (*25*)). PhIP is formed during the heat processing of meat and is a suspected human carcinogen (*26, 27*). In the next sections an overview is provided of the various topics essential for the studies presented in this thesis including the ABC transporters, their inhibition by flavonoids, and the overall characteristics of the group of heterocyclic amines of which the model carcinogen of the present study PhIP is an important representative.

ATP binding cassette (ABC) transporters

In 1992 the name ABC transporter was introduced by Chris Higgins (*28*). The designation ABC was based on the highly conserved ATP binding cassette, the most characteristic feature of this superfamily.
ABC transporters are involved in several biological processes in humans and animals such as the excretion of endogenous metabolites, prevention of uptake of toxic compounds and protecting vital structures in the body including the brain, the placenta and the testis. They have been shown to play a role in some human

diseases caused by the absence of ABC transporters such as the inborn disease Dubin-Johnson syndrome which is caused by genetic defects in MRP2 (*29*). In addition over-expression of ABC transporters in tumors may lead to multidrug resistance of cancers (reviewed in (*30*)).
This thesis focuses especially on the ABC transporters involved in the efflux of compounds from the intestinal cells either to the basolateral blood side, facilitating absorption, or back into the intestinal lumen, reducing bioavailability. Table 1 presents an overview of the various ABC transporters and their major characteristics.

Active transport proteins involved in efflux of chemicals

The intestinal ABC transporters involved in the efflux of chemicals from the intestinal cells include P-glycoprotein (P-gp/MDR1/ABCB1), multidrug resistance proteins (MRPs/ABCCs) and breast cancer resistance protein (BCRP/ABCG2/ABCP/MXR) (*5, 13, 14, 31*) (Table 1). Most of these transporters are located specifically in the apical (intestinal luminal side) or basolateral (blood/plasma side) membrane of the enterocytes (Figure 1). P-gp/MDR1, MRP2 (ABCC2) and BCRP are localized in the apical membrane (*5, 6, 8*), whereas MRP1 (ABCC1), MRP3 (ABCC3) and MRP5 (ABCC5) are localized in the basolateral membrane of the intestinal enterocytes (*10-13*). Little is known about MRP4 (ABCC4) which could be located in the apical as well as in the basolateral membrane of the intestinal cells (*5, 7, 9*). Finally, MRP6 (ABCC6) seems to be located on the basolateral side (*32, 33*) although it may only be expressed in the mucosal cells of the intestine (*34*). More MRP homologues have been defined (*35-37*), but their function and location of expression is still uncertain.
Examples of the involvement of the transporters in the bioavailability of drugs and bioactive food ingredients have been published using various methodologies. A much appreciated *in vitro* model is the two-compartment cell culture system in which monolayers of epithelial cells (Caco-2, HCT8, MDCK) (*38*) are cultured (Figure 2) (*39*). Some other studies provide *in vivo* data supporting a role for the ABC transporters in bioavailability.

Table 1 Relevant intestinal ABC transporters and literature describing their characteristics.

Transporter	Tissues	Physiological substrates	References
P-gp (MDR1) (ABCB1)	Adrenal gland, blood-tissue barriers, brain, choroid plexus, epithelia, heart, intestine, kidney, liver, lung, ovary, placenta, prostate, skeletal muscle spleen, stomach.	Amphipathic drugs, neutral and positive charged hydrophobic compounds.	(*5, 8, 30, 53, 138*)
MRP1 (ABCC1)	Blood cerebral spinal fluid barrier, intestine, kidney, liver, lung, peripheral blood mononuclear cells, testis.	Anionic drug conjugates, leukotriene C_4, GSH, oxidized GSH (GSSG), many glutathione, glucuronate and sulfate conjugated organic anions.	(*5, 30, 53, 138-140*)
MRP2 (ABCC2)	Brain, intestine, kidney, liver, lung, placenta.	Acidic bile salts, amphipathic organic anions and xenobiotics, anionic drug-conjugates, bilirubin-glucuronides, GSSG, GSH, leukotriene C_4, relative hydrophilic compounds, sulfate conjugates of endogenous and exogenous compounds.	(*5, 8, 30, 53, 138-140*)
MRP3 (ABCC3)	Adrenal cortex, colon, intestine, kidney, liver, lung, pancreas, placenta, prostate, spleen.	Bile salts, endogenous organic anions.	(*5, 30, 53, 139*)
MRP4 (ABCC4)	Adrenal gland, bladder, brain, gall bladder, kidney, liver, lung, ovary, pancreas prostate, skeletal muscle, small intestine, spleen, testis, thymus, tonsil.	cGMP, cAMP, conjugated steroids, bile acids, folic acid, folinic acid, estradiol-17-β-glucuronide.	(*5, 30, 53, 139, 141, 142*)
MRP5 (ABCC5)	Brain, erythrocyte, heart, intestine, kidney, liver, lung, skeletal muscle, testis.	cAMP, cGMP, folate, GSH, organic anions.	(*5, 30, 53, 139-141, 143*)
BCRP (ABCG2) (ABCP) (MXR)	Breast, heart skeletal muscle, intestine, kidney, liver endothelium, lung, ovary, pancreas, placenta, spleen, thymus.	Amphipathic drugs, conjugated organic anions, organic anions, relative hydrophilic anticancer-agents, sulfate-, glucuronide- and gluthatione conjugates of many endogenous and exogenous compounds, weak bases.	(*5, 8, 30, 53, 138, 139, 144-147*)

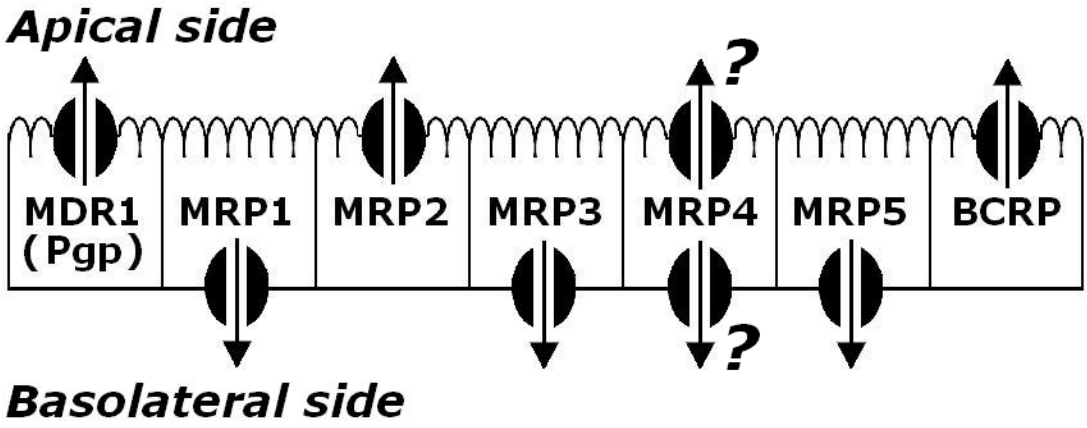


Figure 1 Cellular localization of intestinal ABC transporters. P-glycoprotein (P-gp/MDR1/ABCB1), multidrug resistance proteins MRP2 (ABCC2) and breast cancer resistance protein (BCRP/ABCG2/ABCP) are localized in apical membranes (*5, 6, 8*). MRP1 (ABCC1), MRP3 (ABCC3) and MRP5 (ABCC5) are localized in basolateral membranes of enterocytes (*10-13*). MRP4 (ABCC4) has been suggested to be located in the apical as well as in the basolateral membrane of the intestine (*5, 7, 9*).

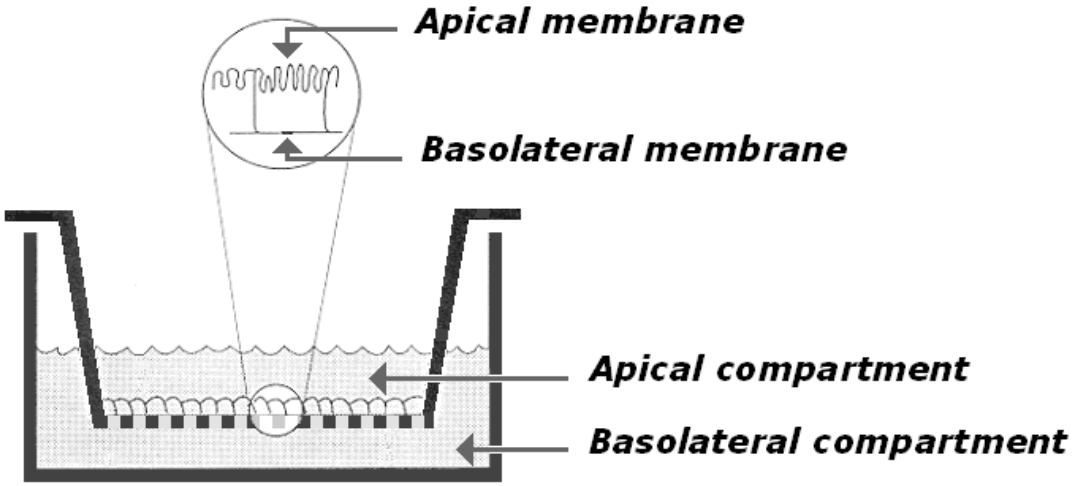


Figure 2 The two-compartment cell culture system consists of a permeable cell culture filter insert that is placed in a well of a normal cell culture plate. The cells are seeded on the filter inserts and are cultured to cover the whole surface area. Caco-2 cells are known to display morphological and biochemical characteristics of human enterocytes after differentiation and form a layer of polarized intestinal cells (*182*). The two compartments are designated the apical (luminal) compartment and the basolateral (serosal) compartment. Several advantages of this system include (*183*): i) that only small amounts of the compound suffice to perform a transport or effect experiment, ii) the experiments are relatively rapid and reproducible compared to other absorption models, iii) and real intestinal epithelial permeation rates can be determined (without gastrointestinal degradation, hepatic metabolism or complicating whole body kinetics).

Transport by ATP dependent transporters has been well recognized as a determinant of drug absorption from the gastrointestinal tract (*40, 41*). Drug administration to *P-gp* knock-out mice demonstrated for example a role for P-gp in reducing the oral bioavailability of several drugs that are known substrates of P-gp, including cyclosporin, digoxin, quinidine, talinolol, vinblastine and HIV protease inhibitors (*40, 41*). Studies in humans indicated a role for intestinal P-gp in limiting the intestinal uptake of cyclosporin (*42*). Furthermore, the reduced oral bioavailability of digoxin upon combination therapy with rifampicin has now been ascribed to rifampicin-mediated induction of P-gp (*40, 43*)
Efficient transport of saquinavir, ritonavir and indinavir by *MRP2* transfected Madin-Darby Canine Kidney (MDCK)II cells, compared to MDCKII cells over-expressing other ABC transporters, indicated an important role for MRP2 in the efflux of these HIV protease inhibitors, thereby presumably limiting their oral bioavailability (*44*). Merino *et al.* (*45*) demonstrated a two-fold increase in the plasma concentration of the fluoroquinolone antibiotic ciprofloxacin after oral administration to Bcrp1 (the murine homologue of human BCRP) deficient *Bcrp1-(-/-)* mice compared to wild-type mice, which suggests that Bcrp1 restricts the oral bioavailability of ciprofloxacin. Also, the oral bioavailability of the NMDA receptor antagonist GV196771 (*46*), and topotecan (*47*), seems affected by Bcrp1 and is increased in *P-gp* knockout mice by GF120918, an inhibitor of both P-gp and Bcrp1 (*48*). In humans co-administration with GF120918, in this case inhibiting BCRP as well as P-gp, increases the bioavailability of oral topotecan as well (*49*).

In addition to studies on the role of ABC transporters in the oral bioavailability of drugs, more recent studies also focus on a role for the ABC transporters in determining the bioavailability of food ingredients, including toxic and bioactive compounds.
In a study using Caco-2 cells it was demonstrated that an apical ABC transporter (not P-gp or MRP2) might be involved in luminal excretion of polar metabolites of the polycyclic aromatic hydrocarbon (PAH) benzo[*a*]pyrene formed by CYP1A1 or CYP1B1 inside the Caco-2 cells (*50*). The authors suggested that this active transport of the intestinal benzo[*a*]pyrene metabolites indicates a biochemical barrier function against potential mutagenic compounds through metabolism and luminally-directed transport. In subsequent studies, Ebert *et al.* (*51*) identified

BCRP as an important transporter of benzo[*a*]pyrene conjugates metabolically formed in Caco-2 cells.
Schrickx *et al.* (*52*) showed that the mycotoxin ochratoxin A is a substrate for MRP2 and BCRP but not for P-gp in Caco-2 monolayers and thereby limiting the efflux to the apical compartment of this mycotoxin.

Given the wide substrate selectivity of the intestinal ABC transporters, an involvement of these transporters on the bioavailability of not only a wide number of drugs, but also of many bioactive food ingredients and/or toxic compounds, is likely and has been demonstrated. Although the substrate specificities of P-gp, BCRP and the MRPs are overlapping, they also differ markedly (Table 1). P-gp has a very broad spectrum of substrates including *e.g.* many anticancer drugs, cardiac drugs, immunosuppressants and antibiotics (*5, 53*). Most P-gp substrates are hydrophobic, neutral or mildly positive lipophilic compounds with a planar structure (*54*), whereas MRPs are able to transport lipophilic anions. MRP1 transports anionic conjugates of lipophilic compounds including glutathione (GSH), glucuronide and sulphate conjugates (*55*), but also some cations and neutral compounds using GSH as a co-factor (*56, 57*). MRP1 substrates also include lipid peroxidation products, herbicides, tobacco specific nitrosamines, mycotoxins, heavy metals, natural product and antifolate anticancer agents (*20*). MRP2 and the other MRPs share similar but not identical substrate specificity with MRP1 (*5*). The importance of MRP4 and MRP5 as drug transporters and their role in intestinal drug disposition is unclear at present. BCRP seems to have an overlapping substrate specificity with P-gp, and like P-gp, substrates for BCRP include many anticancer agents like daunorubicine, doxorubicine, mitoxantrone and topotecan (*5, 47, 53*).

Flavonoids

Flavonoids (Figure 3) consist of a large group of polyphenolic antioxidants found in fruits, vegetables and plant-derived beverages such as tea and red wine (*58*), as well as in dietary supplements. In foods, flavonoids are often present as β-glycosides of aglycones and methylated derivatives. Flavonoids and flavonoid-rich extracts have been implicated as beneficial agents in a multitude of disease states (*59-61*), including cancer (*62-64*), cardiovascular diseases (*65-67*), neurodegenerative disorders (*68-70*) and osteoporosis (*71*).

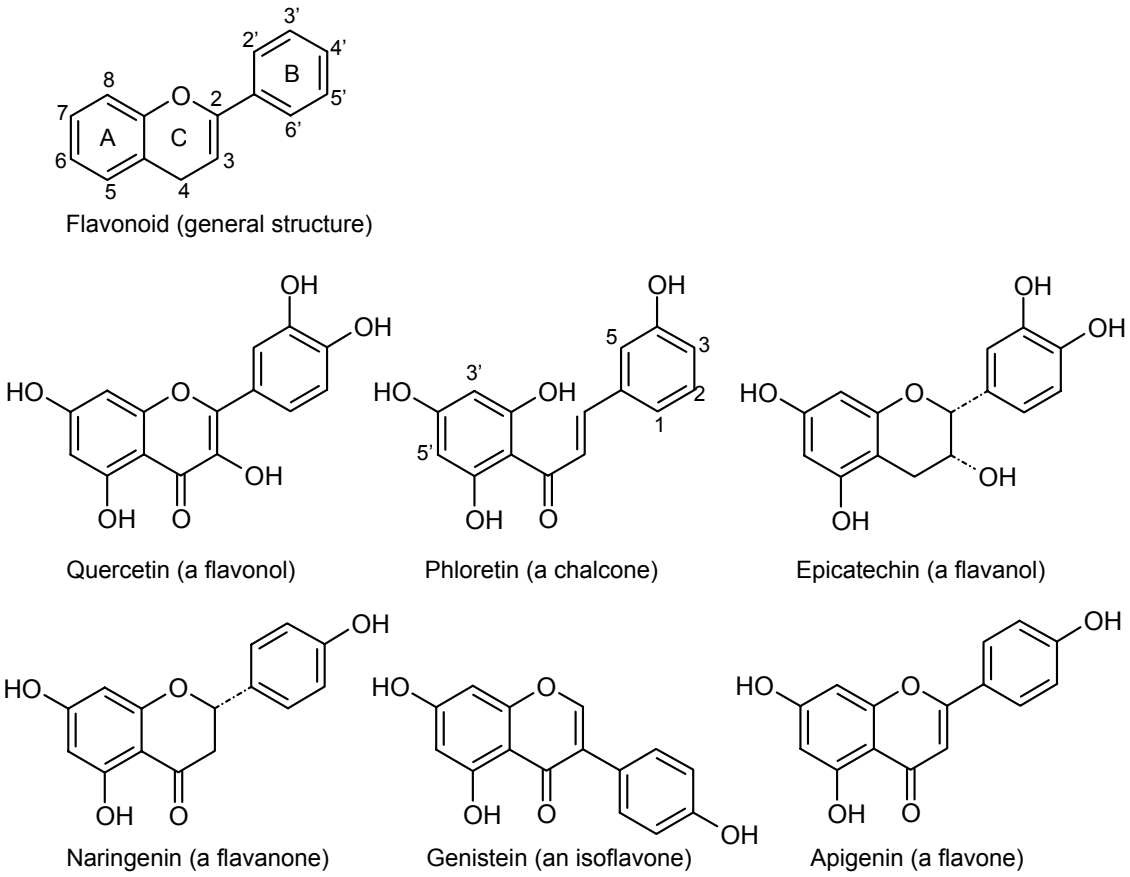


Figure 3 Structural formula of examples of dietary flavonoids representing the major classes of flavonoids (chalcones, flavanols, flavanones, flavones, flavonols and isoflavones).

Several studies have investigated the bioavailability of flavonoids present in the human diet. E.g. Hollman *et al.*(*4*) showed in ileostomy patients that the absorption of orally administered quercetin aglycon was approximately 24% and the absorption of the quercetin glycosides from onions was 52%, suggesting that the presence of a glycoside moiety enhances absorption. In another study Hollman *et al.* (*72*) measured plasma levels of quercetin in healthy volunteers who were fed a single dose of onions, apples or quercetine-3-rutinoside as a pure compound. The bioavailability of quercetin from the apples and the pure quercetin-3-rutinoside was only 30% compared with onions.

Walle *et al.* (*73*) showed that quercetin-4'-glucoside and other quercetin glucosides are hydrolyzed to the quercetin aglycon in the human oral cavity by both bacteria and enzymes from the cytosol of damaged epithelial cells. This results in the presence of the quercetin aglycon at the surface of the oral epithelial cells.

Crespy *et al.* (*74*) demonstrated in Wistar rats, that while approximately 38% of quercetin was absorbed in the stomach, the quercetin glycosides rutin and isoquercitrin were not absorbed in the stomach.
In the small intestine flavonoid glycosides can be deglycosylated by β-glucosidases including lactase phlorizin hydrolase which has specific activity towards flavonoid glycosides (*75, 76*). The resulting flavonoid aglycon can be absorbed via passive diffusion through the enterocytes.
Furthermore, the sodium-dependent glucose transporter-1 (SGLT-1) might be involved in the absorption of flavonoid glycosides. Studies using rat jejunum placed in Ussing chambers showed that SGLT-1 is involved in the uptake of quercetin-4'-glucoside and quercetin-3-O-glucoside (*2, 77*). The flavonoid aglycones are metabolized in the enterocytes into glucuronide-, sulphate- and methylated conjugates (*78-82*).
The absorption of flavonoids in the intestine may be limited by the transport of flavonoids or their metabolites back to the intestinal lumen by ABC transporters. Results from a study with Caco-2 cell monolayers and the MRP-associated transport inhibitor MK-571 suggest that MRP2 plays a role in the transport of the flavonoid genistein-7-glucoside from intestinal cells back into the intestinal lumen, thereby limiting its bioavailability (*83*).
Using MRP2 deficient rats and *in situ* intestinal perfusion, as well as the specific Bcrp1 inhibitor fumitremorgin C (FTC) and MDCKII cells transfected with either human *MRP2* or murine *Bcrp1*, it was demonstrated that especially Bcrp1 and not MRP2 limits the net intestinal absorption of the flavonoid quercetin. This because of an efficient efflux of quercetin by *Bcrp1*-transfected MDCKII cells compared to control and *MRP2*-transfected cells, and of quercetin glucuronide metabolites from intestinal cells of *MRP2* deficient rats back into the intestinal lumen, which could be inhibited by the Bcrp1 inhibitor FTC (*84*).
Using the typical P-gp inhibitor verapamil and Bcap37/MDR1 cells which are transfected with a P-gp gene construct Wang *et al.* (*85*) demonstrated that the flavonoids quercetin, kaempferol and isorhamnetin from *Ginkgo biloba* leaves were substrates for P-gp and that P-gp-mediated efflux of these flavonols might limit their bioavailability.
A similar role for MRP2 in the bioavailability of epicatechin and possible other tea flavonoids is suggested by Vaidyanathan and Walle (*86*) based on studies with Caco-2 cells and the MRP-inhibitor MK-571.

Effect of flavonoids as ABC transporter inhibitors on multidrug resistance and bioavailability of bioactive chemicals

Table 2 presents an overview of dietary flavonoids known to inhibit the activity of the various ABC transport proteins. It has become clear that flavonoids or their metabolites are important modulators or substrates of intestinal membrane bound transport proteins including P-gp, MRPs and BCRP (Figure 4). Their properties to modulate ABC transport proteins and multidrug resistance make them interesting therapeutic candidates (*87*).

Table 2 Overview of selected literature on inhibition of ABC transporters present in the intestine by dietary flavonoids.

Transporter	Flavonoid inhibitor	References
P-gp (MDR1)	biochanin A, epigallocatechin, epicatechin-gallate, epigallocatechin-gallate, hesperetin, isoquercitrin, kaempferol, morin, naringenin, phloretin, quercetin.	(*97, 148-154*)
MRP1	apigenin, baicalein, biochanin A, chalcone, galangin, genistein, hesperetin, kaempferol, luteolin, morin, myricetin, naringenin, phloretin, quercetin, robinetin.	(*22, 23, 91, 155-157*)
MRP2	myricetin, quercetin-4'-glucoside, robinetin.	(*22, 91, 153*)
MRP3	-	
MRP4	daidzin, hesperetin, naringenin, quercetin, resveratrol.	(*23*)
MRP5	daidzin, hesperetin, naringenin, quercetin.	(*23*)
BCRP	acacetin, apigenin, biochanin A, chalcone, chrysin, daidzein, diosmetin, fisetin, flavone, galangin, genistein, hesperetin, kaempferide, kaempferol, luteolin, luteolin-4'-*O*-glucoside, 7-methoxyflavanone, naringenin, naringenin-7-glucoside, phloretin, quercetin, theaflavine, theaflavine-3-*O*-gallate.	(*19, 24, 88, 89, 158-160*)

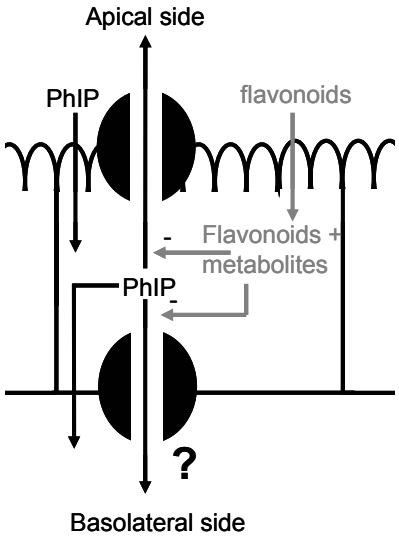


Figure 4 Schematic presentation of the role of flavonoids, or their metabolites formed within intestinal cells, as substrates or inhibitors of apical intestinal ABC transport proteins (P-gp, MRP2 and BCRP) and their possible effect on PhIP bioavailability.

Given the involvement of the transport proteins in the efficiency of intestinal transport, it can be envisaged that the ABC transport inhibitors may not only affect multidrug resistance of tumor cells, but may also affect the bioavailability of a variety of drugs, bioactive food ingredients and/or toxic compounds upon oral uptake. Some examples of such a role for flavonoids have been described in recent literature, including *in vitro* studies on the effect of flavonoids on intracellular accumulation of substrates for ABC transporters using for example BCRP over-expressing MCF-7 MX100 cells (*24, 88, 89*), and *MRP1*- or *MRP2*-transfected MDCKII cells (*22, 90, 91*). Table 3 presents an overview of studies reporting an effect of dietary flavonoids on intracellular accumulation and/or bioavailability of drugs, or food-borne toxins and bioactive ingredients. Some of the studies are discussed in more detail hereafter. For instance, in *MRP1*- and *MRP2*-transfected MDCKII cells, it was shown that myricetin can inhibit cellular vincristine efflux by MRP1 and MRP2 thereby sensitizing the cells towards vincristine (*91*). Further it has been shown that quercetin and 3',4',7-trimethoxyquercetin, but not rutin the naturally occuring 3-rhamosylglucoside of quercetin were able to potentiate the effects of adriamycin on a multidrug resistant MCF-7 human breast cancer cell line, in which the MDR was associated to high levels of P-gp (*92*). Di Pietro *et al.* (*93*) demonstrated the effect of various flavonoids on the intracellular accumulation of daunomycin in P-gp over-expressing K562/R7 cells, concluding that prenylation of

the flavonoids increases both their affinity for P-gp and their effect on the duanomycin accumulation.

In addition to these studies showing the effect of flavonoids on intracellular accumulation of chemicals, several other studies have investigated the effect of flavonoids on the intestinal transport of compounds using Caco-2 cells in transwell dishes as an *in vitro* model for the human intestinal barrier (Table 3). Last, but not least, some studies have shown this effect of flavonoids in *in vivo* models including rats, and healthy volunteers.

In Caco-2 cell monolayers, naringin was reported to increase the apical to basolateral transport of the HIV protease inhibitor saquinavir by inhibition of the P-gp mediated apical saquinavir efflux (*94*). Furthermore, a significant increase in apical to basolateral transport as well as cellular accumulation, of ochratoxin A, a food-borne mycotoxin, in Caco-2 cells, was observed upon co-incubation with chrysin, quercetin, genistein, biochanin A or resveratrol, all at concentrations that can be expected in the gastrointestinal tract (*95*). The authors hypothesise that the polyphenols may exert their effect though competitive inhibition of the MRP efflux pump involved, previously proposed to be MRP2 (*96*). In another study with Caco-2 cells, the apical to basolateral transport of digoxin was significantly increased, whereas the basolateral to apical transport was significantly decreased by biochanin A or silymarin due to inhibition of the P-gp-mediated transport of digoxin by these flavonoids (*97*). This suggests that these flavonoids could increase the absorption and bioavailability of co-administered drugs that are P-gp substrates. Furthermore, botanical ingredients other than flavonoids may result in altered absorption and bioavailability of drugs that are ABC transporter substrates because of their interaction with the ABC transport protein (*98, 99*).

Zhang *et al.* (*89*) was one of the first to study the effect of flavonoid mixtures on mitoxantrone accumulation in MCF-7 MX100 cells. They observed that the flavonoids apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, naringenin, and silymarin act in an additive way in studies characterising the inhibition of the BCRP-mediated efflux of mitoxantrone by mixtures of 2, 3, 5 or 8 flavonoids. Since the human diet consists of multiple flavonoids and humans are thus exposed to mixtures of flavonoids rather than to a single flavonoid it is of interest to study the effect of low levels of flavonoid mixtures on the inhibition of ABC transporters. However, up till now the effect of mixtures of flavonoids has not been studied extensively.

Table 3 Overview of literature on the effect of dietary flavonoids on cellular accumulation, transport or bioavailability of drugs and other xenobiotics in different model systems.

Flavonoids	Model system	Compounds of which cellular accumulation, transport or bioavailability is increased	References
	Cell models		
genistein	BC19/3, MCF-7, MCF-7 VP, BALB/c-3T3, BALB/c-3T3-1000	daunorubicin, rhodamine-123	(*161*)
biochanin A	Caco-2	digoxin, vinblastine	(*97*)
heptamethoxy-flavone, nobiletin, tangeretin	Caco-2	vinblastine	(*162*)
catechin-gallate, epigallocatechin-gallate, epicatechin-gallate	Caco-2, $CH^{R}C5$	rhodamine-123, vinblastine	(*163*)
genistein	GLC4, GLC4 ADR	daunorubicin	(*164*)
quercetin, theaflavine, theaflavine-3-*O*-gallate	HEK-293/*R482-ABCG2*	SN-38	(*160*)
apigenin, galengin, genistein, naringenin	HEK-293/*R482-ABCG2*, HEK-293/*T482-ABCG2*, MDA-MB-231, MDA-MB-231/*T482-ABCG2*	mitoxantrone, rhodamine-6G	(*158*)
kaempferol, naringin	HK-2	calcium-AM, cyclosporine A, vinblastine	(*148*)
acacetin, apigenin, chrysin, diosmetin, genistein, kaempferide, kaempferol, luteolin, luteolin-4'-*O*-glucoside, naringenin, naringenin-7-glucoside	K562, K562/*BCRP*	mitoxantrone, SN-38, topotecan	(*19*)
baicalein, epicatechin-gallate, epigallocatechin, epigallocatechin-gallate, fisetin, kaempferol, morin, myricetin, quercetin	KB-3-1, KB-C2	daunorubicin, rhodamine-123	(*150, 151*)
daidzein, genistein, kaempferol, quercetin	KB-3-1, KB-V1	paclitaxel, rhodamine-123, vinblastine	(*166*)

Table 3 continued

Flavonoids	Model system	Compounds of which cellular accumulation, transport or bioavailability is increased	References
	Cell models		
quercetin	MCF-7, MCF-7 ADM	doxorubicin	(*92*)
biochanin A, morin, naringenin, quercetin	MCF-7, MCF-7 ADR	daunomycin	(*149*)
biochanin A, chalcone, chrysin, genistein, morin, phloretin, quercetin	MCF-7, MCF-7 ADR	daunomycin	(*167*)
chrysin	MCF-7, MCF-7 MX100	topotecan	(*88*)
apigenin, biochanin A, chrysin, genistein, hesperetin, kaempferol, naringenin	MCF-7, MCF-7 MX100	mitoxantrone	(*89*)
biochanin A, morin, phloretin	MCF-7, MCF-7 ADR, MDA435/LCC6, MDA435/LCC6*MDR*	daunomycin, doxorubicin	(*154*)
apigenin, biochanin A, chrysin, fisetin, genistein, hesperetin, kaempferol, luteolin, naringenin, phloretin, quercetin	MCF-7, MCF-7 MX100, NCI-H460, NCI-H460 MX20	mitoxantrone	(*24*)
biochanin A, daidzein, formononetin, genistein	HT-29, MDCKII/*P-gp*, MDCKII/*MRP1*, MDCKII/*MRP2*	(-)-epigallocatechin-3-gallate	(*90*)
chrysin	MDCKII-*Bcrp1* or MDCKII-BCRP	nitrofurantoin	(*168*)
myricetin	MDCKII, MDCKII/*MRP1*, MDCKII/*MRP2*	vincristine	(*22*)
biochanin A, chalcone, chrysin, epigallocatechin, genistein, kaempferol, morin, phloretin, quercetin	Panc-1	daunomycin, vinblastine	(*157*)
galangin, kaempferol, quercetin	rat hepatocytes	rhodamine-123	(169)

Table 3 continued

Flavonoids	Model system	Compounds of which transport or bioavailability is increased	References
	Transport models		
biochanin A	Caco-2, AP to BL	digoxin, vinblastine	(*97*)
biochanin A, chrysin, genistein, quercetin, resveratrol	Caco-2, AP to BL	ochratoxin A	(*95*)
6',7'-dihydroxy-bergamottin, naringin	Caco-2, AP to BL	saquinavir	(*94*)
hesperetin, quercetin, kaempferol, naringin	Caco-2, AP to BL	talinolol	(*152*)
epigallocatechin-gallate	Caco-2, AP to BL	vinblastine	(*163*)
naringenin, naringin	Caco-2, AP to BL	vinblastine	(*162*)
epicatechin-gallate, epigallocatechin-gallate, genistein, genistin, naringenin, quercetin, xanthohumol	Caco-2, MDCKII, MDCKII/*P-gp*, AP to BL	cycolosporin A, digoxin	(*170*)
quercetin	rat everted gut sac	etoposide	(*171*)
flavonoid containing grapefruit juice and orange juice	rat everted gut sac	fexofenadine, rhodamine-123, saquinavir	(*172*)
	In vivo models		
morin, naringin	male SD-rats	diltiazem	(*173, 174*)
flavone, quercetin, morin	male SD-rats	paclitaxel	(*100, 101*), (*175*)
flavonoid containing grapefruit juice	male SD-rats	talinolol	(*176, 177*)
chrysin	female SD-rats	nitrofurantoin	(*168*)
biochanin A	rats	paclitaxel and digoxin	(*178*)
genistein	sheep	enrofloxacin	(*179*)
flavonoid containing grapefruit juice	healthy volunteers	cyclosporine	(*180*)
flavonoid containing grapefruit juice and orange juice	healthy volunteers	Dextro-methorphan	(*181*)

The relative simplicity of *in vitro* models makes them very suitable for screening purposes and mechanistic research. It is however, important to emphasize the importance of confirmation of results obtained *in vitro* using *in vivo* models. Only a few studies actually demonstrate a role for flavonoids as ABC transporter inhibitors or substrates leading to modulation of the *in vivo* bioavailability of other bioactive ingredients (Table 3). Zhang *et al.* (*88*), for example, used Sprague-Dawley (SD) rats and *mdr1a/1b(-/-)* mice to investigate the bioavailability of topotecan in the presence and absence of the flavonoids chrysin or 7,8-benzoflavone. Neither chrysin nor 7,8-benzoflavone altered topotecan bioavailability in rats or in *mdr1a/1b(-/-)* mice after oral co-administration. The authors indicate that this might be due to the fact that the two flavonoids are only weak inhibitors of mouse or rat Bcrp1-mediated topotecan transport. This suggestion was based on the observation that chrysin and 7,8-benzoflavone inhibited the human BCRP mediated transport of topotecan in human BCRP over-expressing MCF-7 MX100 cells to a level comparable to that observed for the potent BCRP inhibitor FTC, but not in MDCK/Bcrp-1 cells over-expressing mouse Bcrp-1 (*88*).

In the same study, co-administration of topotecan with GF120918, a potent BCRP and P-gp inhibitor (*48*), to the SD rats or *mdr1a/1b(-/-)* mice appeared to significantly increase the bioavailability of topotecan by more than 4-fold, indicating the possibility to increase oral bioavailability *in vivo* by inhibitors of ABC transport proteins.

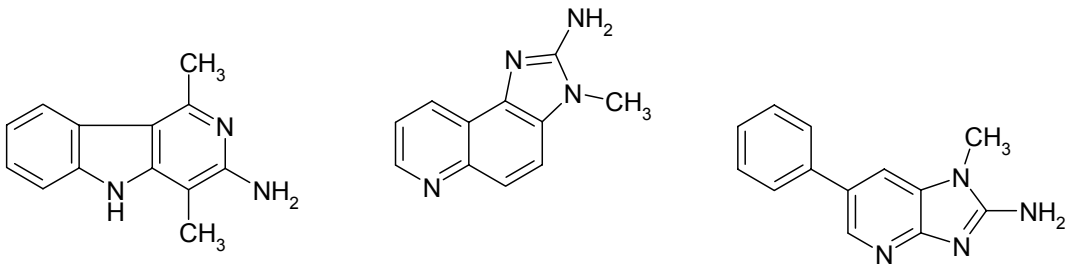
Choi *et al.* (*100*) showed that the pre-treatment with quercetin in male SD rats significantly increased the bioavailability of paclitaxel or its water soluble prodrug. Furthermore, co-administration with flavone resulted in a significant increase of the bioavailability of this drug and the authors suggest that this might be caused by the inhibition of P-gp or CYP3A (*101*).

These examples clearly illustrate the importance of *in vivo* validation of *in vitro* data on the effect of ABC transporter inhibitors on bioavailability. This validation is also important because of possible consequences of the extensive *in vivo* phase II metabolism of the flavonoids for their ultimate activity as ABC transporter inhibitors and/or substrates.

Heterocyclic aromatic amines

In 1939 Widmark (*102*) reported that carcinogenic compounds were present in food after he had applied extracts of grilled horse meat on the back of mice which induced tumors in the mammary glands of the mice. In 1977 Nagao *et al.* (*103*) were one of the first to report high mutagenic activity in cooked foods as tested in the Ames test. Since then around twenty mutagens/carcinogens, known as heterocyclic amines have been identified in cooked foods.

The identified heterocyclic amines can be divided in two different classes based on the temperatures at which they are formed. At cooking temperatures below 300 ^{o}C the most common class of heterocyclic amines, the amino-imidazoazaarenes (IQ-like heterocyclic amines), are formed (*104*), whereas at temperatures above 300 ^{o}C pyrolysis of proteins occurs and pyrido-imidazoles or indoles (non-IQ-type hetercocyclic amines) are formed (*105, 106*). Figure 5, depicts examples of the two classes of heterocyclic amines, the pyrolytic mutagen 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) which is a non-IQ-type heterocyclic amine and the imidazoquinoline 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ) and PhIP which are IQ-type heterocyclic amines.



TrP-P-1 (non-IQ-type heterocyclic amine) | IQ (IQ-type heterocyclic amine) | PhIP (IQ-type heterocyclic amine)

Figure 5 Structural formula of examples of heterocyclic amines representing the major classes of heterocyclic amines (IQ-type and non-IQ type).

Heterocyclic amines are formed during the heating of meat and fish and are probably formed from creatinine and Maillard reaction products as shown in experiments using model systems (*107*). The most important factors affecting the formation of heterocyclic amines are cooking temperature, cooking time, cooking method and the content of precursors and inhibitors present in the food (reviewed in (*108*)).

In 1986 Felton *et al.* (*27*) isolated 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) from fried beef. PhIP is formed from the reaction of its precursors phenylalanine (*109*), leucine, isoleucine or tyrosine (*110, 111*) with creatine (which is a precursor of creatinine) at cooking temperatures below 300 ^{o}C. The amount of PhIP formed during the frying of meat at temperatures below 300 ^{o}C is approximately 16.4 ng/g meat. From the amount of heterocyclic amines formed in meat during laboratory experiments and the daily intake of meat as estimated from food frequency questionnaires the human exposure to heterocyclic amines was estimated to be between 0.1-10 µg HCA/person/day (*112, 113*)
Determination of HCAs in fried beef showed that 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-dimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) and PhIP account for approximately 60% of the mutagenicity of fried beef (*114*).

Metabolism and bioactivation of heterocyclic amines

Heterocyclic amines are pro-carcinogens and/or pre-mutagens which after metabolic activation can act as carcinogens. PhIP was found to be mutagenic after metabolic activation with S9 mix in the Ames test (*115, 116*) and carcinogenic in rodents (*117*) and non human primates (*118*). Most HCA are mutagenic in the Ames test but are only weakly positive in mammalian cell based mutagenicity assays. PhIP, however, which is more abundantly present in fried meat, is a potent inducer of DNA damage in mammalian cells (*119, 120*) and a mutagen *in vivo* (*121*).
Ohgaki *et al.* (*122*) showed that ten different HCA, including PhIP, are carcinogenic in rats and mice. The target organs for HCA induced tumours are the liver, and in CDF_1 mice lung, forestomach, lymphomas, and blood vessel tumors in the brown adipose tissue, whereas in F344 rats, the Zymbal gland, skin, clitoral gland, small and large intestine, oral cavity and mammary gland were also target organs (*122*).
Figure 6 depicts a schematic overview of the metabolic pathways for PhIP bioactivation. In rodents PhIP is C- and N-oxidized by cytochrome P450 1A2. The C-oxidation leads to detoxification whereas the oxidation of the exocyclic amine group leads to the formation of the genotoxic N-hydroxy-PhIP metabolite. The N-hydroxy-PhIP-metabolite may bind with the DNA or it may be further metabolized by phase II enzymes including N-acetyltransferase or sulfotransferases which form

unstable esters that readily react with DNA (123, 124). After heterolytic cleavage of the N-acetoxy-PhIP metabolite the nitrenium ion is formed which can bind directly to the DNA bases, which results in DNA adducts (*127*).
The N-hydroxy-PhIP metabolite may also react with GSH, either chemically or catalyzed by GST which leads to its chemical reduction back to the parent amine (*125, 126*).

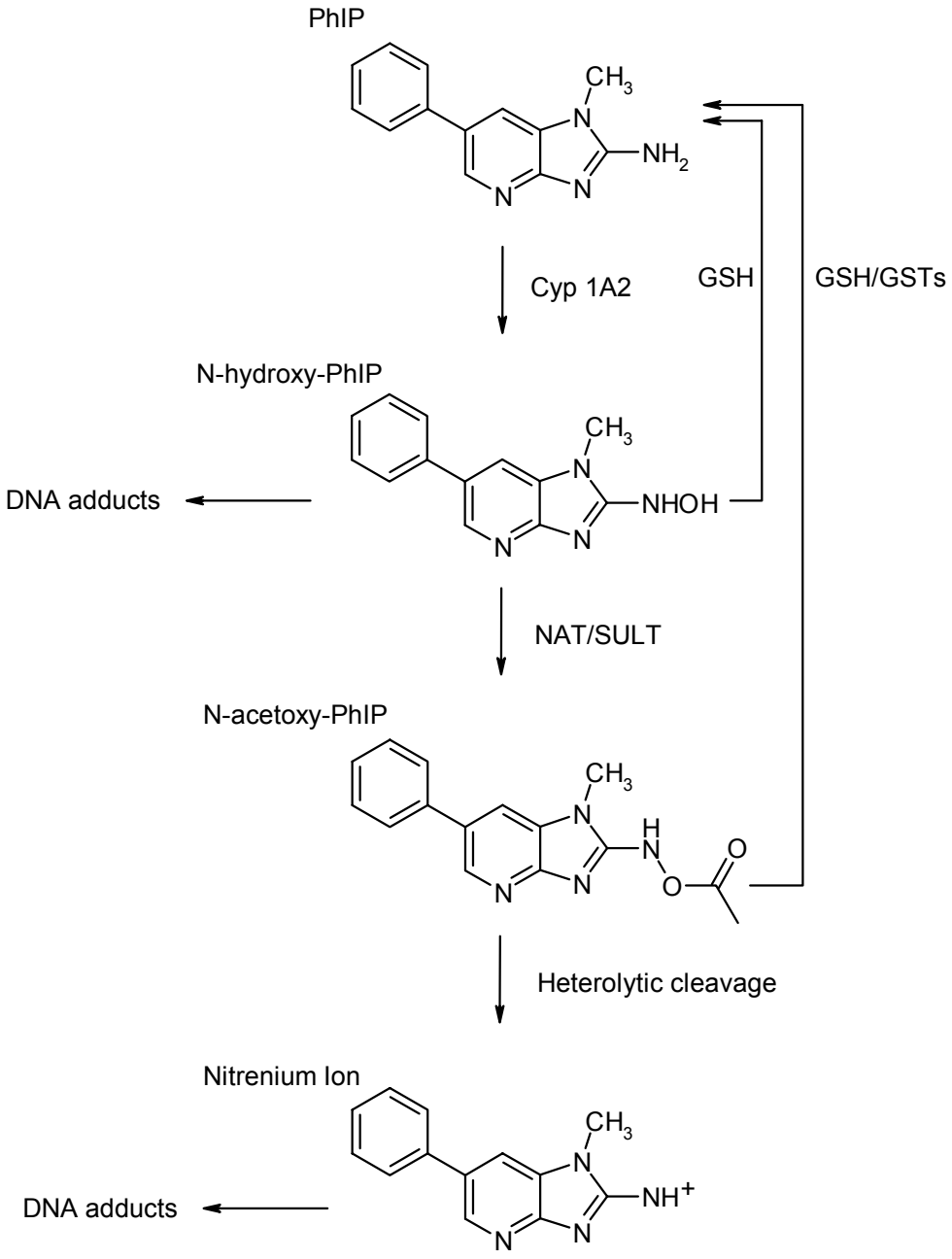


Figure 6 Schematic overview of metabolic activation of PhIP in humans

ABC transporters involved in the excretion of heterocyclic amines

Some studies have investigated the involvement of ABC transporters in the excretion of PhIP. Studies using Caco-2 monolayers in a two-compartment system and typical P-gp or MRP inhibitors showed that the ABC transporters P-gp and MRP2 are involved in the transport of PhIP (*15*). Basolateral to apical PhIP transport was increased in MDCKII-*bcrp1* transfected cells suggesting the involvement of *Bcrp1* in the excretion of PhIP (*16*). In rodents it was shown that PhIP absorption was increased in MRP2 deficient rats (*17*), *MRP2* knockout mice (*18*), and Bcrp1(-/-) mice (*16*), demonstrating that in rodents MRP2 and *Bcrp1* are involved in the excretion of PhIP back to the intestinal lumen limiting its bioavailability. Furthermore, Vlaming *et al.* (*18*) showed in *MRP2* knockout mice that not only the heterocyclic amine PhIP is excreted by MRP2 but that also the heterocyclic amine 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) is transported back to the intestinal lumen by MRP2.

Upon oral administration in male Fisher rats after 24 hours approximately 15% of PhIP was excreted in the urine and 78% in the feces. Of the PhIP excreted in the feces 66% was unmetabolized. After 48 hours 98% of the administered PhIP was excreted (*128*). Twelve hours after oral administration of radiolabeled PhIP, high levels were observed in the colon and cecum, whereas the liver, kidney, pancreas and small intestine showed lower levels of radioactivity (*128*).

Jonker *et al.* (*47*) showed in *bcrp(-/-)* mice that PhIP is actively excreted by *bcrp* into mouse milk. Furthermore, Diettrich *et al.* (*129*) showed in Wistar rats that the excretion of PhIP in the bile was approximately 4 times higher in wild type than in MRP2 deficient rats. The total body elimination of radioactivity was significantly lower in MRP2 deficient rats compared with Wistar rats, suggesting that MRP2 is involved in the excretion of PhIP.

Altogether it can be concluded that following uptake in the intestine, PhIP has been demonstrated to be transported back into the lumen by apical ABC transporters, thereby providing the first line of defence against this harmful compound.

Caco-2 monolayers

Cultured Caco-2 cell monolayers represent a generally accepted *in vitro* model for studying intestinal transport processes (*130, 131*). Caco-2 cells are derived from a human colon carcinoma (*132*). When the Caco-2 cells reach confluence the cells spontaneously differentiate and when grown on a filter insert the cells become

polarized. After 14-21 days the cells obtain the characteristics of the enterocytes. Artursson and Karlsson (*39*) showed that a good correlation exists between human oral absorption data and apparent permeability values obtained in Caco-2 monolayers for drugs and peptides.
Several studies have investigated the expression of ABC transporters in the human intestine and in Caco-2 monolayers. Prime-Chapman *et al.* (*11*) showed that MRP1, MRP2, MRP3, MRP4, MRP5 and MRP6 are significantly expressed in the human jejunum and ileum and that lower expression levels of the ABC transporters were observed in the duodenum. They also studied the expression of the ABC transporters MRP1 through MRP6 in Caco-2 monolayers grown on filter inserts. All tested transporters were expressed in the Caco-2 monolayers with MRP2 and MRP6 being the most abundant, followed by MRP3 and MRP4 and the expression of MRP1 and MRP5 being the lowest. Xia *et al.* (*133*) studied the expression and localization of BCRP in Caco-2 cells. They showed that BCRP is expressed in Caco-2 cell monolayers grown on filter inserts and that when the cells reach confluence the BCRP transporter is localized on the apical membrane of the Caco-2 monolayer.
Taipalensuu *et al.* (*13*) compared the expression levels of P-gp, MRPs, BCRP, lung resistance-related protein and CYP3A4 between jejunum biopsies of healthy human volunteers and Caco-2 monolayers. They showed that in general the expression of the genes of the efflux transporters present in the Caco-2 monolayers are in good agreement with their expression in the human jejunum. For MDR1, MDR3 and MRP1 through MRP6 the expression levels of these genes differed less than 2.5-fold in the Caco-2 monolayers compared to the human jejunum, whereas for BCRP the transcript level in Caco-2 monolayers was 100 fold less than in human jejunum.
Based on these observations it can be concluded that Caco-2 monolayers are an adequate model to study the role of these ABC transporters in apical to basolateral intestinal transport.

In silico modelling to describe the effect of compounds on ABC transporters

At present several *in silico* models have been developed to describe the effect of compounds on a single transporter, e.g Gao *et al.* (*134*) developed an assay to determine the apparent inhibition constant (Ki) for inhibitors like quinidine, daunorubicin, verapamil, doxorubicin, vinblastine, etoposide, and celiprolol on the

taxol binding site of P-gp. This model was extended by Zhang and Morris (*97*) to study the effects of the flavonoids biochanin A and sylimarin. Doppenschmitt *et al.* (*135*) developed an *in silico* model based on the passive diffusion of the substrate through the cell membrane and its binding affinity to P-gp. In addition Gonzales-Alvarez *et al*. (*136*) developed a kinetic model which combines *in situ* rat intestinal segments perfusion data and *in vitro* cell culture data of the P-gp substrate CNV97100 to be able to predict the absorption of this compound and the P-gp mediated efflux. The developed models have in common that they are capable of predicting the effect of the substrate or inhibitor on a single ABC transporter in this case P-gp.
Whereas most models are based on the effect of one efflux transporter, Ofer *et al.* (*137*) developed a model to predict bidirectional membrane transport of talinolol and the effect of flavonoids on this transport. The model is based on the presence of an efflux transporter and a carrier which is capable of bidirectional transport. The model describes that the flavonoids not only inhibit P-gp mediated excretion of talinolol but also inhibit a transport mediated influx.

Outline of this thesis

The aim of this thesis was to investigate the interactive effects of (mixtures of) flavonoids on the absorption and bioavailability of PhIP using *in vitro*, *in vivo* and *in silico* model systems. Initially, the effect of the flavonoids myricetin was studied on the transport of PhIP through Caco-2 monolayers (chapter 2). In chapter 3 the effect of ten different flavonoids on the transport of PhIP through Caco-2 monolayers was studied and an *in silico* kinetic model for this transport was developed. For the flavonoids quercetin, myricetin, luteolin and naringenin the apparent inhibition constants for the apparent transporter involved in PhIP transport were determined. In chapter 4 the effect of flavonoid mixtures containing two or five flavonoids on the transport of PhIP through Caco-2 monolayers was studied and the kinetic model from chapter 3 was extended to be capable of predicting the effect of flavonoid mixtures. In chapter 5, the effect of quercetin on the absorption and bioavailability of PhIP in the rat was studied to confirm the observed *in vitro* effects in an *in vivo* study. Finally, chapter 6 summarizes the results of this thesis and presents a general discussion.

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2

Myricetin stimulates the absorption of the pro-carcinogen PhIP

Maaike E. Schutte, Johannes J. M. van de Sandt, Gerrit M. Alink, John P. Groten and Ivonne M.C.M. Rietjens

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Abstract

The effect of the flavonoid myricetin on the transport of the pro-carcinogen 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP) through differentiated Caco-2 monolayers, a model for the intestinal epithelium, is described. Myricetin causes an increase of the transport of PhIP from the apical to the basolateral compartment. This effect was observed at physiologically relevant concentrations of PhIP and myricetin. Cyclosporin A (MRP2 inhibitor) but not PSC833 (P-gp inhibitor) showed a similar effect on PhIP transport.

The results indicate that myricetin induces an increased basolateral uptake of the pro-carcinogen PhIP, in part through inhibition of the MRP2 mediated excretion of PhIP from the intestinal cells back to the lumen.

Introduction

The intestinal epithelium is the major determinant for the oral absorption of ingested food ingredients, pharmaceuticals and environmental contaminants. The predominant absorption route for small and/or lipophilic compounds is through transcellular transport. A first line of defense against absorbed xenobiotics is the excretion of the toxicants from the epithelial cells back into the intestinal lumen. Both uptake into the bloodstream as well as excretion back into the intestinal lumen is mediated by transport proteins such as the ATP binding cassette (ABC) transporters (*1, 2*). In the intestine relevant ABC transporters, such as the multidrug resistance protein 1 (MRP1) and MRP3, are localized in the basolateral membrane, whereas MRP2, P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) are localized in the apical membrane of the intestinal enterocytes (*3*).

One class of compounds for which it is shown that ABC transporters may be involved in their transport are heterocyclic amines such as 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP). The apical ABC transporters MRP2, BCRP and P-gp may be involved in the transport of PhIP back to the intestinal lumen, thus reducing the bioavailability of this pro-carcinogen (*4-6*). PhIP is the most prevalent heterocyclic amine formed in heat-processed meat (*7*). PhIP by itself is not a mutagen, however when metabolized by the liver cytochromes P450 1A2 and 1A1 the mutagenic and carcinogenic metabolite 2-hydroxy-amino-PhIP is formed (*8, 9*). The intake of PhIP is associated with the prevalence of diseases as colorectal carcinoma (*10*).

Another class of compounds in our diet of which it is known that they interfere with ABC transporters are flavonoids (*11-13*). Given the protective role of MRP2, in among others limiting the bioavailability of PhIP, it is of interest to notice that flavonoids are inhibitors of the ABC transporters including MRP2 as shown in research regarding multidrug resistance (*11, 12*). Especially the flavonol myricetin, present in tea, wine, grapes, and broad beans (*14, 15*), was recently shown to inhibit MRP2 (*16*). Since apparently both PhIP and myricetin interact with the MRP2 transporter, the aim of the present study was to investigate the possible interaction between myricetin and PhIP on the transport across the intestinal epithelium.

The model system used in the present study to investigate this possible transport interaction between myricetin and PhIP consisted of Caco-2 monolayers. The adenoma carcinoma cell line Caco-2 differentiates into enterocytes when grown on a filter (*17*), and when grown polarized, expresses several ABC transporters

including P-gp, MRP1, MRP2, MRP3 and BCRP (*3, 18, 19*). The Caco-2 monolayer system represents a generally accepted *in vitro* model for studying intestinal transport processes (*20, 21*).

Materials and methods

Cell culture

The human colon carcinoma cell line Caco-2 (American Type Culture Collection) was cultured in Dulbecco's modified eagle medium containing 25 mM Hepes (GibcoBRL), supplemented with 1% MEM non essential amino acids (NEAA) (GibcoBRL), 10% heat inactivated fetal bovine serum (FBS) (GibcoBRL) and 0.2% gentamycin (50 mg/ml) (GibcoBRL) and cultured in a humidified atmosphere of 5% CO_2/95% air at 37 ºC. Cell cultures were split 2:10 when 70-90% confluent, using trypsin/EDTA. Cells at passages 35-45 were used for the transport experiments.

Transwell incubations

For the transport studies 1×10^5 cells per cm^2 were seeded in 12 wells transwell plates with a pore size of 0.4 µm and a growth area of 1.13 cm^2 (Costar). The medium was changed three times a week and the experiment was performed after 17-21 days post seeding. Before the experiment was performed the integrity of the monolayers was checked by measuring the transepithelial electrical resistance values with a millicell-ERS volt/ohmmeter (Millipore). Only monolayers that demonstrated a resistance value exceeding 500 $\Omega\cdot cm^2$ but below 1000 $\Omega\cdot cm^2$ were used for the transport experiments. The transport experiments were carried out with transport medium consisting of the culture medium but without FBS, phenol red and gentamycin or in Hank's balanced salt solution (HBSS) supplemented with 1% NEAA, as indicated.

Transport of PhIP from the apical to the basolateral compartment

During the transport experiment the apical compartment was filled with 0.5 ml exposure medium containing the model compound PhIP (at 5 µM final concentration) (98% purity, Toronto Research Chemicals, Inc), myricetin (95% purity, Acros Chemicals) at final concentrations of 0, 5, 10, 20 or 50 µM (as

indicated) and 1 mM ascorbic acid (Sigma) (to prevent auto oxidation of myricetin). The basolateral compartment was filled with 1.8 ml transport medium containing 1 mM ascorbic acid. After 0, 30, 60, 90, 120, 150, 180 and 210 minutes 200 µL aliquots were taken from the basolateral compartment and the volume was replenished to the initial volume with transport medium containing 1 mM ascorbic acid. The samples were stored at –20 ºC until high performance liquid chromatography (HPLC) analysis. The concentrations of myricetin and PhIP used were not toxic as demonstrated using the LDH leakage test (data not shown) (*22*).
To determine the effect of 20 µM myricetin on the transport of lower concentrations of PhIP (0.04, 0.2, 1 and 5 µM) through Caco-2 monolayers similar transport experiments were performed with the following modifications; the transport medium consisted of HBSS supplemented with 1% NEAA and 1 mM ascorbic acid, the initial concentration of PhIP was determined and all medium (1.8 ml) from the basolateral compartment was collected after either 60 or 120 minutes using a monolayer for every measured time point. The samples were freeze-dried and dissolved in 90 µl nanopure water after which they were analyzed with HPLC.

Determination of the apparent permeability coefficients

The apparent permeability coefficients (Papp) were determined under sink conditions (<10% of the substrate is transported to the receiving compartment). The apparent permeability coefficients were calculated according to:

$$Papp = \frac{\frac{dQ}{dt}}{A \times C_0} \qquad \text{(Equation 1)}$$

in which dQ/dt is the initial transport velocity (nmol/s), A the surface area of the membrane insert (cm^2) and C_0 the initial concentration of PhIP in the donor chamber (nmol/ml) (*17*).
The ratio $Papp_{ba}/Papp_{ab}$ represents the ratio between the Papp for the two transport directions, and was calculated by dividing the apparent permeability coefficient of transport from the basolateral compartment to the apical compartment ($Papp_{ba}$) by the apparent permeability coefficient of transport from the apical compartment to the basolateral compartment ($Papp_{ab}$).
The effect of myricetin on the Papp of PhIP was assessed in two manners: i) by exposing the cells to myricetin at the apical side only, and ii) by exposing the cells

to myricetin at both apical and basolateral sides. Following the second approach the transport of PhIP from both apical to basolateral direction ($Papp_{ab}$) and basolateral to apical direction ($Papp_{ba}$) was determined. The same approach was used to assess the effect of the typical MRP2 inhibitor cyclosporin A (25 μM) (Fluka) and the typical P-gp inhibitor PSC833 (0.1 μM) (kind gift from Novartis Pharma AG) on the $Papp_{ab}$ and $Papp_{ba}$ of PhIP.

High Performance Liquid Chromatography analysis

The HPLC system consisted of a Spectra Physics Analytical P2000 pump, an AS3000 automated sample processor/injector, and a UV6000LP diode array UV detector.

For PhIP analysis an Alltima C18 5μ 150 × 4.6 mm (Alltech) reverse phase column was used with a mobile phase of 35% (v/v) acetonitrile (Lab Scan Analystical Chemicals) in 50 mM ammonium acetate (J.T. Baker) (pH 5) with a flow rate of 1.0 ml/min and UV detection at 315 nm. PhIP had a retention time of 5.8 minutes. For the analysis of myricetin an Alltima C18 5μ 150 × 4.6 mm (Alltech) reverse phase column was used with a mobile phase of 20% (v/v) acetonitrile in 0.1% (v/v) trifluoro acetic acid (0-3 min) with a linear gradient to 55% (v/v) acetonitrile in 0.1% (v/v) trifluoro acetic acid (3-18 min) and a linear gradient to the starting conditions (20% (v/v) acetonitrile in 0.1% (v/v) trifluoro acetic acid) (18-22 min) followed by an equilibration period of 5 minutes with a flow rate of 1.0 ml/min and UV detection at 370 nm. Myricetin had a retention time of 11.1 minutes. For quantification calibration curves were made with commercially available PhIP and myricetin.

Statistical analysis

The statistical significance of differences between treatments was assessed using a two-tailed paired Student's *t*-test with a significance level of $P<0.05$.

Results

The effect of apical myricetin on the cumulative absorption of PhIP through Caco-2 monolayers

To mimic the *in vivo* exposure the Caco-2 monolayers were exposed to both myricetin and PhIP in the apical compartment. Figure 1A shows that in the presence of 20 µM myricetin transport of PhIP through Caco-2 cell monolayers from the apical to the basolateral compartment is increased compared to cells exposed to PhIP in the absence of myricetin. The $Papp_{ab}$ of PhIP in the presence of 20 µM myricetin ($19.8\times10^{-6} \pm 0.33\times10^{-6}$ cm/s) is 3.6 fold higher than the $Papp_{ab}$ for PhIP transport in the absence of myricetin ($5.6\times10^{-6} \pm 1.1\times10^{-6}$ cm/s). Figure 1B shows the effect of increasing concentrations of myricetin on the cumulative (two hours) transport of 5 µM PhIP compared to Caco-2 cells exposed to PhIP in the absence of myricetin. The total amount of PhIP transported to the basolateral compartment increases in the presence of an increasing amount of myricetin in the apical compartment to a level finally amounting to 9.3 times the value in the absence of myricetin at 50 µM of the flavonoid.

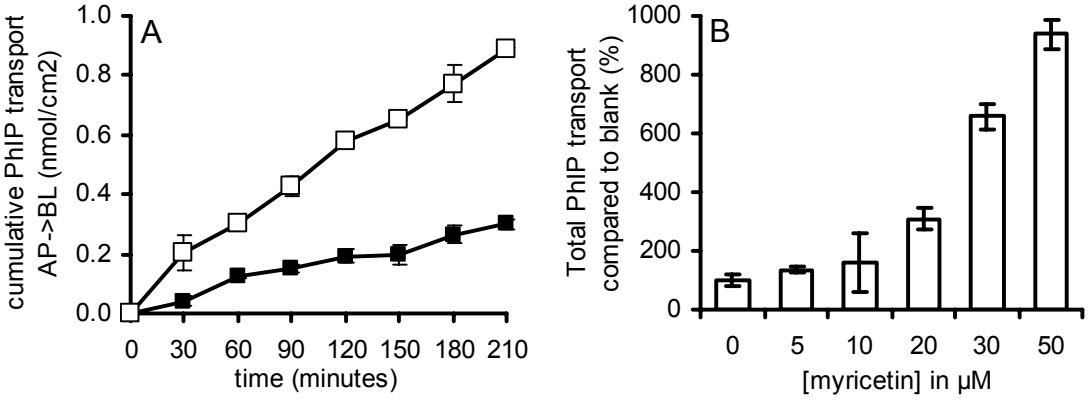

Figure 1 (A) PhIP transport through Caco-2 monolayers from the apical to the basolateral compartment. Caco-2 monolayers were exposed to 5 µM PhIP in the presence (□) or absence (■) of 20 µM myricetin in the apical compartment. (B) The influence of different concentrations of myricetin on the cumulative (2 hours) transport of PhIP from the apical to the basolateral compartment. Plotted is the percentage PhIP transported compared to transport in a solvent control incubation. Solvent control was set at 100% (n=3, mean ± SD).

Figure 2 depicts the amount of PhIP transported through Caco-2 monolayers after one hour of exposure to 0.2, 1 and 5 μM PhIP and after two hours of exposure to 0.04 μM PhIP all in the presence or absence of 20 μM myricetin. At concentrations of PhIP lower than 5 μM, its transport through Caco-2 monolayers is higher (3.6-4.9 fold) in the presence of 20 μM myricetin.

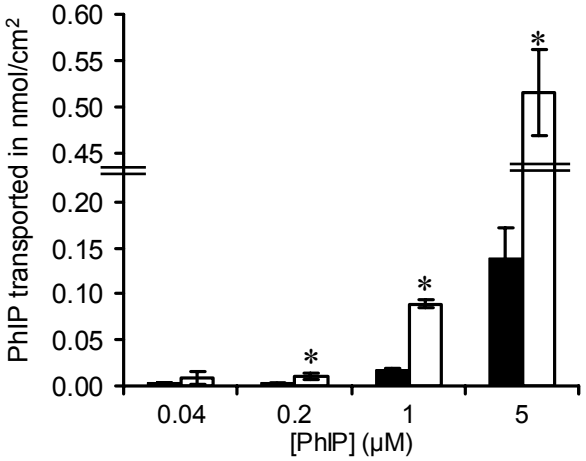

Figure 2 The amount of PhIP transported through Caco-2 monolayers exposed to different concentrations of PhIP in the presence of 20 μM myricetin (open bars) or solvent control (solid bars) from the apical to the basolateral compartment. Bars represent the amount of PhIP in nmol/cm^2 transported after one hour (for monolayers exposed to 0.2, 1 and 5 μM PhIP) or after two hours (for monolayers exposed to 0.04 μM PhIP). * Significantly different (P<0.05) from Caco-2 cells exposed to PhIP in the absence of 20 μM myricetin.

The effect of myricetin on the apparent permeability coefficients of Caco-2 monolayers for PhIP

In pharmaceutical research, potential inhibitors of transporters are most often tested by exposing epithelial cells at both the apical and the basolateral side, thereby ensuring that a constant concentration is maintained during the experiment. To test the effect of myricetin on the transport of PhIP under these optimized conditions, Caco-2 monolayers were exposed to 5 μM PhIP in the donor compartment but with myricetin in both compartments.

Figure 3A depicts the apparent permeability coefficients from the apical to basolateral compartment of Caco-2 monolayers for 5 μM PhIP with increasing concentrations of myricetin. A concentration dependent increase of the $Papp_{ab}$ from 7.1×10^{-6} cm/s for the control to 25×10^{-6} cm/s in the presence of 50 μM myricetin is

observed. This result is in agreement with the experiment in which Caco-2 monolayers were exposed to myricetin only in the apical compartment (Figure 1A). Figure 3B depicts the $Papp_{ba}$ of Caco-2 monolayers for the transport of 5 µM PhIP in the opposite direction, from basolateral to apical, at increasing concentrations of myricetin. A concentration dependent decrease in the $Papp_{ba}$ from 49×10^{-6} cm/s for the control to 23×10^{-6} cm/s in the presence of 50 µM myricetin is observed. The ratio $Papp_{ba}/Papp_{ab}$ shows a concentration dependent decrease from a $Papp_{ba}/Papp_{ab}$ value of 6.9 for Caco-2 monolayers exposed to PhIP in the absence of myricetin to a $Papp_{ba}/Papp_{ab}$ value of 0.90 for Caco-2 monolayers exposed to 5 µM PhIP in the presence of 50 µM myricetin (Figure 3C). In the presence of 50 µM myricetin the dominating transport direction for PhIP changed from basolateral to apical to the opposite direction e.g. apical to basolateral.

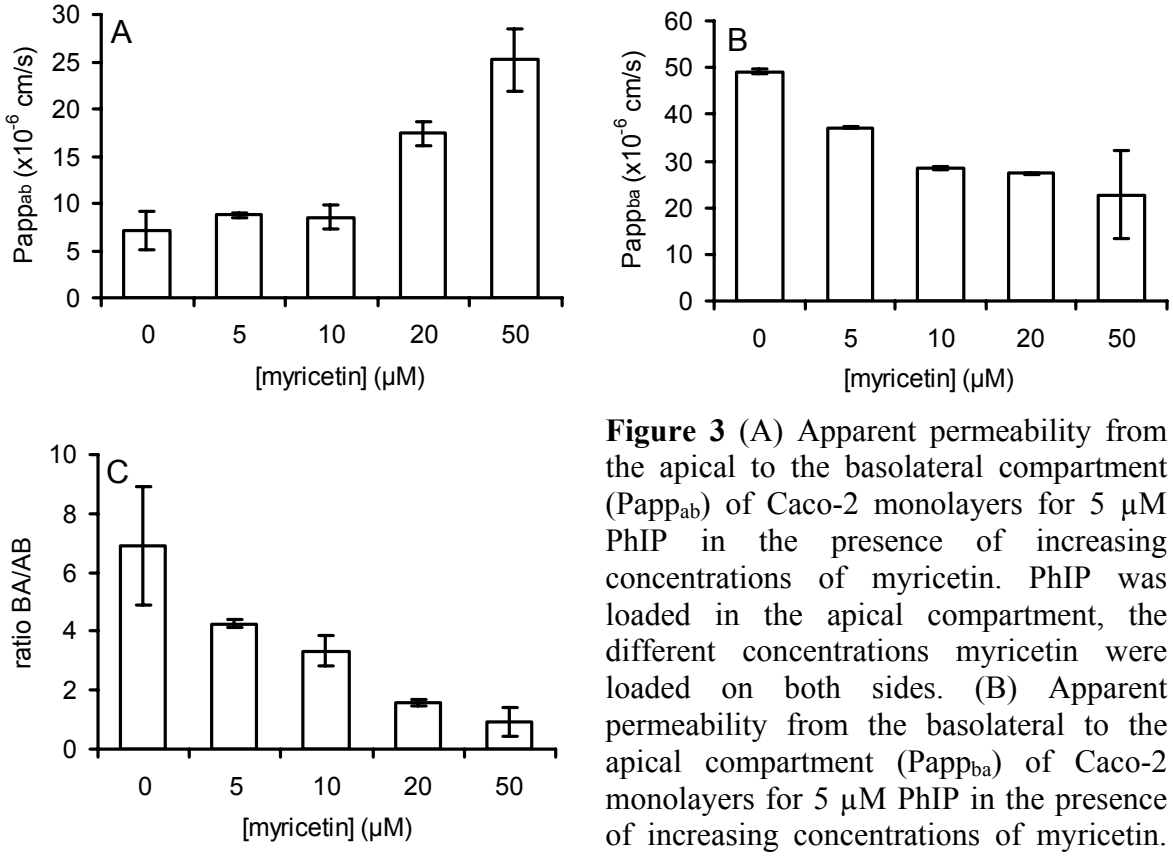

Figure 3 (A) Apparent permeability from the apical to the basolateral compartment ($Papp_{ab}$) of Caco-2 monolayers for 5 µM PhIP in the presence of increasing concentrations of myricetin. PhIP was loaded in the apical compartment, the different concentrations myricetin were loaded on both sides. (B) Apparent permeability from the basolateral to the apical compartment ($Papp_{ba}$) of Caco-2 monolayers for 5 µM PhIP in the presence of increasing concentrations of myricetin. PhIP was loaded on the basolateral compartment, the different concentrations myricetin were loaded on both sides. (C). Apparent permeability ratios ($Papp_{ba}$/ $Papp_{ab}$) of Caco-2 monolayers exposed to 5 µM PhIP and increasing concentrations of myricetin. Data are mean ± SD from one typical experiment.

The effect of typical transporter inhibitors on the apparent permeability coefficient of Caco-2 monolayers for PhIP

Table 1 reflects the apparent permeability coefficients for both apical to basolateral transport ($Papp_{ab}$) and basolateral to apical transport ($Papp_{ba}$) of Caco-2 monolayers exposed to 5 μM PhIP in the presence of typical transporter inhibitors on both sides. The addition of the typical P-gp inhibitor PSC833 had no statistically significant effect on the transport of PhIP trough Caco-2 monolayers as reflected by the fact that the $Papp_{ba}/Papp_{ab}$ ratio was not significantly changed.
The addition of the typical MRP2 inhibitor cyclosporin A resulted in a decrease of the transport of PhIP from the basolateral to the apical compartment (reflected by a statistically significant decrease in the $Papp_{ba}$) accompanied by an increase of the amount of PhIP transported from the apical to the basolateral compartment (reflected by a statistically significant increase of the $Papp_{ab}$). Together these changes result in a decrease in the $Papp_{ba}/Papp_{ab}$ ratio for PhIP in the presence of cyclosporin A.

Table 1 The effect of typical P-gp or MRP2 transporter inhibitors (PSC833 or cyclosporin A) on the transport of 5 μM PhIP, with PhIP in the donor compartment and the inhibitors in both compartments. Data are the mean ± SD from one typical experiment.

	$Papp_{ab}$ (±SD) (cm/s)	$Papp_{ba}$ (±SD) (cm/s)	$Papp_{ba}/Papp_{ab}$ (±SD)
5 μM PhIP	2.6×10^{-6} (0.15×10^{-6})	48×10^{-6} (4.0×10^{-6})	18.5 (2.6)
5 μM PhIP + 0.1 μM PSC833	2.7×10^{-6} (0.12×10^{-6})	42×10^{-6} (8.7×10^{-6})	15.2 (3.9)
5 μM PhIP + 25 μM cyclosporin A	9.2×10^{-6} (0.46×10^{-6})*	40×10^{-6} (0.51×10^{-6})*	4.3 (0.27)

* Significantly different ($P<0.05$) from Caco-2 cells exposed to PhIP in the absence of the inhibitor.

Discussion

The aim of this study was to investigate the possible interaction between myricetin and PhIP on the transport across the intestinal epithelium. Flavonoids, like myricetin are bioactive food ingredients of interest in the field of novel foods for their various possible beneficial health effects (*23*). The results of the present study, however, point at a possible adverse effect of the flavonoid myricetin, known from previous studies to be an inhibitor of several so-called ABC transport proteins including the multidrug resistance proteins MRP1 and MRP2 (*12, 16*). Especially MRP2 is known to be one of the transport proteins located in the apical side of the intestinal enterocytes, where it is involved in the first line of defense against absorbed xenobiotics, by means of excretion of the xenobiotics from the intestinal enterocytes back into the intestinal lumen (*1*). A natural occurring food contaminant for which such a protective effect of MRP2 may exist is the pro-carcinogen PhIP, because studies using Caco-2 monolayers showed the involvement of MRP2 in PhIP transport (*4*) and studies in MRP2 deficient rats suggested the involvement of MRP2 in the extrusion of PhIP (*5*). The results obtained in the present study with the Caco-2 monolayer intestinal model demonstrate that in the presence of myricetin the apical to basolateral transport of PhIP through the Caco-2 monolayer is significantly enhanced, ultimately resulting in a situation where, at 50 μM myricetin, the $Papp_{ba}/Papp_{ab}$ is lower than 1. This reflects a myricetin-mediated reversal of the preferential direction of transport of the pro-carcinogen, significantly favoring basolateral transport of the compound. Since absorption data of drugs from Caco-2 cells correlate well with human absorption data (*17*), the results of the present study indicate that the flavonoid myricetin may stimulate the *in vivo* uptake of the pro-carcinogen PhIP from the intestinal lumen.

In theory this effect of myricetin can be due to several mechanisms, including i) the inhibition of the apical transport of PhIP by MRP2, ii) the inhibition of other apical PhIP transporters known to be present in the apical membrane of intestinal enterocytes, including P-glycoprotein and breast cancer resistance protein which may also be sensitive towards inhibition by flavonoids (*13, 24*), iii) stimulation of basolateral transporters including MRP1 and/or iv) a combination of all these effects. Additional results from the present study using the typical MRP2 transporter cyclosporin A and the typical P-gp inhibitor PSC833, revealed that especially cyclosporin A was able to influence the PhIP transport through the Caco-

2 cells in a way similar to what was observed for myricetin. Furthermore, previous data revealed myricetin to be an inhibitor, not a stimulator of MRP1 activity (*16*). Therefore, it is concluded that of all mechanisms suggested, at least inhibition of MRP2 by myricetin seems to be involved in the mechanism of myricetin mediated stimulation of PhIP uptake.

Finally, it is also important to note that the effect of myricetin on the transport of PhIP was already detected at physiological relevant concentrations since intake of 100 mg myricetin a representative amount used in human supplements may result in an intestinal concentration of 30 μM. Thus, the results of the present study reveal an unexpected adverse effect of the supposed beneficial food ingredient myricetin, which appears able to increase the absorption of the pro-carcinogen PhIP.

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3

An *in vitro* and *in silico* study on the flavonoid mediated modulation of the transport of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) through Caco-2 monolayers

Maaike E. Schutte, Andreas P. Freidig, Johannes J. M. van de Sandt, Gerrit M. Alink, Ivonne M.C.M. Rietjens and John P. Groten

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Abstract

The present study describes the effect of different flavonoids on the absorption of the pro-carcinogen PhIP through Caco-2 monolayers and the development of an *in silico* model describing this process taking into account passive diffusion and active transport of PhIP. Various flavonoids increased the apical to basolateral PhIP transport. Using the *in silico* model for flavone, kaempferol and chrysoeriol the apparent Ki value for inhibition of the active transport to the apical side was estimated to be below 53 μM and for morin, robinetin and taxifolin between 164 and 268 μM. For myricetin, luteolin, naringenin and quercetin the apparent Ki values were determined more accurately and amounted to 37.3, 12.2, 11.7 and 5.6 μM respectively. Additional experiments revealed that the apical to basolateral PhIP transport was also increased in the presence of a typical BCRP or MRP inhibitor with apparent Ki values in the same range as those of the flavonoids. This observation together with the fact that flavonoids are known to be inhibitors of MRPs and BCRP, corroborates that inhibition of these apical membrane transporters is involved in the flavonoid-mediated increased apical to basolateral PhIP transport.

Based on the apparent Ki values obtained, it is concluded that the flavonols, at the levels present in the regular Western diet, are capable of increasing the transport of PhIP through Caco-2 monolayers from the apical to the basolateral compartment. This points to flavonoid-mediated stimulation of the bioavailability of PhIP and, thus, a possible adverse effect of these supposed beneficial food ingredients.

Introduction

The intestinal epithelium is the major determinant for the absorption of ingested food, pharmaceuticals and environmental contaminants. Uptake of compounds into the bloodstream as well as excretion back into the intestinal lumen is often mediated by transport proteins such as the ATP Binding Cassette transporters (ABC transporters). ABC transporters are known to be capable of efficiently excreting xenobiotics and toxicants out of the cell. In the intestinal cells, the ABC transporters multidrug resistance protein 2 (MRP2/ABCC2), P-glycoprotein (P-gp/ABCB) and breast cancer resistance protein (BCRP/ABCG2) are expressed on the apical side whereas MRP1 (ABCC1), MRP3 (ABCC3) and MRP5 (ABCC5) are expressed on the basolateral side (*1, 2*). Flavonoids, ubiquitously present in our diet, are potent inhibitors of MRP1, MRP2, MRP4, MRP5, P-gp and BCRP (*3-7*). Since epidemiological data suggest that flavonoids may protect against cardiovascular diseases and cancer (reviewed in (*8*)) they are also increasingly consumed as food supplements.

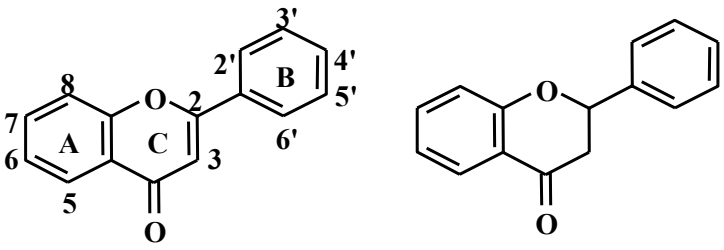
Previously, we showed that the flavonoid myricetin is capable of stimulating the absorption of the pro-carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) through Caco-2 monolayers (*9*). PhIP is formed out of Maillard reaction products during heat processing of meat and is a suspected human carcinogen (*10, 11*). PhIP is not a mutagen by itself, but after metabolism in the liver by cytochromes P450 1A1 and 1A2 the carcinogenic and mutagenic metabolite 2-hydroxy-amino-PhIP is formed (*12, 13*).

Studies in rodents showed that the bioavailability of PhIP is decreased in the presence of the ABC transporters BCRP (*14*) and MRP2 (*15, 16*). Thus, the ABC transporters BCRP and MRP2 may be involved in protecting the body against PhIP, and flavonoids or mixtures thereof acting as BCRP and MRP2 inhibitors may play an unexpected but pivotal role in the regulation of the bioavailability of PhIP.

The aim of the present study was to specify the effect of various flavonoids on the transport of the pro-carcinogen PhIP through Caco-2 monolayers and to develop an *in silico* model to describe the transepithelial transport and the interactive effects of flavonoids during this process. The flavonoids used in this study (Table 1) were selected for their ability to inhibit several ABC transporters (*3-5*) (Table 2). The *in silico* model was used to calculate from the experimental data the passive diffusion constant (*P*), and also the apparent maximum velocity (Vmax) and the apparent Michaelis-Menten constant (Km) for the active transport of PhIP through the

Caco-2 monolayers as well as the apparent inhibition constant (Ki) of the flavonoids for the inhibition of the active transport of PhIP through Caco-2 monolayers. With these apparent constants, we aim to capture the cumulative effect of several (even unknown) transporters. The results provide insight in the ability of the flavonoids to increase the overall transport of PhIP through Caco-2 monolayers from the apical to the basolateral compartment. An increase in apical to basolateral transport caused by flavonoids may reflect an increased bioavailability of PhIP. The PhIP transport through Caco-2 monolayers exposed to PhIP in the presence of the typical inhibitors GF120918, a BCRP and P-gp inhibitor, and MK571, a MRP inhibitor, was also determined in order to obtain insight in the possible involvement of these transporters, also known to be inhibited by the selected flavonoids, in the transport of PhIP through Caco-2 monolayers.

Table 1 The model flavonoids used in this study, with their hydroxylation and methoxylation pattern.



Flavone **Flavanone**

Flavonoid	Class	Hydroxylation pattern	Methoxylation pattern
Flavone	Flavone	-	
Kaempferol	Flavone	3, 5, 7, 4’	
Luteolin	Flavone	5, 7, 3’, 4’	
Morin	Flavone	3, 5, 7, 2’, 4’	
Quercetin	Flavone	3, 5, 7, 3’, 4’	
Robinetin	Flavone	3, 7, 3’, 4’, 5’	
Myricetin	Flavone	3, 5, 7, 3’, 4’, 5’	
Chrysoeriol	Flavone	5, 7, 4’	3’
Naringenin	Flavanone	5, 7, 4’	
Taxifolin	Flavanone	3, 5, 7, 3’, 4’	

Table 2 Overview of selected literature data of the model flavonoids and their ability to inhibit different transporters.

		Transporter affected [a]				
Flavonoid	Substrate	P-gp	MRP1	MRP2	BCRP	Reference[b]
Flavone	Calcein-AM		+	+		(*4*)
	Mitoxantrone				+	(*38*)
Kaempferol	Calcein-AM		+	+		(*4*)
	SN-38				+	(*39*)
	Mitoxantrone				+	(*39*)
	Calcein-AM		+	+		(*4*)
	Calcein-AM	+				(*27*)
	LTC4		+			(*3*)
	DNM		+			(*40*)
Luteolin	Mitoxantrone				+	(*5*)
	Calcein-AM		+	○		(*4*)
Morin	Mitoxantrone				○	(*5*)
	Calcein-AM		+	+		(*4*)
	DNM		+			(*40*)
Quercetin	Mitoxantrone				+	(*5*)
	Calcein-AM		+	○		(*4*)
	LTC4		+			(*3*)
	DNM		+			(*5*)
Robinetin	Calcein-AM		+	+		(*4*)
Myricetin	Mitoxantrone				○	(*5*)
	Calcein-AM		+	+		(*4*)
	LTC4		+			(*3*)
Chrysoeriol	Calcein-AM		+	+		(*4*)
Naringenin	SN-38				+	(*39*)
	mitoxantrone				+	(*39*)
	vincristine	○				(*39*)
	VP-16		○			(*39*)
	Mitoxantrone				+	(*5*)
	Calcein-AM		○	○		(*4*)
	Calcein-AM	+				(*27*)
	LTC4		+			(*3*)
Taxifolin	Calcein-AM		○	○		(*4*)

a) Inhibition of the transporter is indicated with +, no effect is indicated with ○

b) van Zanden *et al*. (4) performed experiments with MDCKII cells transfected with MRP1 or MRP2; Zhang *et al.* (38) performed experiment with MCF7 MX100 cells (MCF7 selected with mitoxantrone) Imai *et al*. (39) performed experiments with K562 cells transfected with BCRP; MDR or MRP1; Zhang *et al*. (5) performed experiments with MCF7 cells selected with MX100 or NCI-H460 MX20 both expressing BCRP; Romiti *et al*. (27) performed experiments with HK-2 cells; Leslie *et al*. (3) performed experiments with membrane vesicles containing MRP1; Nguyen *et al.* (40) performed experiments with Panc-1 cells.

Materials and methods

Cell line and chemicals

The human cell line Caco-2 originating from a human colorectal adenocarcinoma was purchased from the American Type Culture Collection (Manassas, USA). Dulbecco's modified Eagles medium, containing 25 mM HEPES, 4500 mg/L glucose and pyridoxine with or without phenol red, MEM non essential amino acids 100×, fetal bovine serum, phosphate buffered saline and gentamycin 50 mg/ml were all purchased from Gibco Ltd. Life Technologies (Paisley, United Kingdom).

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (98% purity) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). The flavonoids flavone (99% purity), morin hydrate (95% purity) were obtained from Aldrich Chemicals (Milwaukee, USA). Naringenin (98% purity) was obtained from Sigma-Aldrich (Steinheim, Germany). Chrysoeriol, luteolin, kaempferol, robinetin (all HPLC grade) were purchased from Extrasynthese (Genay, France). Myricetin (95% purity) and quercetin dihydrate (95% purity) were obtained from Across Organics (New Jersey, USA) and taxifolin was purchased from ICN biomedicals Inc (Autora, Ohio, USA).

MK571 was purchased from Biomol (Plymouth Meeting, PA) and GF120918 was a generous gift from GlaxoSmithKline (Hertfordshire, United Kingdom).

Ascorbic acid, acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (Steinheim, Germany). Ammonium acetate (HPLC grade) was obtained from J.T. Baker (Deventer, The Netherlands). Ethylenedinitrilotetraacetic acid (EDTA) and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany) and Across Organics (New Jersey, USA) respectively.

Transwell incubations

The Caco-2 cells were maintained in culture as described previously (*9*). For the transport studies, Caco-2 cells were seeded in a density of 1×10^5 cells per cm^2 in 12 well transwell plates with a pore size of 0.4 μm and a growth area of 1.13 cm^2 (Corning Incorporated, NY). The medium was changed three times a week and the experiments were performed after 17-20 days post seeding. Caco-2 cells were used at passages 39-45.

Before and after the experiment was performed the integrity of the monolayers was checked by measuring the transepithelial electrical resistance values with a

millicell-ERS volt/ohm meter (Millipore Corporation (Bedford, MA)). Studies from Duizer *et al*. (*17, 18*) have demonstrated a good correlation between TEER values and mannitol fluxes. Only monolayers that demonstrated a resistance value between 500 $\Omega \cdot cm^2$ and 1000 $\Omega \cdot cm^2$ were used for the transport experiments and data processing. The transport experiments were carried out in transport medium consisting of Dulbecco's modified Eagle medium with 25 mM HEPES and 1% MEM non essential amino acids.

Determination of the passive diffusion constant (P), and the apparent Km and apparent Vmax values for the transport of PhIP through Caco-2 monolayers

During the transport experiments the apical compartment was filled with 0.5 ml exposure medium containing the compound PhIP (at 5, 10, 25, 50, or 100 µM final concentration added as a 200 times concentrated stock solution in DMSO) and 1 mM ascorbic acid in both compartments (to prevent auto-oxidation of the flavonoids). The basolateral compartment was filled with 1.8 ml transport medium. After 0, 30, 60, and 120 minutes, 200 µl aliquots were taken from the basolateral compartment and the volume was replenished to the initial volume with transport medium. The concentration of PhIP was determined in the donating compartment at the beginning and at the end of the experiment.
From these data the passive diffusion constant (P), the apparent Km and the apparent Vmax values were calculated using the *in silico* model that is described below.

Initial screening phase: determination of the apparent permeability coefficients for transport of PhIP through the Caco-2 monolayer in the presence and absence of several flavonoids and model inhibitors

The apparent permeability coefficients (Papp) for transport of PhIP through Caco-2 monolayers were determined under sink conditions (<10% of PhIP is transported to the receiving compartment). The apparent permeability coefficients were calculated according to:

$$Papp = \frac{\frac{dQ}{dt}}{A \times C_0} \qquad \text{(Equation 1)}$$

in which dQ/dt is the initial transport velocity (nmol/s), A the surface area of the membrane insert (cm^2) and C_0 the initial concentration of PhIP in the donor compartment (nmol/ml) (*19, 20*).
In an initial set of screening experiments the effect of the selected flavonoids (Table 1) (20 µM final concentration added as 0.1% (v/v) from a 1000 times concentrated stock solution in DMSO) on the $Papp_{ab}$ of PhIP (5 µM final concentration added as 0.1% (v/v) from a 1000 times concentrated stock solution in DMSO) was assessed by exposing the cells to PhIP in the apical or the basolateral compartment in the presence of the flavonoid in both the apical and the basolateral compartment. The Caco-2 cells were exposed to PhIP in the presence of the flavonoid in both compartments to ensure that a constant flavonoid concentration was maintained during the experiment. The apical compartment was filled with 0.5 ml and the basolateral compartment with 1.8 ml exposure medium. After 0, 30, 60, and 120 minutes 200 µl aliquots were taken from the receiving compartment and the volume was replenished to the initial volume with transport medium containing the model flavonoid. All experiments were performed in the presence of 1 mM ascorbic acid in both compartments to prevent auto-oxidation of the flavonoids. The transport of PhIP from the apical to the basolateral compartment ($Papp_{ab}$) and from the basolateral to the apical compartment ($Papp_{ba}$) was determined.
The same approach was used to assess the effect of the typical BCRP and P-gp inhibitor GF120918 (0, 0.1, 1 and 10 µM final concentration) (*21-23*) and the typical MRP inhibitor MK571 (0, 3 and 30 µM final concentration) (*24*) on the $Papp_{ab}$ and $Papp_{ba}$ of PhIP.
All samples were stored at –20 ºC until high performance liquid chromatography (HPLC) analysis.
Using the model developed and the experimental data obtained with 0 and 20 µM flavonoid and the typical inhibitors GF120918 and MK571 an initial rough estimate of the apparent inhibition constant (Ki) for inhibition of PhIP transport by the flavonoids, GF120918 or MK571 was made.

Confirmation phase: determination of the apparent inhibition constant (Ki) of selected flavonoids

Based on the initial set of screening experiments at 20 µM of the model flavonoids, four flavonoids were selected for more detailed characterization of their effects on

PhIP transport by a more accurate determination of their apparent inhibition constant.

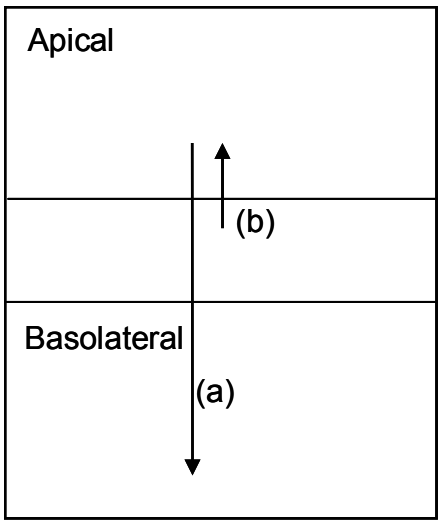
To determine the apparent inhibition constant (Ki) of myricetin, luteolin, quercetin and naringenin on the transport of PhIP, a transport experiment was performed as described above; the flavonoid myricetin (at 0, 5, 10 and 25 µM final concentration all in the presence of 0.5% DMSO) or luteolin (at 0, 5 and 15 µM final concentration all in the presence of 0.5% DMSO) or quercetin (0, 2.5, 5 and 7.5 µM final concentration all in the presence of 0.5% DMSO) or naringenin (0, 2.5, 5 and 7.5 µM final concentration all in the presence of 0.5% DMSO) was added in both compartments. After 0, 30, 60 and 120 minutes 200 µl aliquots were taken from the basolateral compartment and the volume was replenished to the initial volume with transport medium consisting of the concentration of the respective flavonoid and 1 mM ascorbic acid. The samples were stored at –20 ºC until high performance liquid chromatography (HPLC) analyses. The concentrations of PhIP, myricetin, luteolin, quercetin and naringenin used were not cytotoxic as demonstrated using the LDH leakage test (data not shown) (*25*).

In silico predictions of trans-epithelial transport of PhIP

The aim of the *in silico* predictions was to describe a model that is able to simulate the transport of various concentrations of PhIP in the absence and presence of various flavonoids. The model was based on the following assumptions (Figure 1); i) transport of PhIP from the apical to the basolateral compartment consists of a passive diffusion minus an apparent active transport component back to the apical compartment, ii) all the apical and basolateral efflux transporters present in the Caco-2 cells can be modeled as one apparent transporter localized in the apical membrane, iii) the active transport of PhIP from the intracellular to the apical compartment follows Michaelis-Menten kinetics, iv) the concentration of PhIP in the Caco-2 cells is equal to the concentration of PhIP in the apical compartment, v) the passive diffusion from the basolateral to the apical compartment is neglected since experiments were performed under sink conditions (<10% of PhIP is transported from the apical to the basolateral compartment). Together these assumptions result in the following Equation for the model presented:

$$v = P \times [S] - V\max \times \frac{[S]}{[S] + Km \times (1 + \frac{[I]}{Ki})} \quad \text{(Equation 2)}$$

With:
v: flux (µmol/cm^2/s)
P: passive diffusion constant (cm/s)
[S]: initial PhIP concentration (µmol/ml)
*V*max: apparent maximum velocity of active PhIP transport (µmol/cm^2/s)
Km: apparent Michaelis-Menten constant (µmol/ml)
[I]: initial flavonoid concentration (µmol/ml)
Ki: apparent inhibition constant (µmol/ml)



Equation (2):

$$v = P \times [S] - V\max \times \frac{[S]}{[S] + Km \times (1 + \frac{[I]}{Ki})}$$

(a) Passive diffusion ($P \times [S]$)

(b) Active transport ($V\max \times \frac{[S]}{[S] + Km \times (1 + \frac{[I]}{Ki})}$)

Figure 1 Schematic presentation of the model used. The model consists of three compartments: the apical compartment, the basolateral compartment and the Caco-2 monolayer. Active transport from the Caco-2 monolayer to the apical compartment and passive diffusion from the apical to the basolateral compartment are depicted with an arrow. In the *in silico* model the overall effect of flavonoids on the transport of PhIP is described by one apparent transporter and a component for passive diffusion.

The model describes the flux of PhIP based on the assumption that the cumulative effect of all epithelial transporters involved in PhIP uptake can be described by one apparent transporter.
Equation 2 is a first approximation in which the transport across the Caco-2 monolayer is described by two processes; passive diffusion ($P \times [S]$) towards the

basolateral compartment and active transport by an apparent transporter ($V\max\times\frac{[S]}{[S]+Km\times(1+\frac{[I]}{Ki})}$) towards the apical compartment.

The passive diffusion constant, the apparent Vmax and the apparent Km for the uninhibited transport were determined on the basis of experimental data of the transport of PhIP through Caco-2 monolayers exposed to 5, 10, 25, 50 and 100 µM PhIP in the absence of a flavonoid.

Next, the model was used to make a first rough estimate of the apparent inhibition constant Ki of selected flavonoids on the basis of experimental data for the transport of PhIP through Caco-2 cells exposed to 5 µM PhIP in the presence of 20 µM flavonoid.

Finally, the model was validated by studying four flavonoids in more detail. The apparent inhibition constant was determined for myricetin, quercetin, luteolin and naringenin based on the experimental data for the transport of PhIP through Caco-2 cells exposed to 5, 10, 25, 50 and 100 µM PhIP in the presence of 0, 5, 10 and 25 µM myricetin or 0, 5 and 15 µM luteolin or 0, 2.5, 5 and 7.5 µM quercetin or 0, 2.5, 5 and 7.5 µM naringenin. For every individual experiment the passive diffusion constant, the apparent Vmax and the apparent Km were fitted in the model on the basis of the results for uninhibited transport of PhIP through Caco-2 cells exposed to PhIP in the absence of a flavonoid.

Using the fitted passive diffusion constant and the fitted apparent Vmax value and apparent Km value as predicted by the model of the uninhibited transport, the experimental data of the transport of PhIP in the presence of different concentrations flavonoid were fitted to Equation 3.

$$v = P\times[S] - V\max\times\frac{[S]}{[S]+Km_i} \qquad \text{(Equation 3)}$$

with Km_i being the apparent Km in the presence of a specific inhibitor concentration [*I*], which, according to Equation 2, can be described as follows:

$$Km_i = Km\times\left(1+\frac{[I]}{Ki}\right) \qquad \text{(Equation 4)}$$

From Equation 4 it follows that a plot of Km_i against [*I*] gives a straight line with an intercept Km (the apparent Michaelis-Menten constant in the absence of the inhibitor) and a slope of Km/Ki from which the Ki can be determined.

High Performance Liquid Chromatography analysis

The HPLC system consisted of a Waters 600 controller, a 600 pump, a 717plus autosampler and a 2996 photodiode array detector. For PhIP analysis an Alltima C18 5µ 150 x 4.6 mm reverse phase column (Alltech, Bergen op Zoom The Netherlands) was used with a mobile phase of 35% (v/v) acetonitrile in 50 mM ammonium acetate (pH 5) with a flow rate of 1.0 ml/min and UV detection at 315 nm. Under these conditions PhIP had a retention time of approximately 4.5 minutes. For quantification calibration curves were made with commercially available PhIP.

Statistical analysis

The statistical significance of differences between treatments was assessed using a one-tailed paired Student's *t*-test with a significance level of $P<0.05$.

Results

Kinetic constants for passive diffusion and active transport for trans-epithelial transport of PhIP through Caco-2 monolayers

To describe the transport of PhIP in an *in silico* model, it is assumed that the transport of PhIP consists of passive diffusion and active transport by an apparent transporter. This apparent transporter describes the activity of all efflux transporters present in the Caco-2 cell in both the apical and the basolateral membrane by combining them in one apparent transporter located in the apical membrane (Figure 1). Passive diffusion from the basolateral to the apical compartment was neglected since Papp values were determined under sink conditions (<10% of PhIP is transported from the apical to the basolateral compartment). To determine the passive diffusion constant, and the apparent Km and the apparent Vmax values for the active transport of PhIP through the Caco-2 monolayers, the transport of PhIP was measured at increasing concentrations of PhIP in the absence of an inhibitor. Figure 2 depicts the transport of PhIP through Caco-2 monolayers at increasing

concentrations of PhIP. The total amount of PhIP transported to the basolateral compartment can be divided in i) the passive diffusion of PhIP from the apical compartment to the basolateral compartment which is linear dependent on the PhIP concentration in the apical compartment and ii) the active transport of PhIP back to the apical compartment which follows Michaelis-Menten kinetics (Figure 1).
The data obtained were fitted to Equation 2 to derive the passive diffusion constant, the apparent Km value and the apparent Vmax value of the transport of PhIP for the apparent transporter. For the transport of PhIP through Caco-2 monolayers in the absence of an inhibitor, the model predicts a passive diffusion constant of 3.5×10^{-5} cm/s, an apparent Vmax value of 4.2×10^{-6} µmol/cm^2/s and an apparent Km value of 117 µM. Additional experiments, described below, revealed similar data (Table 4). Overall the average value (n=5) for the passive diffusion constant was 3.7×10^{-5} ($\pm0.2\times10^{-5}$) cm/s, for the apparent Vmax value the average value amounted to 3.6×10^{-6} ($\pm0.5\times10^{-6}$) µmol/cm^2/s and for the apparent Km the average value was 106 (±12) µM.
Based on these kinetic constants, Figure 2 also depicts the concentration dependence of the two transport processes contributing in our model to the transport of PhIP through the Caco-2 monolayers. This graphic presentation of the passive diffusion and the active transport separately reveals that at low concentrations of PhIP the passive diffusion of PhIP to the basolateral compartment is almost completely counteracted by the active transport of PhIP back to the apical compartment, whereas at higher concentrations diffusion exceeds active transport.

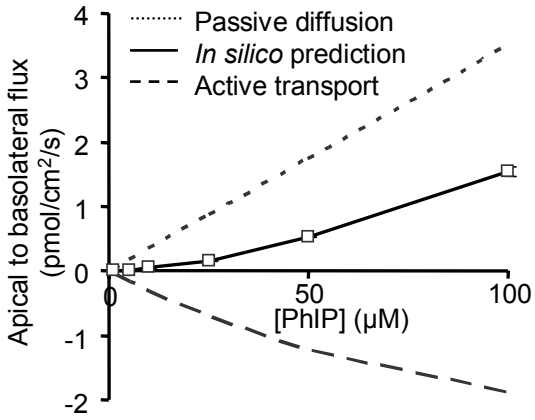


Figure 2 The flux of PhIP through the Caco-2 monolayer in the absence of an inhibitor is plotted. Plotted are the observed fluxes (□) as well as the concentration dependent flux predicted by the *in silico* model. In addition the concentration dependent passive diffusion of PhIP from the apical to the basolateral compartment and the active transport of PhIP from the Caco-2 monolayer to the apical compartment as calculated by the model are plotted. Apical to basolateral PhIP transport is plotted as a positive flux, whereas basolateral to apical PhIP transport is plotted as a negative flux. Data are mean values ± SD (n=3), error bars are contained within the symbol.

Initial screening phase: the effect of different flavonoids on the apparent permeability coefficient of PhIP through Caco-2 monolayers

To determine the effect of a series of flavonoids (Table 1) on the transport of PhIP, Caco-2 cells were exposed to 5 μM PhIP in the donating compartment and the selected flavonoid at 20 μM in both compartments. Figure 3A depicts the effect of flavonoids on the apparent permeability coefficient for PhIP through Caco-2 monolayers from the apical to the basolateral compartment ($Papp_{ab}$). Exposing Caco-2 cells to 5 μM PhIP in the absence of a flavonoid resulted in a $Papp_{ab}$ value of 4.7×10^{-6} ($\pm0.8\times10^{-6}$) cm/s. The transport of PhIP through Caco-2 cells from the apical to the basolateral compartment showed a significant increase in the presence of most of the flavonoids tested. The increase in the apical to basolateral transport of PhIP in the presence of flavonoids as compared to the solvent control was significant ($P<0.05$) for flavone, kaempferol, luteolin, quercetin, myricetin, chrysoeriol, and naringenin but not for morin, robinetin and taxifolin.

Figure 3B depicts the effect of the flavonoids on the apparent permeability coefficient for PhIP through Caco-2 monolayers from the basolateral to the apical compartment ($Papp_{ba}$). Caco-2 cells exposed to 5 μM PhIP in the absence of a flavonoid showed a basolateral to apical apparent permeability coefficient $Papp_{ba}$ of 56×10^{-6} ($\pm15\times10^{-6}$) cm/s. The transport of PhIP from the basolateral to the apical compartment showed a significant decrease in the presence of several of the selected flavonoids. The presence of the flavonoids flavone, kaempferol, luteolin, morin, quercetin, myricetin, chrysoeriol and naringenin significantly ($P<0.05$) decreased the transport of PhIP from the basolateral to the apical compartment; however, the flavonoids robinetin and taxifolin did not alter the transport of PhIP from the basolateral to the apical compartment significantly.

Note that in these experiments $Papp_{ba}$ is much larger than $Papp_{ab}$ whereas under normal physiological conditions, and also in the transport experiments in Figure 2 PhIP is added only at the apical side and the PhIP transport is measured under sink conditions with low PhIP concentrations at the basolateral side. This results in the transport from the basolateral to apical side being much smaller than the apical to basolateral transport of PhIP.

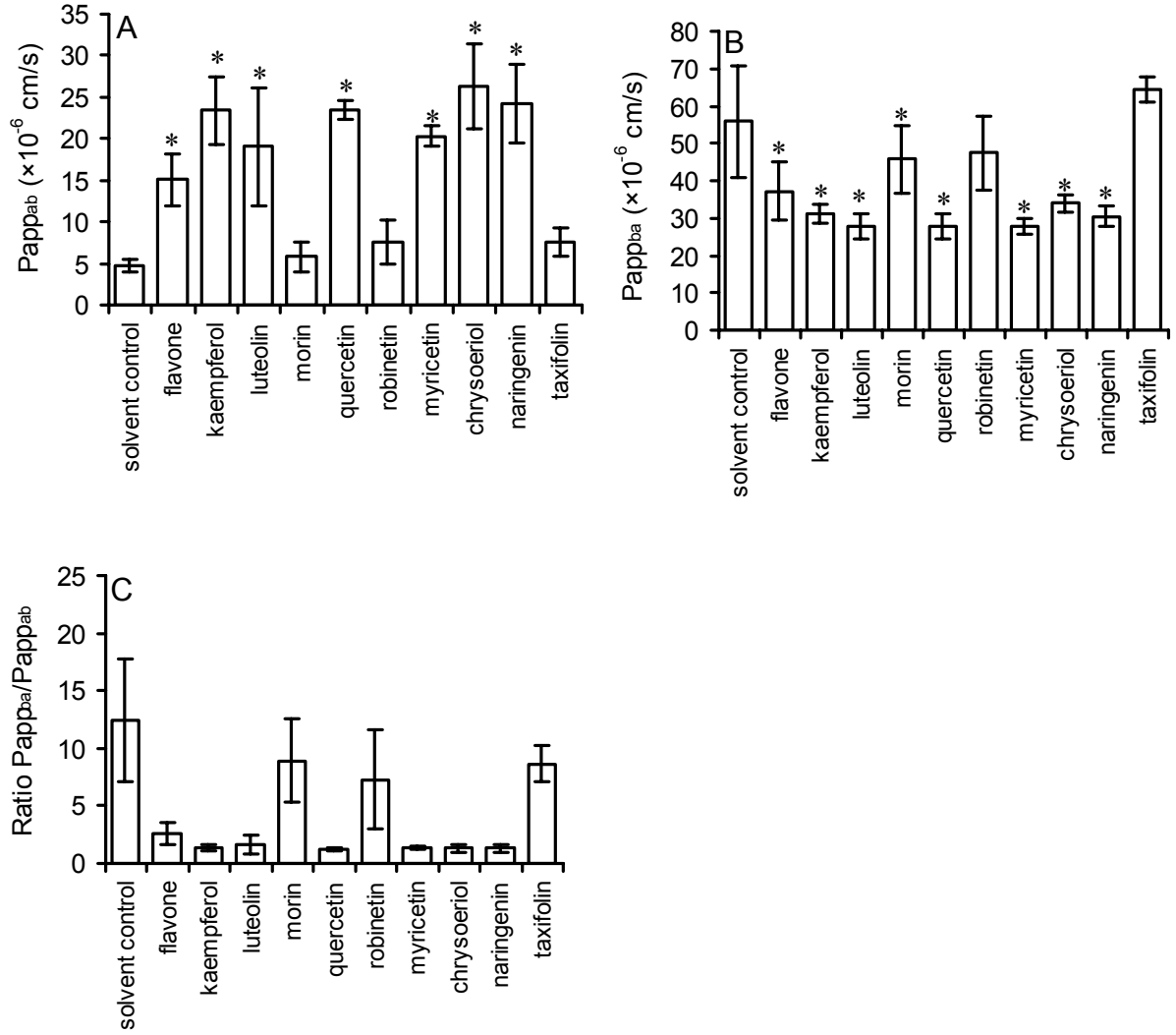


Figure 3 Apparent permeability coefficients of PhIP transported through Caco-2 monolayers in the presence of 20 μM flavonoid, (A) from the apical to the basolateral compartment ($Papp_{ab}$), (B) from the basolateral to the apical compartment ($Papp_{ba}$) and (C) the resulting apparent permeability ratio's ($Papp_{ba}/Papp_{ab}$). Data are the mean values ± SD (n=6-8).
*Significantly different (P<0.05) from solvent control.

Figure 3C depicts the ratio $Papp_{ba}/Papp_{ab}$ for the transport of PhIP through Caco-2 monolayers in the presence of the different flavonoids. The presence of a flavonoid resulted in a decrease of the $Papp_{ba}/Papp_{ab}$ value from 12.5 for the solvent control to 7.3-8.9 for morin, robinetin and taxifolin, 2.6 for flavone and 1.2-1.7 for kaempferol, luteolin, quercetin, myricetin, chrysoeriol and naringenin. This suggests that in the presence of these flavonoids the transport of PhIP is modified in favor of apical to basolateral transport.

Initial screening phase: range finding of the apparent Ki for ten different flavonoids

Using the kinetic parameters of the *in silico* model described above and the *in vitro* experiments described in the previous paragraph, a first rough estimate of the apparent Ki could be obtained for the transport of PhIP through Caco-2 monolayers (Table 3). These first estimates are based on the data for transport of PhIP after exposing the Caco-2 cells to 5 μM PhIP in the presence of 20 μM flavonoid, so these first estimates are based on data obtained at one concentration of substrate (5 μM) and one concentration of inhibitor (20 μM).

Based on these apparent Ki estimates of the tested flavonoids, the flavonoids can be divided in two groups; naringenin, kaempferol, luteolin, myricetin, chrysoeriol, flavone and quercetin as a group of potent inhibitors of the apparent transporter (apparent Ki values between 4.6 and 53 μM), whereas the flavonoids, robinetin, morin and taxifolin (apparent Ki values between 164 and 268 μM), were less potent inhibitors of the apparent transporter.

Table 3 Initial screening phase: estimates of the apparent Ki values of different flavonoids for their effect on the active transport part of the transport of PhIP through Caco-2 monolayers, predicted by the model, and determined using one concentration of flavonoid (20 μM) and one concentration of PhIP (5 μM).

Flavonoid	Ki[a] in μM
Flavone	41 (± 32)
Kaempferol	10 (± 6.3)
Luteolin	25 (± 10)
Morin	257 (± 200)
Quercetin	53 (± 43)
Robinetin	164 (± 89)
Myricetin	28 (± 24)
Chrysoeriol	29 (± 27)
Naringenin	4.6 (± 3.3)
Taxifolin	268 (± 138)

[a] Data are presented as mean±SD of n=3-4 repeated experiments

Confirmation phase: the effect of increasing concentrations of selected flavonoids quercetin, myricetin, luteolin and naringenin on the apparent permeability coefficient of different concentrations of PhIP through Caco-2 monolayers

For more detailed measurements of accurate apparent Ki values, four flavonoids were selected including quercetin, luteolin, naringenin and myricetin. Quercetin and luteolin were selected because they are present in relatively high levels in a Western diet, whereas naringenin is present in grapefruit juice and known to be a potent inhibitor of the BCRP transporter (*5*) and the flavonoid myricetin was shown to be a potent inhibitor of MRP1 and MRP2 (*4*).

For Caco-2 cells exposed to PhIP in the presence of quercetin, luteolin, naringenin and myricetin the apparent permeability coefficients for transport from the apical to the basolateral compartment were determined ($Papp_{ab}$) at several concentrations of PhIP and flavonoid. Figure 4A depicts the $Papp_{ab}$ values for the transport of increasing concentrations of PhIP through the Caco-2 monolayer in the presence of increasing concentrations luteolin. The flavonoid luteolin caused an increase in the $Papp_{ab}$ value of PhIP in a concentration dependent manner up to 71 µM PhIP. At 124 µM PhIP, the concentrations of luteolin used (≤ 15 µM) were no longer capable of increasing the PhIP transport to a significant extent. Figure 4B depicts the effect of increasing concentrations of quercetin on the transport of increasing concentrations of PhIP through the Caco-2 monolayers. At concentrations of PhIP below 35 µM the flavonoid quercetin caused a dose dependent increase in the $Papp_{ab}$ value of PhIP. At higher concentrations of PhIP, the concentrations of quercetin used (≤ 7.5 µM) were no longer capable of increasing PhIP transport. Figure 4C depicts the $Papp_{ab}$ values for the transport of increasing concentrations of PhIP through the Caco-2 monolayers exposed to PhIP in the presence of increasing concentrations of myricetin. An increasing concentration of myricetin resulted in a concentration dependent increase in the $Papp_{ab}$ value of PhIP from the apical to the basolateral compartment up to 57 µM PhIP. At 96 µM PhIP, the concentrations of myricetin used (≤ 25 µM) were no longer capable of increasing the PhIP transport. Figure 4D depicts the effect of increasing concentrations of naringenin on the $Papp_{ab}$ value of PhIP transport through Caco-2 monolayers. Naringenin is capable of stimulating the $Papp_{ab}$ value of PhIP in a concentration dependent manner up to 51 µM PhIP. At 98 µM PhIP, the concentrations of naringenin (≤ 7.5 µM) used were no longer capable of affecting the transport to a significant extent.

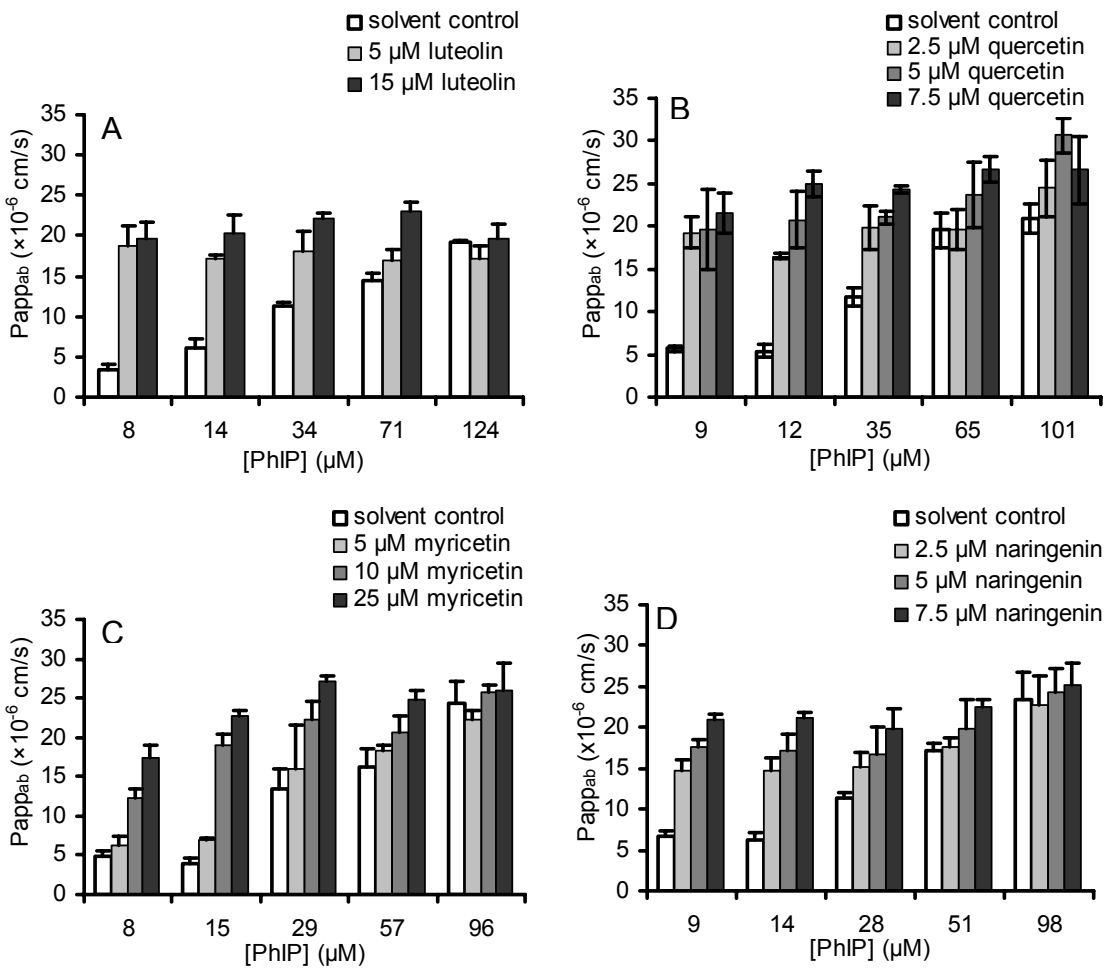


Figure 4 The apparent permeability coefficient of PhIP transported through Caco-2 monolayers from the apical to the basolateral compartment ($Papp_{ab}$), for Caco-2 cells exposed to an increasing concentration PhIP in the presence or absence of several concentrations flavonoid including (A) 0, 5 and 15 μM luteolin, (B) 0, 2.5, 5 and 7.5 μM quercetin. The 9 μM PhIP data of quercetin reflect the average values of two datasets. (C) 0, 5, 10 and 25 μM myricetin and (D) 0, 2.5, 5 and 7.5 μM naringenin.

Confirmation phase: determination of the apparent Ki value for quercetin, myricetin, luteolin and naringenin using in silico predictions

Using the *in vitro* data depicted in Figure 4 and the model, the apparent Ki value for the four flavonoids was determined more accurately.

The apparent Ki value for myricetin, luteolin, quercetin and naringenin was determined by fitting the experimental flux values to the kinetic Equations 2-4. First the parameters for the passive diffusion constant, the apparent Vmax and the apparent Km in the absence of an inhibitor were determined for each data set (Figure 4A-D respectively and Table 4) by fitting the experimental data with the Equation for transport of PhIP in the absence of an inhibitor. Using the respective passive diffusion constants, apparent Vmax and apparent Km values and the data for the transport of PhIP in the presence of a flavonoid through Caco-2 monolayers, the model predicts an apparent Ki value of 12.2 μM for luteolin, 5.6 μM for quercetin, 37.3 μM for myricetin and 11.7 μM for naringenin. For quercetin the accurately determined apparent Ki value varies significantly from the estimated apparent Ki value in Table 3. This is due to the fact that with an apparent Ki value of 5.6 μM, experiments at 20 μM quercetin are saturating and not suitable for proper kinetics.

Table 4 Confirmation phase: the kinetic constants for passive diffusion and active transport of PhIP as determined from five individual series of experiments. The passive diffusion constant (P), the apparent Vmax and the apparent Km were determined for the transport of PhIP through Caco-2 monolayers exposed to PhIP in the absence of an inhibitor. Apparent Ki values determined for PhIP transport in the presence of the flavonoid inhibitors indicated are also presented.

	Figure 2	Figure 4A	Figure 4B	Figure 4C	Figure 4D	Average (± SD)
P (cm/s)	3.5×10^{-5}	3.4×10^{-5}	3.8×10^{-5}	3.9×10^{-5}	3.8×10^{-5}	3.7×10^{-5} ($\pm 0.2\times10^{-5}$)
Vmax (μmol/cm^2/s)	4.2×10^{-6}	3.1×10^{-6}	3.2×10^{-6}	3.9×10^{-6}	3.4×10^{-6}	3.6×10^{-6} ($\pm 0.5\times10^{-6}$)
Km (μM)	117	96	90	117	110	106 (± 12)
Inhibitor		luteolin	quercetin	myricetin	naringenin	
Ki (μM)		12.2	5.6	37.3	11.7	

The effect of typical inhibitors on the apparent permeability coefficient of PhIP through Caco-2 monolayers

The Caco-2 monolayers were exposed to PhIP in the presence of typical inhibitors to determine the possible transporters involved in the transport of PhIP.

Figure 5 depicts the apparent permeability coefficients for both apical to basolateral transport ($Papp_{ab}$) and basolateral to apical transport ($Papp_{ba}$) of Caco-2 cells exposed to PhIP in the presence of the typical inhibitors MK571 (MRP1 and MRP2 inhibitor) and GF120918 (BCRP and P-gp inhibitor). Exposing the Caco-2 cells to 5 μM PhIP in the apical compartment in the presence of increasing concentrations of the typical inhibitor MK571 or GF120918 in both compartments resulted in an increase in the $Papp_{ab}$ values of the transport of PhIP through the Caco-2 monolayer from the apical to the basolateral compartment (Figure 5A). Similar experiments with 5 μM PhIP in the basolateral compartment in the presence of increasing concentrations MK571 or GF120918 in both compartments resulted in a decrease in the $Papp_{ba}$ values of the transport of PhIP through the Caco-2 monolayer from the basolateral to the apical compartment (Figure 5B). For reasons already outlined above, the $Papp_{ba}$ is higher than at physiological relevant conditions.

Figure 5C shows that the $Papp_{ba}/Papp_{ab}$ values of PhIP in the presence of MK571 decreased in a concentration dependent manner. The presence of 1 and 10 μM GF120918 but not 0.1 μM GF120918 decreased the transport ratio of PhIP. This suggests that the BCRP transporter as well as a MRP transporter may be involved in the transport of PhIP from the intracellular compartment back to the apical side of Caco-2 monolayers.

Based on the data presented in Figure 5 and the described model, an apparent Ki value of 10 μM was predicted for GF120918. Exposing the Caco-2 monolayers to PhIP in the presence of 0, 3 and 30 μM MK571 results in an increase in the flux from the apical to the basolateral compartment and an apparent Ki value of 42 μM as predicted by the model. These results show that the calculated apparent Ki values for the typical transporter inhibitors GF120918 and MK571 are in the same range as the apparent Ki values calculated for several of the tested flavonoids (Table 3).

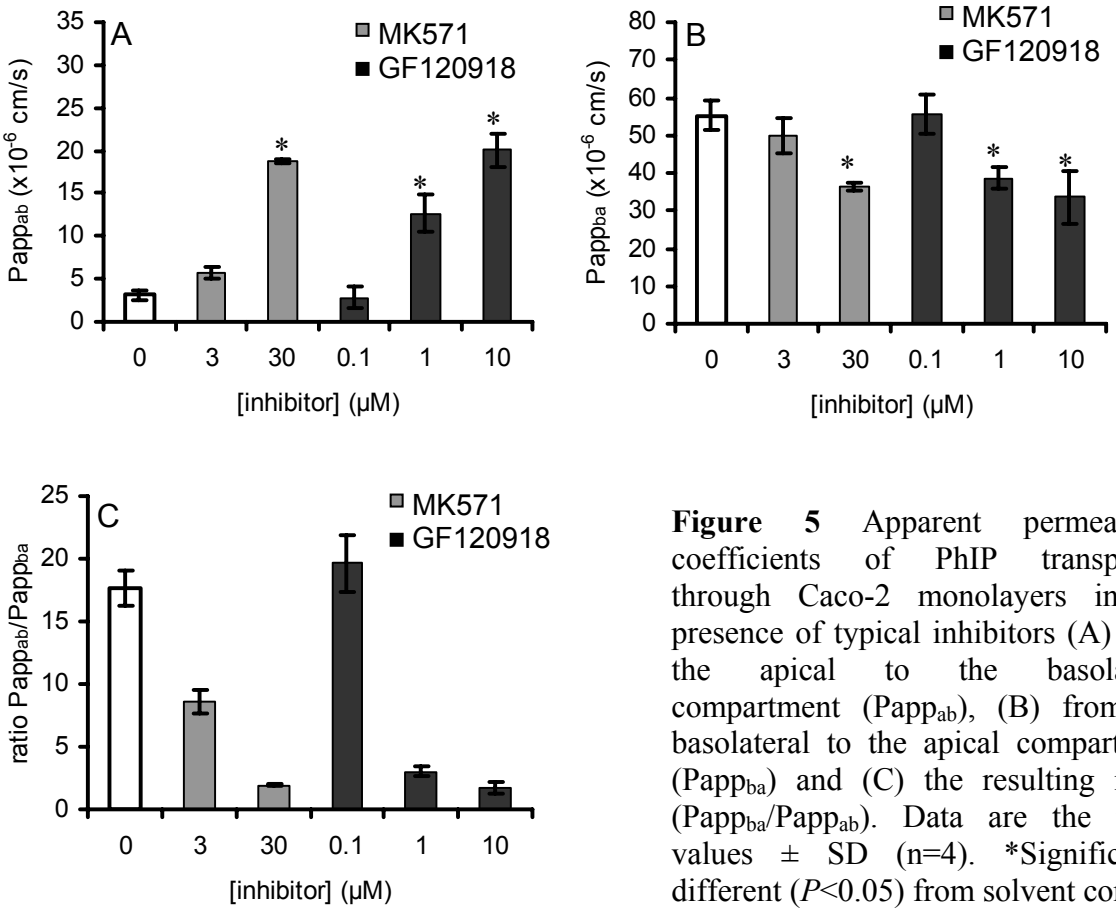


Figure 5 Apparent permeability coefficients of PhIP transported through Caco-2 monolayers in the presence of typical inhibitors (A) from the apical to the basolateral compartment ($Papp_{ab}$), (B) from the basolateral to the apical compartment ($Papp_{ba}$) and (C) the resulting ratios ($Papp_{ba}/Papp_{ab}$). Data are the mean values ± SD (n=4). *Significantly different (P<0.05) from solvent control.

Discussion

The present study shows for the first time that several flavonoids present in a Western diet are capable of increasing the transport of the pro-carcinogen PhIP through Caco-2 monolayers. Eight of ten tested flavonoids (Table 1) (except robinetin and taxifolin) were capable of increasing the transport of PhIP from the apical to the basolateral compartment and inhibiting the transport of PhIP from the basolateral to the apical compartment, resulting in a significant decrease in $Papp_{ba}/Papp_{ab}$ which might indicate an increase in the bioavailability of PhIP.

The increase of the transport of PhIP through Caco-2 monolayers in the presence of a flavonoid might in theory be caused by the inhibition of apical transporters, including the P-gp transporter, the MRP2 transporter and/or the BCRP transporter (Table 2). This suggestion is corroborated by the observation of the present study

that the typical MRP inhibitor MK571 as well as the typical BCRP and P-gp inhibitor GF120918 were capable of stimulating the transport of PhIP from the apical to the basolateral compartment in a similar way as observed for the flavonoids. Furthermore, several studies reported that flavonoids including myricetin are capable of inhibiting the MRP2 and/or BCRP transporter (*4, 5*) (Table 2). Although flavonoids have also been reported to inhibit the apical transporter P-gp (*26, 27, 28, 29*), previous results showed that the P-gp transporter is probably not involved in the transport of PhIP and therefore also not involved in the flavonoid-mediated effect on PhIP transport (*9*). To which extent all of the ABC transporters are also present in Caco-2 cells at physiological relevant levels is not precisely known. However, the ABC transporters P-gp, MRP1, MRP2, MRP3, MRP4, MRP5, MRP6 and BCRP are expressed in Caco-2 cells (*1, 30*).

Various mechanisms for the inhibition of ABC transporters by flavonoids have been reported. The ABC transporters can be influenced by the flavonoids by an effect on their ATPase activity. The flavonoid quercetin is capable of inhibiting the ATPase activity (*3*) whereas the flavonoids kaempferol (*3, 31*) and naringenin (*3*) are capable of stimulating the ATPase activity.

Another possible mechanism for flavonoid-mediated inhibition of the ABC transporters is that the flavonoids act as substrates that might be transported by the ABC transporters thereby causing competitive inhibition towards other substrates. Walgren *et al*. (*32*) showed that the flavonoid quercetin is transported through Caco-2 monolayers with a preferred transport direction to the apical compartment suggesting the involvement of a transporter in the apical membrane of the Caco-2 monolayer (*32*). Sesink *et al*. (*33*) showed that quercetin is transported by the Bcrp1 transporter in MDCKII-Bcrp1 cells (*33*). Thus, the flavonoids and/or their metabolites, by being transported themselves, might inhibit the transport of other xenobiotics by these ABC transporters.

In the *in silico* model, the assumption was made that the flavonoids act as competitive inhibitors of the apparent transporter-mediated PhIP transport. This assumption is corroborated by results from Leslie *et al.* (*3*) who showed that the flavonoids kaempferol, quercetin, myricetin, apigenin and naringenin are capable of inhibiting the transport of Leukotrine C4 in MRP1–enriched HeLa membrane vesicles in a competitive manner. Furthermore, van Zanden *et al.* (*4*) showed that the flavonoid robinetin is capable of inhibiting the transport of calcein-AM in MDCKII-MRP2 cells in a competitive manner (*4*). The apparent Ki values for quercetin, naringenin and myricetin as predicted by the model are in the same order

of magnitude as the Ki values of these flavonoids for the inhibition of the MRP1 transporter (*3*).

Gao *et. al.* (*34*) and Zhang *et. al.* (*29*) describe a model to determine the apparent inhibition constant for flavonoids on P-gp-mediated transport in Caco-2 monolayers. However, since our model consists of one apparent transporter that describes all efflux transporters present in the Caco-2 monolayer, the described model was not applicable for our studies.

It has to be emphasized that all the parameters obtained from the model are apparent parameters for one apparent transporter located in the apical membrane. Although, this apparent transporter does not have a physiological relevant function, modelling all apical and basolateral transporters in one apparent transporter makes it possible to model the overall effect of the flavonoids on the transport of PhIP.

This implies that the observed effect of the flavonoids on the transport of PhIP can not be attributed to one transport protein in particular. Although this apparent transporter is an oversimplification of the transporters present in the Caco-2 monolayer, these apparent parameters are useful to study and model the overall effect of the flavonoids on the transport of PhIP through the Caco-2 monolayer.

The assumption that the concentration of PhIP in the Caco-2 cells equals the concentration of PhIP in the apical compartment is a first approximation. Therefore, we have also modelled our data assuming that the intracellular concentration of PhIP would be 50% of the apically added PhIP concentration. Under this assumption, average value (n=5) for the passive diffusion constant and the apparent Vmax value increased from 3.7×10^{-5} cm/s to 4.3×10^{-5} cm/s and from 3.6×10^{-6} µmol/cm^2/s to 4.9×10^{-6} µmol/cm^2/s respectively; this variation is within the limits of the general experimental variation for determination of this kind of kinetic constants. For the apparent Ki value a systematic increase by about 40% was observed for each flavonoid to compensate for the reduced substrate concentration in line with the used model. Nevertheless, the apparent Ki values were still in the same micromolar range. As expected, the apparent Km value for PhIP decreased by 40%. Assuming a linear PhIP gradient over the Caco-2 cell, it can be assumed that the PhIP concentration close to the apical membrane will be closer to 100% instead of 50% of the apically added PhIP concentration. Nevertheless, these outcomes at 50% apical PhIP concentration provide insight in the sensitivity of this model to this assumption on the intracellular PhIP concentration.

Furthermore, phase II conjugation and phase III efflux capacities are different in Caco-2 cells as compared to human small intestinal cells (*1, 35*), and therefore validation of the present results in an *in vivo* intestinal model will confirm whether our *in vitro* results can be extrapolated to the *in vivo* situation. Since glucuronide- and sulphate conjugates are well known substrates of ABC transporters (*32, 33, 36, 37*) phase II conjugation of the flavonoids could result in metabolites that may still inhibit transport of other substrates by these membrane transporters. This is an interesting subject for further investigation.

Given the apparent Ki values it is of interest to note that the intestinal levels of quercetin, luteolin, naringenin and myricetin that might be reached upon a supplement intake of a 100 to 200 mg capsule with flavonoid may amount to about 35 to 75 μM. The observation that the apparent Ki values obtained in the present study are in a physiologically relevant range indicates that flavonoids or a combination thereof may influence the uptake of pro-carcinogens such as heterocyclic amines.

The model also revealed that at low concentrations PhIP (below 10 μM) the passive diffusion from the apical to the basolateral compartment of PhIP through Caco-2 monolayers in the absence of a flavonoid is almost completely counteracted by the active transport of PhIP from the intracellular to the apical compartment. It is therefore anticipated that transporters on the apical membrane are, in principle, capable of protecting the body against this food mutagen. However, the presence of flavonoids disturbs this balance between the transport directions, inhibiting especially the apical transporters MRP2 and BCRP, thereby inhibiting the transport back to the apical side upon cellular uptake of PhIP, and as such leading to an increase of the apical to basolateral transport. If we extrapolate these findings to the *in vivo* situation at physiological low doses, these results indicate that the absorption and thus the bioavailability of the pro-carcinogen PhIP might be affected by flavonoids and mixtures thereof. This points to a possible adverse effect of these food ingredients which are also considered beneficial to human health. Our hypothesis remains to be studied *in vivo* to be able to extrapolate our *in vitro* results to the *in vivo* situation.

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4

Effects of flavonoid mixtures on the transport of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) through Caco-2 monolayers: an *in vitro* and kinetic modelling approach to predict the combined effects on transporter inhibition

Maaike E. Schutte, Marelle G. Boersma, Dorien A.M. Verhallen, John P. Groten and Ivonne M.C.M. Rietjens

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Abstract

This study describes and kinetically models the effect of flavonoid mixtures on PhIP transport through Caco-2 monolayers. Previously, it was shown that quercetin, luteolin, naringenin and myricetin increase the apical to basolateral PhIP transport in Caco-2 monolayers. In this study apigenin was shown to exert a similar effect with an apparent Ki value of 10.8 μM. Additional experiments revealed that several binary flavonoid mixtures and one mixture containing all five model flavonoids increased the apical to basolateral PhIP transport through the Caco-2 monolayer. Assuming competitive inhibition of the apparent active transporter by the flavonoids and concentration-additivity for their inhibiting effect, the kinetic model previously developed to describe the effect of the individual flavonoids on PhIP transport, could be extended and thus adequately describes the experimental values obtained for the flavonoid mixtures. We conclude that combinations of flavonoids increase the transport of PhIP and do so by interacting in an additive way with the active transport of PhIP. This flavonoid-mediated increase in PhIP transport through Caco-2 monolayers may point at a possible increased bioavailability of PhIP in the presence of flavonoid mixtures in the *in vivo* situation. This would imply an adverse effect of these supposed beneficial food ingredients.

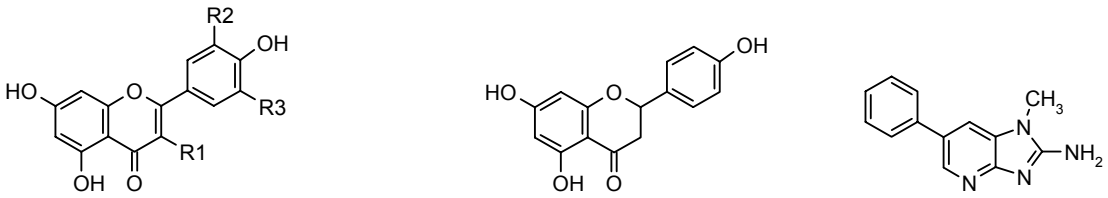
Introduction

The transport of food ingredients across the intestinal epithelium is an important factor determining the absorption upon oral intake. This includes the absorption of food ingredients, pharmaceuticals and also toxic and xenobiotic compounds. For small and lipophilic compounds the predominant route for intestinal absorption is through transcellular transport. Furthermore, the intestinal epithelium is also capable of transporting compounds from the intracellular enterocyte to the lumen or serosal side of the enterocyte by active transport proteins. The active transport proteins involved in the efflux of compounds from the enterocytes include the ATP Binding Cassette transporters (ABC transporters). The ABC transporter super family consists of among others the multidrug resistance proteins (MRPs/ABCC), P-glycoprotein (P-gp/ABCB1) and breast cancer resistance protein (BCRP/ABCG2). In the intestinal epithelium several ABC transporters are present in the apical membrane of the enterocyte, catalyzing the efflux of compounds to the intestinal luminal side, and these include P-gp, MRP2 (ABCC2), and BCRP (*1-5*). MRP1 (ABCC1), MRP3 (ABCC3) and MRP5 (ABCC5) are localized in the basolateral membrane of the enterocyte catalyzing the efflux of compounds to the serosal side (*6-10*) MRP4 (ABCC4) could be located in the apical as well as in the basolateral membrane of the intestinal cells but this is not yet fully elucidated (*1, 4, 5*). Finally, MRP6 (ABCC6) is probably located in the basolateral membrane (*11, 12*) although it may only be expressed in the mucosal cells of the intestine (*11*).

The presence of ABC transporters in the intestinal cells provides possibilities for their interaction with food components. Several examples of interactions of food ingredients with the ABC transporters are known. Research regarding multidrug resistance showed that flavonoids, present in fruits, vegetables, nuts, wine and tea (*15*), are capable of inhibiting the cellular efflux of substrates by ABC transporters (reviewed in (*13*)). Estimates of average flavonoid intake from a Western diet vary widely from approximately 1 g/day (*14*) to approximately 23 mg/day (*15*). Since epidemiological data suggest that flavonoids may protect against cardiovascular diseases and cancer (*16*) they are also available as food supplements which may lead to daily intakes up to 1 gram.

The flavonoids used in this study (Figure 1) are all present in the Western diet and capable of inhibiting the cellular efflux of substrates by several ABC transporters. The flavonoid apigenin is present in parsley and is capable of inhibiting BCRP (*17*), and to a lesser extent MRP1 (*18, 19*). Naringenin which is present in

grapefruit juice is capable of inhibiting P-gp (*20*), MRP1 (*21*) and BCRP (*22, 23*). Luteolin is present in red bell pepper (*24*) and is capable of inhibiting BCRP (*23*) and MRP1 (*19*). Myricetin is present in broad beans (*24*) and is capable of inhibiting MRP1 and MRP2 (*19*). Quercetin is present in tea, onions, red wine, French beans and tomato (*24*) and is capable of inhibiting the ABC transporters P-g (*25*), MRP1 (*19, 21*), MRP4 (*26*), MRP5 (*26*) and BCRP (*23*).



Quercetin; R1=OH, R2=OH, R3=H
Myricetin; R1=OH, R2=OH, R3=OH
Apigenin; R1=H, R2=H, R3=H
Luteolin; R1=H, R2=OH, R3=H

Naringenin

PhIP

Figure 1 Structure of PhIP and of the model flavonoids used in the present study

The inhibition of ABC transporters by food-borne ingredients like flavonoids is of interest especially because the ABC transporters are capable of transporting toxic compounds back to the intestinal lumen and thereby protecting the body. For example the polar metabolites of benzo[a]pyrene (*27*) and the food born pro-carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Figure 1) are all excreted by ABC transporters (*28-30*). Studies with transfected cells (*29*) and studies in rodents (*28, 29, 31*) showed that the pro-carcinogen PhIP is transported by MRP2 and BCRP. As a result, MRP2 and BCRP may be involved in protecting the body against the absorption of PhIP. PhIP is formed during heat processing of meat and fish and the amount of PhIP formed is approximately 16.4 ng/g meat (*46*). PhIP is metabolized in the liver by cytochromes P450 1A1 and 1A2 to its carcinogenic metabolite N-hydroxy PhIP. Previous studies showed that individual flavonoids are capable of increasing the transport of the pro-carcinogen PhIP through Caco-2 monolayers (*32, 33*).

Caco-2 monolayers are an accepted *in vitro* model to study absorption in the intestine (*34*). When grown on a filter the Caco-2 cells form monolayers, and spontaneously differentiate in monolayers of polarized enterocytes (*34*). Several

studies showed that Caco-2 monolayers express the ABC transporters P-gp, MRP1, MRP2, MRP3, MRP4, MRP5, MRP6 and BCRP (*7, 9, 35*).

In most studies on the effect of flavonoids on ABC transporter activity the effect of individual compounds has been studied although the human diet consists of multiple food constituents and humans are exposed to mixtures of compounds, rather than to single compounds. Therefore, it is of interest to study the effect of mixtures of flavonoids on ABC mediated transport of compounds by Caco-2 monolayers.

Recently, Zhang *et al.* (*17*) studied the inhibitory effect of combinations of flavonoids on the BCRP transporter. They showed that mixtures of flavonoids act additively in inhibiting BCRP. The aim of the present study is to quantify the effect of mixtures of flavonoids as compared to the effect of the individual flavonoids on the transport of PhIP through Caco-2 monolayers.

An additional aim of the present study was to investigate whether the previously developed *in silico* kinetic model that describes the transport of the pro-carcinogen PhIP through Caco-2 monolayers, assuming passive diffusion counteracted by a flavonoid sensitive apparent active transporter, can be extended to a kinetic model that describes the effects of flavonoid mixtures.

For the flavonoids naringenin, quercetin, myricetin and luteolin the apparent Ki values for inhibition of the apparent active transporter of PhIP in the Caco-2 model were determined previously and were defined to be 11.7 μM, 5.6 μM, 37.3 μM and 12.2 μM respectively (*32*). In the present study the model was used to determine the apparent Ki value for a fifth model flavonoid, apigenin, and then extended to investigate whether it could be used to describe the effect of binary flavonoid mixtures and even the effect of a mixture containing all five flavonoids on PhIP transport over the Caco-2 monolayer.

The results obtained provide insight in the ability of flavonoid mixtures to increase the transport of PhIP through the Caco-2 monolayer from the apical to the basolateral compartment, and reveal that their mixture interactions for this effect are additive in nature.

Materials and Methods

Cell line and Chemicals

The human cell line Caco-2 originating from a human colorectal adenocarcinoma was purchased from the American Type Culture Collection (Manassas, USA). Dulbecco's modified Eagles medium, containing 25 mM HEPES, 4500 mg/L glucose and pyridoxine with or without phenol red, MEM non essential amino acids 100×, fetal bovine serum, phosphate buffered saline and gentamycin 50 mg/ml were all purchased from Gibco Ltd. Life Technologies (Paisley, United Kingdom).
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (98% purity) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). The flavonoids naringenin (98% purity) and apigenin (>95%) were obtained from Sigma-Aldrich (Steinheim, Germany), luteolin (HPLC grade) was purchased from Extrasynthese (Genay, France) and myricetin (95% purity) and quercetin dihydrate (95% purity) were obtained from Across Organics (New Jersey, USA).
Ascorbic acid, acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (Steinheim, Germany). Ammonium acetate (HPLC grade) was obtained from J.T. Baker (Deventer, The Netherlands). Ethylenedinitrilotetraacetic acid (EDTA) and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany) and Across Organics (New Jersey, USA) respectively.

Determination of the cumulative flux and apparent permeability coefficient ($Papp_{ab}$) for apical to basolateral transport of PhIP through the Caco-2 monolayer in the presence and absence of apigenin

The Caco-2 cells were maintained in culture and transport experiments were performed as described previously (*32, 33*). In short, Caco-2 cells (passages 39-45) were seeded in a density of 1×10^5 cells per cm^2 in 12 well transwell plates with a pore size of 0.4 µm and a growth area of 1.13 cm^2 (Corning Incorporated, NY, USA). The medium was changed two times a week and the experiments were performed after 17-20 days post seeding. During the transport experiments the Caco-2 cells were exposed in the apical compartment to PhIP (at 5, 10, 25, 50, or 100 µM final concentration all in the presence of 0.25% DMSO) and in both compartments to apigenin (at 0, 2.5, 5.0 and 7.5 µM final concentration all in the presence of 0.5% DMSO) and 1 mM ascorbic acid (to prevent auto-oxidation of the flavonoids). After 0, 30, 60, and 120 minutes, 200 µl aliquots were taken from the

basolateral compartment and the volume was replenished to the initial volume with transport medium containing the relevant apigenin concentration and 1 mM ascorbic acid. The concentration of PhIP was determined in the donating compartment at the beginning and at the end of the experiment. Only monolayers with a transepithelial electrical resistance value of >500 $\Omega \times cm^2$ at the end of the experiment were included.
The apparent permeability coefficient (Papp) was calculated according to:

$$Papp = \frac{\frac{dQ}{dt}}{A \times C_0} \qquad \text{Equation 1}$$

In which dQ/dt is the initial transport velocity (nmol/s), A the surface area of the membrane insert (cm^2) and C_0 the initial concentration of PhIP in the donor compartment (nmol/ml) (*34, 36*).
The samples were stored at –20 ºC until analysis for PhIP by high performance liquid chromatography (HPLC).
The concentrations of PhIP and apigenin used were not cytotoxic as demonstrated using the LDH leakage test (data not shown) (*37*).

Determination of the cumulative flux for apical to basolateral transport of PhIP through the Caco-2 monolayer in the presence and absence of flavonoid mixtures

To determine the cumulative flux for apical to basolateral PhIP transport in the presence of binary mixtures of flavonoids through the Caco-2 monolayer, transport experiments were performed as described above. PhIP was added to the apical side whereas the flavonoids were added to both compartments. For Caco-2 cells exposed to 10 µM PhIP in the presence of a binary flavonoid mixture the flavonoid concentrations used amounted to 0.01, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 times their respective apparent Ki values (Table 1). For Caco-2 cells exposed to 50 and 100 µM PhIP in the presence of a binary flavonoid mixture, the flavonoid concentrations used amounted to 0.1, 0.2, 0.3, 0.4 and 0.5 times their respective apparent Ki values. Table 1 gives an overview of the flavonoid concentrations used.

Table 1 Concentration of the individual flavonoids used in transport studies in which Caco-2 monolayers were exposed to several concentrations PhIP in the presence of different binary flavonoid mixtures.

	Apparent Ki (μM)	Samples in binary mixture studies							
		0	0.01 ×Ki	0.05 ×Ki	0.1 ×Ki	0.2 ×Ki	0.3 ×Ki	0.4 ×Ki	0.5 ×Ki
Flavonoid		**Concentrations (μM) in binary mixtures**							
Myricetin	37.3	0	0.37	1.9	3.7	7.5	11.2	14.9	18.7
Luteolin	12.2	0	0.12	0.61	1.2	2.4	3.7	4.9	6.1
Quercetin	5.6	0	0.06	0.28	0.6	1.1	1.7	2.2	2.8
Naringenin	11.7	0	0.12	0.58	1.2	2.3	3.5	4.6	5.8
Apigenin	10.8	0	0.07	0.35	0.7	1.4	2.1	2.8	3.5

To study the effect of a combination which contained all five model flavonoids, Caco-2 cells were exposed to 10 μM PhIP in the presence of all five model flavonoids at concentrations that amounted to respectively 0.004, 0.02, 0.04 and 0.08 times their respective apparent Ki values, for Caco-2 cells exposed to 50 and 100 μM PhIP in the presence of five flavonoids the flavonoid concentrations used amounted to 0.04, 0.08, 0.12, 0.16 and 0.2 times their respective apparent Ki value and the concentrations thus defined and used for the flavonoid mixtures are summarized in Table 2. All flavonoids were added from DMSO stock solutions, resulting in overall DMSO concentrations ≤0.5%.

The samples taken at 0, 30, 60 and 120 minutes time intervals from the basolateral compartment were stored at –20 ºC until analysis of their PhIP content by high performance liquid chromatography (HPLC) analysis. Also for these experiments basolateral media were replenished to the initial volume with transport medium containing the flavonoids at the test concentrations. The concentrations of myricetin, luteolin, quercetin and naringenin and their combinations used were not cytotoxic as demonstrated using the LDH leakage test (data not shown) (*37*).

Table 2 Concentration of the individuals flavonoid used in transport studies in which Caco-2 monolayers were exposed to several concentrations PhIP in the presence of a mixture of five flavonoids.

	Apparent Ki (μM)	Sample in mixture study							
		0	0.004 ×Ki	0.02 ×Ki	0.04 ×Ki	0.08 ×Ki	0.12 ×Ki	0.16 ×Ki	0.2 ×Ki
Flavonoid		**Concentrations (μM) in mixture**							
Myricetin	37.3	0	0.15	0.75	1.5	3.0	4.5	6.0	7.5
Luteolin	12.2	0	0.049	0.24	0.49	0.98	1.5	2.0	2.4
Quercetin	5.6	0	0.022	0.11	0.22	0.45	0.67	0.90	1.1
Naringenin	11.7	0	0.046	0.23	0.46	0.93	1.4	1.9	2.3
Apigenin	10.8	0	0.028	0.14	0.28	0.56	0.84	1.1	1.4

Kinetic modelling of trans-epithelial transport of PhIP

To model the effect of flavonoid mixtures on PhIP transport through Caco-2 monolayers the model previously described for the effect of one inhibitor on the transport of PhIP (*32*) was modified to describe the effect of two respectively five competitive flavonoid inhibitors. The original model was based on several assumptions and extending the model to accommodate two or even five instead of one competitive flavonoid inhibitor adds an additional assumption to the model, namely that the flavonoids act independently but on the same apparent active site of the apparent active transporter as competitive inhibitors. All together the assumptions underlying the model described can be summarized as follows: i) the apical to basolateral PhIP transport consists of a passive diffusion component minus an apparent active transport component back to the apical compartment, ii) all the efflux transporters both apical and basolateral present in the Caco-2 cells can be modelled as one apparent efflux transporter localized in the apical membrane, iii) the active transport of PhIP from the intracellular to the apical compartment by the apparent transporter follows Michaelis-Menten kinetics, iv) the intracellular concentration of PhIP is equal to the concentration of PhIP in the apical compartment, v) the passive diffusion from the basolateral to the apical compartment can be neglected since experiments are performed under sink

conditions (<10% of PhIP is transported from the apical to the basolateral compartment), and vi) the flavonoids inhibit the apparent transporter as independent competitive inhibitors and thus act by concentration-addition.
To summarize the previously developed model, Equation 2 describes the data without a flavonoid inhibitor and Equation 3 the situation with one flavonoid inhibitor (*32*)

$$v = P \times [S] - V\max \times \frac{[S]}{[S] + Km} \qquad \text{Equation 2}$$

$$v = P \times [S] - V\max \times \frac{[S]}{[S] + Km \times (1 + \frac{[I]}{Ki})} \qquad \text{Equation 3}$$

For the situation with two independent competitive inhibitors for the same apparent active site it can be derived that Equation 4 describes the situation:

$$v = P \times [S] - V\max \times \frac{[S]}{[S] + Km \times (1 + \frac{[I]_1}{Ki_1} + \frac{[I]_2}{Ki_2})} \qquad \text{Equation 4}$$

And for the effect of five flavonoids on the apical to basolateral PhIP transport the data can be modeled according to Equation 5:

$$v = P \times [S] - V\max \times \frac{[S]}{[S] + Km \times (1 + \frac{[I]_1}{Ki_1} + \frac{[I]_2}{Ki_2} + \frac{[I]_3}{Ki_3} + \frac{[I]_4}{Ki_4} + \frac{[I]_5}{Ki_5})} \qquad \text{Equation 5}$$

With:
v: flux (µmol/cm^2/s)
P: passive diffusion constant (cm/s)
[S]: initial PhIP concentration (µM)
*V*max: apparent maximum velocity of active PhIP transport (µmol/cm^2/s)
Km: apparent Michaelis-Menten constant (µM)
[I]: initial flavonoid concentration in the medium (µM)
Ki: apparent inhibition constant (µM)

The passive diffusion constant, the apparent Vmax and the apparent Km for the uninhibited transport were determined on the basis of experimental data for the transport of PhIP through Caco-2 monolayers exposed to 5, 10, 25, 50 and 100 μM PhIP in the absence of flavonoids. Using the passive diffusion constant and the apparent Vmax value and apparent Km value as determined by the model on the basis of the uninhibited transport, the experimental data for the transport of PhIP in the presence of different concentrations apigenin were analyzed by the model to determine the apparent Ki value for inhibition of the apparent transporter by apigenin.

For experiments with the binary mixtures and the mixtures containing all five model flavonoids, experimental PhIP transport was compared to the PhIP transport predicted by Equation 4 and 5 using the flavonoid concentrations in the mixture experiments, the P value, apparent Vmax value and apparent Km value determined in the uninhibited PhIP transport experiments and the apparent Ki of the different flavonoids determined in the experiments with one flavonoid inhibitor.

High performance liquid chromatography analysis

The amount of PhIP was quantified using the HPLC method described previously (*32*). Briefly, an Alltima C18 5μ 150 x 4.6 mm reverse phase column (Alltech, Bergen op Zoom, The Netherlands) was used with a mobile phase of 35% (v/v) acetonitrile in 50 mM ammonium acetate (pH 5) with an isocratic flow rate of 1.0 ml/min, UV detection at 315 nm and a calibration curve made with commercially available PhIP.

Statistical analysis

The statistical significance of differences between treatments was assessed using a one-way ANOVA ($P<0.05$) and a Bonferroni Post-Hoc test.

Results

Kinetic constants for passive diffusion and active transport for transepithelial transport of PhIP through Caco-2 monolayers

To describe the transport of PhIP in our kinetic model, it is assumed that the transport of PhIP consists of passive diffusion minus active transport by an apparent transporter (see also (*32*)). This virtual apparent transporter is localized in the apical membrane and comprises the activity of both the apical and the basolateral located efflux transporters present in the Caco-2 cell. To determine the passive diffusion constant, and the apparent Km and the apparent Vmax values for the active transport of PhIP through the Caco-2 monolayers, the transport of PhIP was measured at increasing concentrations of PhIP in the absence of a flavonoid. In Figure 2 the $Papp_{ab}$ and flux of PhIP in the presence and absence of apigenin is depicted. By fitting the data of the uninhibited PhIP flux to Equation 2 the passive diffusion constant, the apparent Vmax value and the apparent Km value of the transport of PhIP for the apparent transporter were determined.

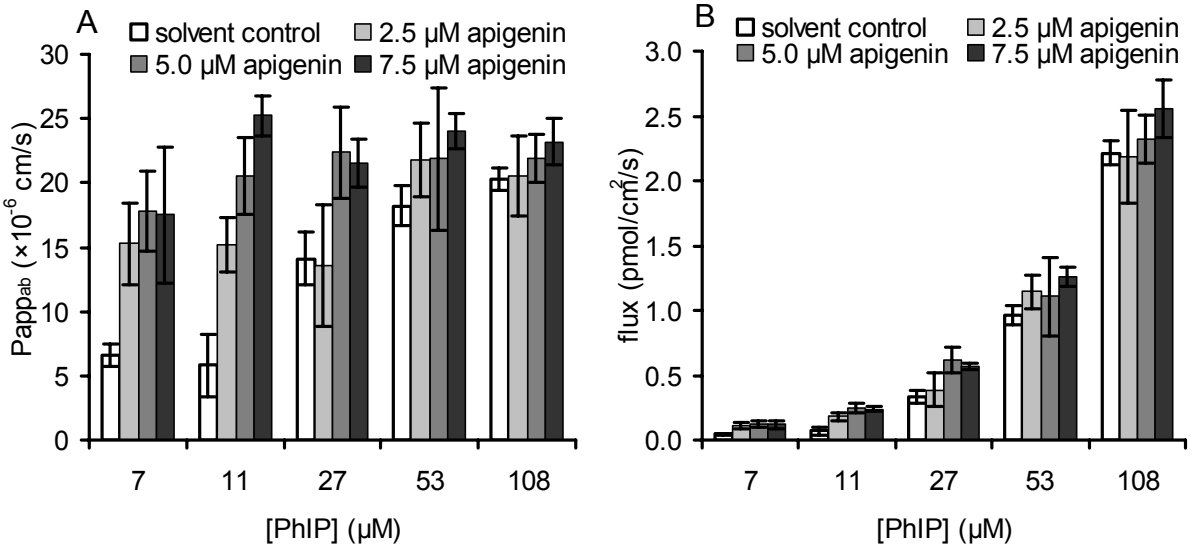


Figure 2 The effect of the presence of 0, 2.5, 5.0, or 7.5 µM apigenin on the transport of increasing concentrations of PhIP through the Caco-2 monolayers, represented by (A) the apparent permeability coefficient of PhIP transported through Caco-2 monolayers from the apical to the basolateral compartment ($Papp_{ab}$) and (B) the cumulative flux of PhIP through the Caco-2 monolayer from the apical to the basolateral compartment.

For the uninhibited PhIP flux through the Caco-2 monolayer the model predicts a passive diffusion constant of 3.7×10^{-5} cm/s, an apparent Vmax value of 2.9×10^{-6} µmol/cm^2/s and an apparent Km value of 86.4 µM. Additional experiments (n=16) described below, each also providing data for the uninhibited PhIP transport, revealed an overall average value for the passive diffusion constant of 3.8×10^{-5} ($\pm0.4\times10^{-5}$) cm/s, for the apparent Vmax value the average value amounted to 3.0×10^{-6} ($\pm0.5\times10^{-6}$) µmol/cm^2/s and for the apparent Km the average value was 89 (±12) µM.

The effect of increasing concentrations of apigenin on the transport of different concentrations of PhIP through Caco-2 monolayers

Figure 2A depicts the $Papp_{ab}$ values for the transport of increasing concentrations of PhIP through the Caco-2 monolayer in the presence of increasing concentrations apigenin. The flavonoid apigenin caused an increase in the $Papp_{ab}$ value of PhIP in a concentration dependent manner up to 53 µM PhIP. At 108 µM PhIP the concentrations of apigenin used (≤ 7.5 µM) were no longer capable of increasing the PhIP transport to a significant extent. Figure 2B presents the flux data of PhIP in the absence and presence of apigenin. The flavonoid apigenin increased the transport of PhIP in a concentration dependent manner up to 108 µM PhIP.
The apparent Ki value for apigenin was determined by fitting the flux values to the kinetic Equations 2 and 3. Using the respective passive diffusion constant, apparent Vmax and apparent Km values and the flux values for the transport of PhIP in the presence of apigenin (Figure 2B), the model predicts an apparent Ki value of 10.8 µM for apigenin.

The effect of different binary flavonoid mixtures on the transport of PhIP through Caco-2 monolayers

Figures 3A-D depict the flux of 10 µM PhIP in the presence of increasing concentrations of naringenin and luteolin (NL), naringenin and myricetin (NM), naringenin and quercetin (NQ) and naringenin and apigenin (NA). The effects of the individual flavonoids on the transport of PhIP for quercetin, naringenin, luteolin and myricetin are described in Schutte *et al.* (*32*) and for apigenin in Figure 2.
The presence of the different flavonoid mixtures caused an increase in the flux of 10 µM PhIP in a concentration dependent manner up to 0.3×Ki value for the

individual flavonoid. At higher flavonoid concentrations PhIP transport was no longer increased in a concentration dependent manner.

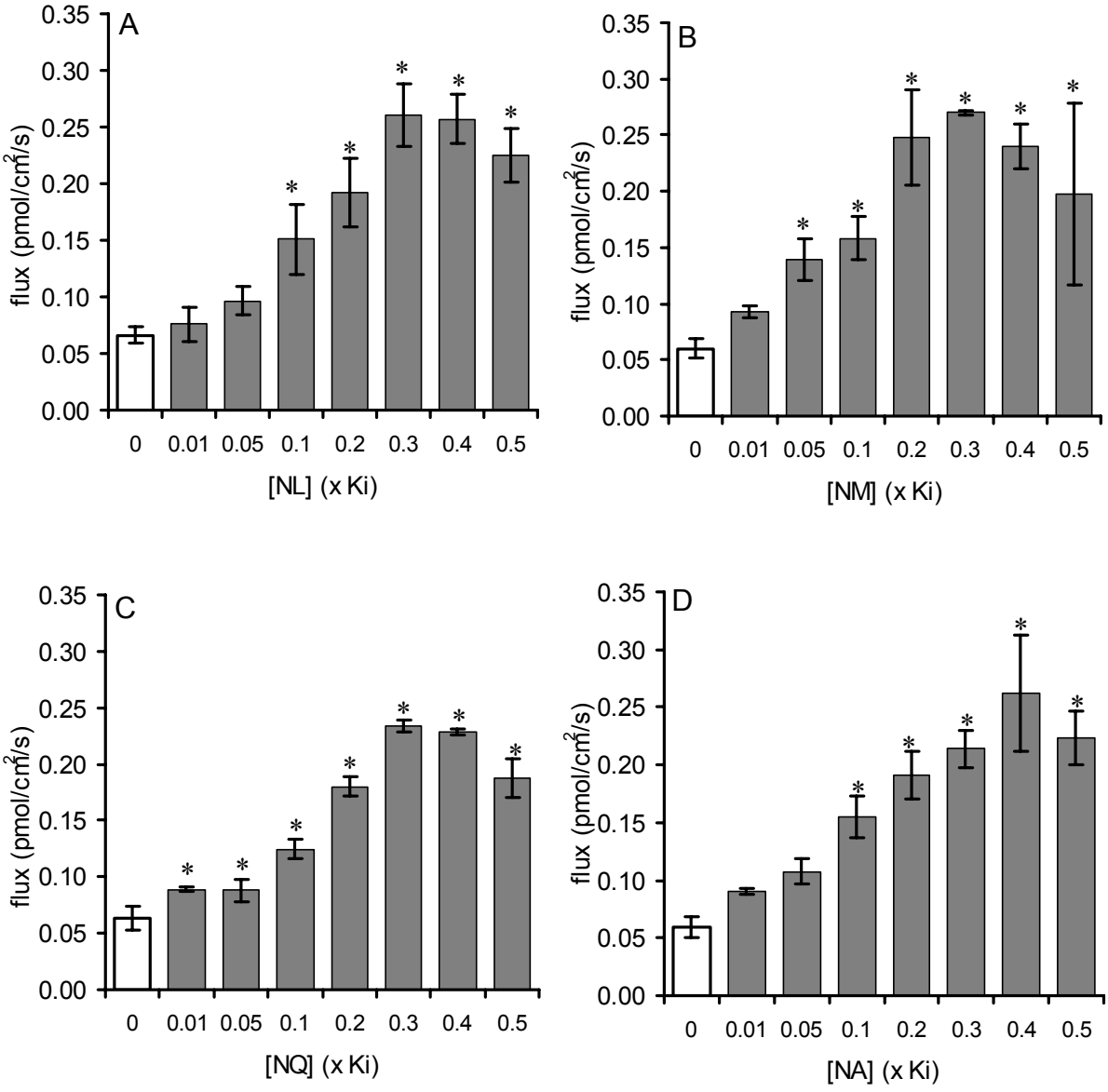


Figure 3 Concentration dependent effects of binary flavonoid mixtures on cumulative flux values for transport of 10 μM PhIP from the apical to the basolateral compartment through the Caco-2 monolayer. Data are presented as mean ± SD from (n=3). NL: the combination of naringenin and luteolin; NM: the combination of naringenin and myricetin; NQ: the combination of naringenin and quercetin; NA: the combination of naringenin and apigenin.

Kinetic modelling of the transport of PhIP in the presence of combinations of two flavonoids

To be able to describe the nature of the interaction of the two flavonoids on the transport of PhIP the experimental data were used for kinetic modelling.
Using the P, apparent Vmax and apparent Km values obtained from the uninhibited PhIP transport, the apparent Ki values for the inhibition of the apparent transporter by the individual flavonoids and Equation 4, the PhIP flux in the presence of several binary flavonoid mixtures can be predicted. Figure 4 depicts the experimentally observed flux for three concentrations PhIP (10, 50 and 100 μM) in the presence of several concentrations of a mixture of two flavonoids plotted against the flux predicted by the kinetic model. For the four flavonoid combinations tested the model adequately predicts the experimental PhIP flux. The correlation between these data points varies between r^2=0.94 for NL and r^2=0.98 for NA.

The effect of a mixture of five flavonoids on the transport of PhIP through the Caco-2 monolayer

Figure 5A depicts the effect of increasing concentrations of the combination of all five flavonoids (NQMLA) on the transport of 10 μM PhIP. The presence of increasing concentrations of NQMLA caused a concentration dependent increase in the flux of PhIP at all tested concentrations of the combination.
Figure 5B depicts the experimentally observed PhIP flux versus the PhIP flux predicted by the model. The predicted PhIP flux was determined using Equation 5 and the kinetic constants P, the apparent Vmax and apparent Km as determined for the uninhibited PhIP transport and the apparent Ki values for every individual flavonoid. The predictions for 10 and 50 μM PhIP are in good correspondence with the experimentally observed flux, whereas at 100 μM PhIP the predictions by the model are somewhat less accurate but still adequate because the deviation of the predicted flux as compared to the experimentally observed flux is between 0.5 and 16% (Figure 5B). The overall correlation between the predicted and the observed flux is r^2=0.90. Altogether this shows that the model predicts the PhIP flux and that our data can be fitted adequately assuming competitive inhibition and that the effect of flavonoid mixtures can be described by concentration-additivity.

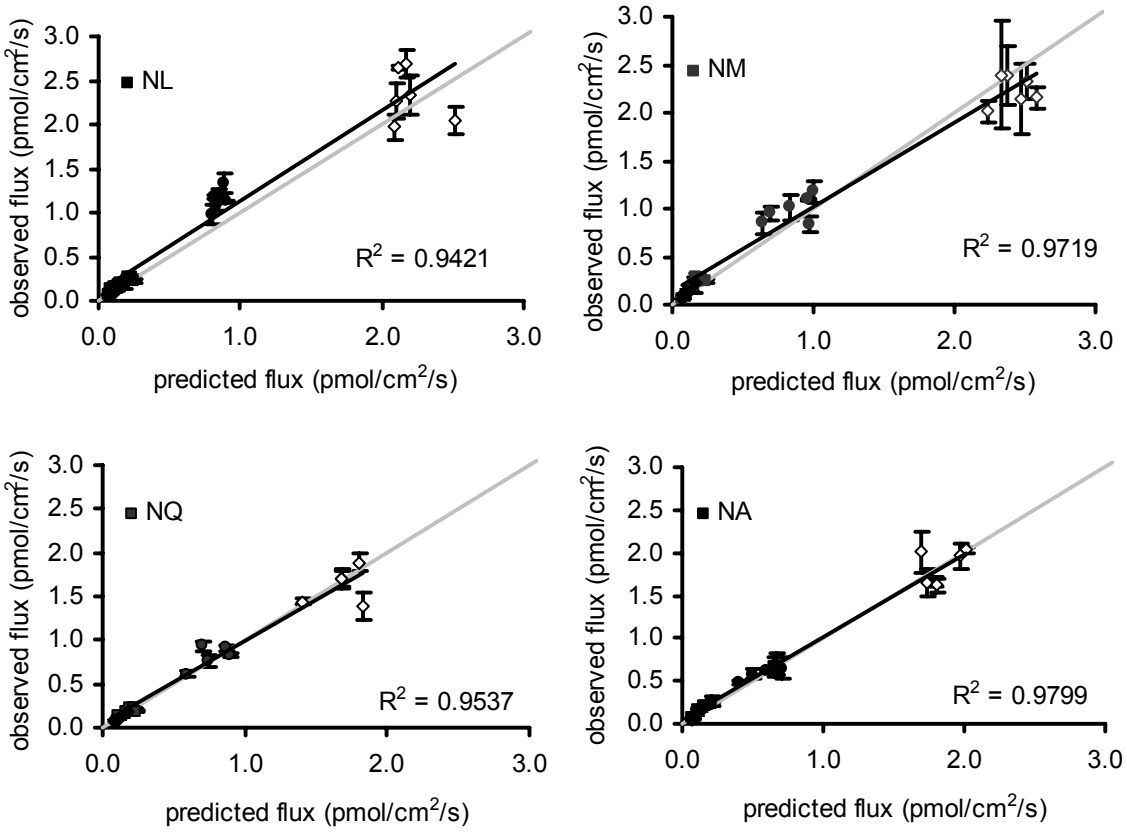


Figure 4 The experimentally observed PhIP flux (10 (■), 50 (•) and 100 (◊) μM PhIP) in the presence of different binary mixtures of flavonoids, plotted against the PhIP flux predicted by the model. The grey line presents the theoretical relationship when the observed and predicted flux are equal, whereas the black line presents the relationship calculated on the bases of the actual data points. Data are presented as mean ± SD from a triplicate experiment. NL: the combination of naringenin and luteolin; NM: the combination of naringenin and myricetin; NQ: the combination of naringenin and quercetin; NA: the combination of naringenin and apigenin.

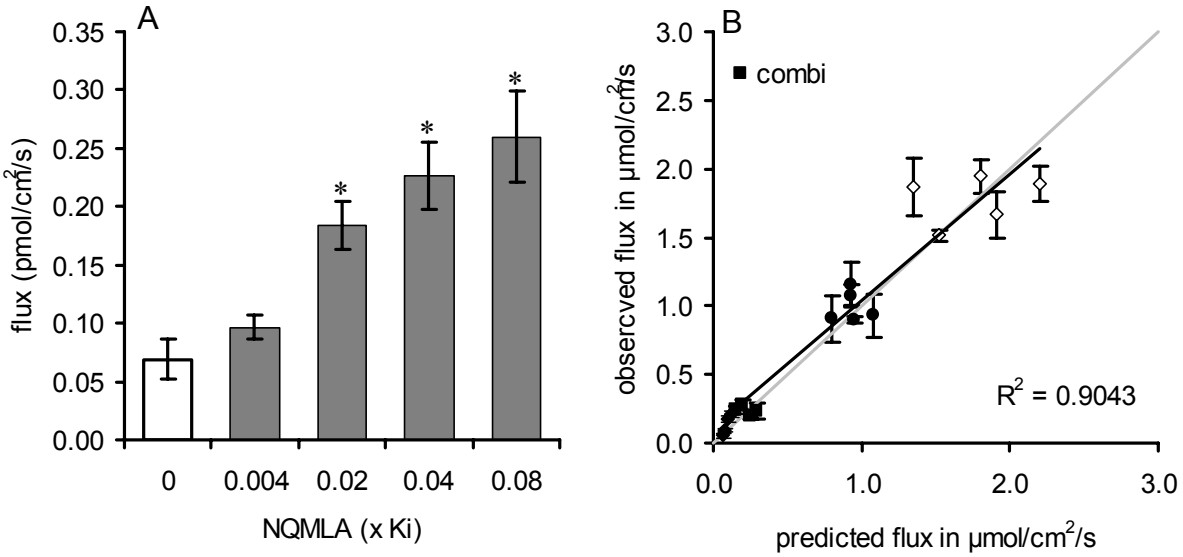


Figure 5 The effect of the presence of a combination of the five model flavonoids on the transport of PhIP through the Caco-2 monolayer represented by (A) the cumulative flux of 10 µM PhIP in the presence of a combination of five flavonoids, and (B) the experimentally observed PhIP flux (10 (■), 50 (•) and 100 (◊) µM PhIP) plotted against the PhIP flux predicted by the model. The grey line presents the theoretical relationship when the observed and predicted flux are equal, whereas the black line presents the relationship calculated on the bases of the actual data points. Data are presented as mean ± SD from a triplicate experiment.

Discussion

The present study shows that flavonoid mixtures increase the apical to basolateral transport of PhIP through Caco-2 monolayers and presents a kinetic model to describe the effect of flavonoid mixtures on PhIP transport. The kinetic model was based on a previously developed *in silico* kinetic model for the transport of the pro-carcinogen PhIP through Caco-2 monolayers assuming passive diffusion counteracted by a flavonoid sensitive apparent active transporter. The kinetic model is based on the following assumptions: the transport of PhIP over the Caco-2 monolayer consists of passive diffusion minus active transport by an apparent transporter. This apparent transporter describes the activity of all efflux transporters present in the Caco-2 cell in both the apical and the basolateral membrane by combining them in one apparent transporter located in the apical membrane. Furthermore, in the kinetic model passive diffusion from the basolateral to the apical compartment was neglected since PhIP transport was determined under sink conditions (<10% of PhIP is transported from the apical to the basolateral

compartment). Also, it is assumed that the flavonoids act as competitive inhibitors and the combined effect on the apparent transporter can be described in a concentration-additive way.

This model can be used to determine apparent Ki values for inhibition of the apparent transporter by flavonoids resulting in a net increase in apical to basolateral transport of PhIP. In the present study the effect of a combination of flavonoids on the transport of PhIP was determined since the human diet contains several flavonoids originating from fruit, vegetables, nuts and tea. All tested flavonoid mixtures were capable of increasing the PhIP transport in a concentration dependent manner.

This increase in PhIP transport from the apical to the basolateral compartment may in theory be caused by the inhibition of apical transporters which are responsible for transport of PhIP back to the intestinal lumen thereby reducing apical to basolateral transport. Rodent studies showed that the Bcrp1 transporter in mice (*29*) and the MRP2 transporter in rats (*28*) and mice (*31*) are involved in the excretion of PhIP back to the intestinal lumen. Studies which used Caco-2 monolayers and typical inhibitors showed that P-gp, BCRP and MRP2 are involved in the transport of PhIP from the cells back to the apical compartment (*30, 32, 33*). Furthermore, several studies reported that flavonoids are capable of inhibiting ABC transporters including MRP2 and/or BCRP (*13, 19, 38*).

In this study the apparent Ki value for the apparent transporter of the flavonoid apigenin was determined to be 10.8 μM and thus similar to the apparent Ki values previously determined for the flavonoids, naringenin, luteolin, quercetin and myricetin. The potency of the model flavonoids of the present study for the inhibition of the apparent transporter decrease in the order: quercetin (5.6 μM) > apigenin (10.8 μM) ≈ naringenin (11.7 μM) ≈ luteolin (12.2 μM) > myricetin (37.3 μM).

Zhang *et al*. (*17*) determined the EC_{50} values of flavonoids for their effect on mitoxantrone accumulation in MCF-7 MX100 cells which is a BCRP over-expressing cell line. They showed that apigenin was approximately 20 times as potent as the flavonoid naringenin with EC_{50} values amounting to 1.66 μM and 32 μM respectively. In our study where apparent Ki values instead of EC_{50} values were determined the effect of both flavonoids on the PhIP flux by the apparent transporter was approximately equal. In addition, Ahmed-Belkacem *et al.* (*39*) also reported that apigenin was a more potent inhibitor of the BCRP mediated mitoxantrone efflux compared to naringenin in HEK-293 cells transfected with

BCRP, with IC_{50} values of 16 μM and 37 μM respectively. Leslie *et al.* (*21*) showed in membrane vesicles that flavonoids are capable of inhibiting MRP1, with a potency of the flavonoids to inhibit LTC_4 transport that can be ranked as follows: apigenin > quercetin > myricetin > naringenin. Whereas, van Zanden *et al.* (*19*) showed in MDCKII-MRP1 cells the following rank order in potency: myricetin ≈ quercetin ≈ luteolin > apigenin> naringenin. The difference in potency of apigenin and naringenin in the different studies compared to our study might be caused by the differences in cell system, which might differ in uptake characteristics and contribution of different transport proteins responsible for the effects studied. The study of Zhang *et al*. (2004a) used MCF-7 cells selected for their relatively high level of BCRP expression and Ahmed-Belkacem *et al.* (2005) used BCRP transfected cells, whereas we used Caco-2 cells which are known to express multiple transporters, including P-gp, MRP1 through MRP6 and BCRP (*7, 9, 10, 35*). In addition, the studies above used different substrates namely mitoxantrone used by Zhang *et al*. (2004a) and Ahmed-Belkacem *et al.* (2005) versus PhIP used in our study. Furthermore, our study determined apparent Ki values of the flavonoids for the apparent transporter and this apparent transporter comprises the effect of all transporters present in the Caco-2 monolayer, thus the apparent Ki value comprises the effect of the flavonoid on more than one transporter. Furthermore, whereas we determined apparent Ki values, which are independent of the concentration of the substrate of which the transport is monitored, the other studies reported EC_{50} and IC_{50} values which may vary with the concentration of the molecule of which the transport is investigated e.g. mitoxantrone.

It is also important to note that Caco-2 cells are known to express several metabolizing enzymes which may metabolize the flavonoids into their methylated, sulphated, and glucuronidated metabolites. However not only the flavonols themselves are capable of inhibiting ABC transporters but also flavonoid metabolites are capable of inhibiting the ABC transporters (*40, 41*). This inhibition may be due to the fact that flavonoids but also their metabolites may act as substrates for the ABC transporters themselves, thus also explaining the competitive nature of the inhibition. Walgren *et al*. (*41*) showed that quercetin is transported through the Caco-2 monolayer with a preferred transport direction to the apical compartment suggesting that an apical transporter may be involved. Sesink *et al*. (*40*) showed that quercetin is transported by the Bcrp1 transporter in MDCKII-Bcrp1 cells.

The model described in the present study is a first approximation to model the effect of mixtures of flavonoids on the transport of PhIP through Caco-2 monolayers. The interaction of the flavonoids on the transport of PhIP is assumed to occur following a mechanism that can be described by concentration-addition. This result is in line with a study reported by Zhang *et al.* (*17*) that showed that the flavonoids apigenin, biochanin A and chrysin act additively on mitroxantrone accumulation.

In the literature several other models have been described to predict the absorption of drugs or xenobiotics in cells (*42-44*). However, all the models described so far take into account the effect of the inhibition of only one transporter, and use models in which one transporter is known to dominate the transport. The model presented in this study is capable of determining the effect of inhibitors on the sum of all transporters involved in a transport process and is capable of modelling the overall effect of the flavonoids on the transport of PhIP.

We showed that flavonoid mixtures are capable of increasing the transport of the pro-carcinogen PhIP from the apical to the basolateral compartment in Caco-2 monolayers. The flux observed experimentally appeared to correlate well with the flux predicted by the model (Figure 4 and Figure 5B). Therefore it can be concluded that the model accurately predicts the PhIP flux and that our data can be fitted assuming concentration-addition between the flavonoid inhibitors.

The results found in studies using Caco-2 monolayers are usually in good correlation with human absorption studies (*34*). However, we have to be careful extrapolating these findings from the *in vitro* situation to the *in vivo* situation, since discrepancy exists between the expression of ABC transporters in the Caco-2 monolayer and their expression in rodents or humans (*9, 10, 45*).

Given the intestinal flavonoid concentrations that might be reached upon supplement intake and the concentrations at which the flavonoid mixtures were able to affect PhIP transport in the present study, it can be concluded that the interaction of the flavonoids with ABC transporters occurs at physiologically plausible flavonoid concentrations. Thus the flavonoid-mediated increase in PhIP transport through Caco-2 monolayers reported in the present study may point at a possible increased bioavailability of PhIP in the presence of flavonoid mixtures in the *in vivo* situation. This would imply an adverse effect of these supposed beneficial food ingredients since an increase in the bioavailability of PhIP might lead to an increase in the carcinogenic PhIP metabolite formed. Therefore, validation of the present results in an *in vivo* intestinal model will confirm whether

our *in vitro* results can be extrapolated to the *in vivo* situation. These studies are currently underway in our laboratory.

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Quercetin increases the bioavailability of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in rats

Maaike E. Schutte, Gerrit M. Alink, Andreas P. Freidig,
Bert Spenkelink, Judith Vaessen, Johannes J. M. van de Sandt,
John P. Groten and Ivonne M.C.M. Rietjens

Submitted

Abstract

This study investigates whether the previous observation that quercetin increases the transport of PhIP through Caco-2 monolayers *in vitro* could be confirmed in an *in vivo* rat model.

Co-administration of 0.09 mg PhIP and 10 mg/kg/bw quercetin significantly increased the blood $AUC_{(0\text{-}8hr)}$ of PhIP in rats to 131±14% of the $AUC_{(0\text{-}8hr)}$ for rats dosed with PhIP alone. Significantly increased blood PhIP levels were detected at 15 minutes (247% of control), 30 minutes (169% of control), 45 minutes (247% of control) and 180 minutes (187% of control). At 4 and 8 hours post-dosing a difference in the PhIP levels in the blood between the two treatment groups was no longer observed.

In vitro and *in silico* modelling of PhIP transport using a Caco-2 transwell system and a previously developed kinetic model for PhIP transport through the Caco-2 cells revealed that the relative increase in PhIP transport induced by quercetin is dependent on the actual concentration of the two compounds. When substituting the PhIP and quercetin concentrations used in the *in vivo* rat experiment in the kinetic *in silico* model, the model predicted an effect of quercetin on PhIP transport that matches the actual effect of 131% observed *in vivo*. At physiologically relevant concentrations of PhIP and quercetin the effect was predicted to amount to 392%.

It is concluded that quercetin increases the bioavailability of the pro-carcinogen PhIP in rats pointing at a potential adverse effect of this supposed beneficial food ingredient.

Introduction

The transport of food components, drugs and xenobiotics across the intestinal epithelium is an important factor determining the bioavailability upon oral intake. Uptake of compounds in the intestine may be facilitated by transport proteins e.g. SGLT1, but may also be influenced by ATP binding cassette transporters (ABC transporters). ABC transporters are known to efficiently excrete physiological substrates and xenobiotics out of the cell. In the intestinal epithelium several ABC transporters are present in the apical membrane of the enterocyte, catalyzing the efflux of compounds to the intestinal luminal side, and these include P-gp, MRP2 (ABCC2), and BCRP (ABCG2) (*1-5*). MRP1 (ABCC1), MRP3 (ABCC3) and MRP5 (ABCC5) are localized in the basolateral membrane of the enterocyte catalyzing the efflux of compounds to the serosal side (*6-10*).

Previously, we showed that the flavonoid quercetin (Figure 1A) increases the transport of the food-born pro-carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP, Figure 1B) through Caco-2 monolayers (*11*). It was also demonstrated that this increase in the transport of PhIP through the Caco-2 monolayer is probably due to the quercetin-mediated inhibition of BCRP and possibly MRP2 (*11, 12*). The role of ABC transporters in the bioavailability of PhIP is corroborated by results from studies with Caco-2 cell monolayers using typical inhibitors revealing that P-gp and MRP2 are involved in the efflux of PhIP (*12, 13*). Also studies using MDCKII-*bcrp1* transfected cells have showed that BCRP is involved in the efflux of PhIP to the apical compartment (*14*). In addition, studies in rodents showed that MRP2 (*15, 16*) and Bcrp1 (*14*) are involved in the efflux of PhIP back to the intestinal lumen.

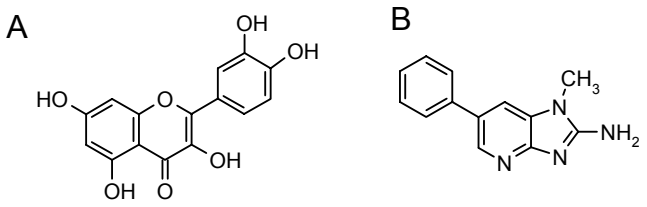

Figure 1 Structure of (A) quercetin and (B) PhIP.

The flavonoid quercetin is present in fruits and vegetables, especially in apples and onions (*17*). Results from epidemiological studies point at protective effects of flavonoids against cardiovascular diseases and certain forms of cancer (*18-20*). Quercetin is capable of inhibiting ABC transporters *in vitro*, including P-gp (*21*) MRP1 (*22-24*), MRP4 (*25*), MRP5 (*25*) and BCRP (*26*). Furthermore, Sesink *et al.* (*26*) showed that quercetin is transported by Bcrp1 in rats. Given the supposed beneficial health effects of flavonoids including quercetin and the commercial availability of quercetin supplements, it is of interest to study whether the previously observed effect of quercetin on PhIP transport through Caco-2 monolayers could be confirmed in an *in vivo* model.

Therefore the aim of the present study was to investigate whether in an *in vivo* rat model quercetin would increase the bioavailability of PhIP when quercetin and PhIP would be co-administered orally as a single dose. To study this Wistar Hannover rats were co-administered with ^{14}C labelled PhIP in the absence or presence of quercetin and the plasma levels of PhIP were determined at time intervals for up to 8 hours following oral administration.

The results provide insight in the ability of quercetin to modulate the bioavailability of a pro-carcinogen *in vivo* and in the possibility of extrapolating *in vitro* results to the *in vivo* situation.

Materials and Methods

Materials

Radioactive ^{14}C-labeled 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP; 98% purity) with specific activity (10 mCi/mmol) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). The flavonoid quercetin dihydrate (95% purity) and dimethyl sulfoxide (DMSO; 99.9% purity) were obtained from Across Organics (Geel, Belgium). Hydroxypropyl-β-cyclodextrin (Molecular Substitution 0.6), corn oil and 30% hydrogen peroxide solution were obtained from Sigma Aldrich (Steinheim, Germany). Soluene-350 and Hionic Fluor were obtained from PerkinElmer (Boston, MA).

Animals

The experimental protocol was approved by the Animal Welfare Committee of Wageningen University. Male Wistar Hannover rats (275 ± 7.5 g) were obtained at 8 weeks of age from Harlan, The Netherlands and housed at the Laboratory Animal Centre (Wageningen University, The Netherlands). The rats were kept under standard conditions, temperature of 22±1 °C, 12 hours light/dark cycle and a humidity of 50% and fed *ad libitum* with a standard diet (Hope farms, Woerden, The Netherlands). The rats were acclimatized for one week before the experiments.

Determination of the systemic bioavailability of PhIP

Rats were fasted for four hours prior to the experiments; drinking water remained available during the fasting period and the experiments. The experiments were started by oral gavage of 1 mL per rat of the test solution. Rats were administered with 0.09 mg (4 μCi, 1.45 μmol/kg bw) radioactive labelled PhIP (0.8% final concentration DMSO) in the presence or absence of 10 mg/kg bw (30 μmol/kg bw; 20-fold excess) quercetin in 15 w/v% hydroxypropyl-β-cyclodextrin. Both solutions had a pH of 6.3. The experimental groups exposed to PhIP or PhIP in the presence of quercetin consisted of 8 and 9 animals per group, respectively. Blood samples of 0.15 mL blood were taken from the tail vein prior to exposure to the test compounds and at 15, 30, 45, 60, 75, 120, 180, 240 and 480 minutes after dosing. After the last blood sample the rats were sacrificed by CO_2 gas and the liver, small intestine, colon, cecum and kidneys were collected from the rat. The blood samples were stored at 4 ^{o}C and the organs were stored at -80 ^{o}C until analysis.

Tissue sample analyses

To the blood samples 1 mL of soluene-350 was added, the samples were incubated for two hours at 50 ^{o}C to dissolve the blood samples and obtain a homogeneous solution. The samples were cooled to room temperature and 1.5 mL 30% hydrogen peroxide solution was added, after which the samples were incubated for 30 minutes at room temperature followed with a 30 minute incubation at 50 ^{o}C to bleach the samples and prevent quenching of the samples in the scintillation counter. Hereafter, 12 mL counting liquid was added and after 12 hours (to allow the formation of a stable mixture) the samples were counted in a Packard 1600 liquid scintillation counter (LSC, 1600 TR, Packard, Downer's grove, IL).

To determine the PhIP content in the liver, kidney, small intestine, colon and cecum, the tissues were weighed, 1 mL distilled water was added to the liver and kidney and 2 ml distilled water was added to the small intestine, colon and cecum, and the samples were homogenized using an Ultra Turrax T25 (Janke & Kunkel Ika-Labortechnik). Then, 0.1 mL of the tissue homogenate was dissolved in 1 mL soluene-350 to obtain a homogenized solution, the samples were incubated for two hours at 50 ^{o}C. The samples were cooled to room temperature and 0.7 mL 30% hydrogen peroxide solution was added, after which the samples were incubated for 30 minutes at room temperature followed with a 30 minute incubation at 50 ^{o}C to bleach the samples and prevent quenching during the radioactivity measurement. Hereafter, 12 mL counting liquid was added and after 12 hours the samples were counted in a Packard 1600 liquid scintillation counter (LSC, 1600 TR, Packard, Downer's grove, IL).

Determination of the flux for apical to basolateral transport of PhIP through the Caco-2 monolayer in the presence and absence of quercetin

The Caco-2 cells were maintained in culture and transport experiments were performed as described previously (*11, 12*). During the transport experiments the Caco-2 cells were exposed in the apical compartment to PhIP (at 1.1 or 2.7 µM final concentration all in the presence of 0.25% DMSO) and in both compartments to quercetin (at 0, 25 and 40 µM final concentration all in the presence of 0.5% DMSO) and 1 mM ascorbic acid (to prevent auto-oxidation of the flavonoids). After 0, 30, 60, and 120 minutes, 200 µl aliquots were taken from the basolateral compartment and the volume was replenished to the initial volume with transport medium containing the relevant quercetin concentration and 1 mM ascorbic acid. The concentration of PhIP was determined in the donating compartment at the beginning and at the end of the experiment. Only monolayers with a transepithelial electrical resistance value of >500 $\Omega \times cm^2$ at the end of the experiment were included.
The flux was calculated according to:

$$Flux = (dQ/dt)/A \qquad \text{Equation 1}$$

In which dQ/dt is the initial transport velocity (nmol/s) and A the surface area of the membrane insert (cm^2) (*27, 28*).

The samples were stored at –20 ºC until analysis for PhIP by high performance liquid chromatography (HPLC).
The concentrations of PhIP and quercetin used were not cytotoxic as demonstrated using the LDH leakage test (data not shown) (*29*).

Kinetic modelling of trans-epithelial transport of PhIP through Caco-2 monolayers

Previously, we developed a kinetic model which describes the effect of quercetin on apical to basolateral PhIP transport through Caco-2 monolayers (*11*). This model was based on the following assumptions: i) the apical to basolateral PhIP transport consists of a passive diffusion component minus an apparent active transport component back to the apical compartment, ii) all the efflux transporters, both apical and basolateral, present in the Caco-2 cells can be modelled as one apparent efflux transporter localized in the apical membrane, iii) the active transport of PhIP from the intracellular to the apical compartment by the apparent transporter follows Michaelis-Menten kinetics, iv) the intracellular concentration of PhIP is equal to the concentration of PhIP in the apical compartment, and v) the passive diffusion from the basolateral to the apical compartment can be neglected since experiments are performed under sink conditions (<10% of PhIP is transported from the apical to the basolateral compartment). To summarize the previously developed model, Equation 2 describes the data without a flavonoid inhibitor and Equation 3 the situation with one flavonoid inhibitor (*11*)

$$v = P \times [S] - V\max \times \frac{[S]}{[S] + Km} \qquad \text{Equation 2}$$

$$v = P \times [S] - V\max \times \frac{[S]}{[S] + Km \times (1 + \frac{[I]}{Ki})} \qquad \text{Equation 3}$$

With:
v: flux (µmol/cm^2/s)
P: passive diffusion constant (cm/s)
$[S]$: initial PhIP concentration (µM)
Vmax: apparent maximum velocity of active PhIP transport (µmol/cm^2/s)
Km: apparent Michaelis-Menten constant (µM)

[I]: initial flavonoid concentration in the medium (μM)
Ki: apparent inhibition constant (μM)

The passive diffusion constant, the apparent Vmax and the apparent Km for the uninhibited transport were determined on the basis of experimental data for the transport of PhIP through Caco-2 monolayers exposed to 1.1, 2.7, 5.8, 20 and 39 μM PhIP in the absence of quercetin. Using the passive diffusion constant and the apparent Vmax value and apparent Km value as determined by the model on the basis of the data for uninhibited transport, and the previously determined apparent Ki value for quercetin of 5.6 μM (*11*), it was possible to model the effect of 20-fold quercetin excess on the transport of PhIP through Caco-2 monolayers at different concentrations of PhIP, as well as the relative quercetin mediated changes in PhIP transport to be expected at other quercetin to PhIP ratios and at physiological concentrations of PhIP and quercetin.

High performance liquid chromatography analysis

The amount of PhIP in the samples from the Caco-2 cell incubations was quantified using the HPLC method described previously (*11*). Briefly, an Alltima^{TM} C18 Reversed-Phase column (5μ; 150 x 4.6 mm reverse phase column; Alltech Applied Sciences, Breda, The Netherlands) was used with an isocratic mobile phase of 35% (v/v) acetonitrile in 50 mM ammonium acetate (pH 5), a flow rate of 1.0 ml/min, UV detection at 315 nm and a calibration curve made with commercially available PhIP.

Statistical analysis

Results are presented as mean ± SEM. After the normal distribution of the data was confirmed by the Kolmogorov-Smirnov test statistical evaluation of data was performed by repeated measures ANOVA ($P<0.05$).
The significant difference in radioactivity levels in the organs between treatments was assessed using a one tailed Student's *t*-test ($P<0.05$).

Results

Systemic bioavailability of PhIP in rats exposed to PhIP in the absence and presence of quercetin.

Figure 2 depicts the time dependent concentration of PhIP in the tail vein blood of rats orally dosed with 0.09 mg PhIP (1.45 μmol PhIP/kg bw) either in the absence or in the presence of 10 mg/kg bw (30 μmol/kg bw) quercetin.

Upon dosing with PhIP for both experimental groups a time dependent increase in PhIP levels in the blood was observed followed by a decrease. For rats dosed with PhIP alone the peak blood level of PhIP (Cmax) was detected at 67±4.3 minutes after administration, whereas the peak blood level of PhIP of rats dosed with PhIP in the presence of quercetin was detected at 53±10 minutes. The results depicted in Figure 2 also reveal that quercetin co-administration at a dose of 10 mg/kg bw significantly increased the bioavailability of PhIP. The blood $AUC_{(0\text{-}8hr)}$ in rats dosed with PhIP in the presence of quercetin amounted to 3.0±0.3 nmol/min which was 131±14% ($p<0.02$) of the $AUC_{(0\text{-}8hr)}$ for control rats dosed with PhIP alone which amounted to 2.3±0.4 nmol/min. Significantly increased plasma PhIP levels were detected at 15 minutes (247% of control, $P<0.03$), 30 minutes (169% of control, $P<0.0003$), 45 minutes (247% of control, $P<0.01$) and 180 minutes (187% of control, $P< 0.03$). At 4 and 8 hours post-dosing a difference in the PhIP levels in the blood between the two treatment groups was no longer observed.

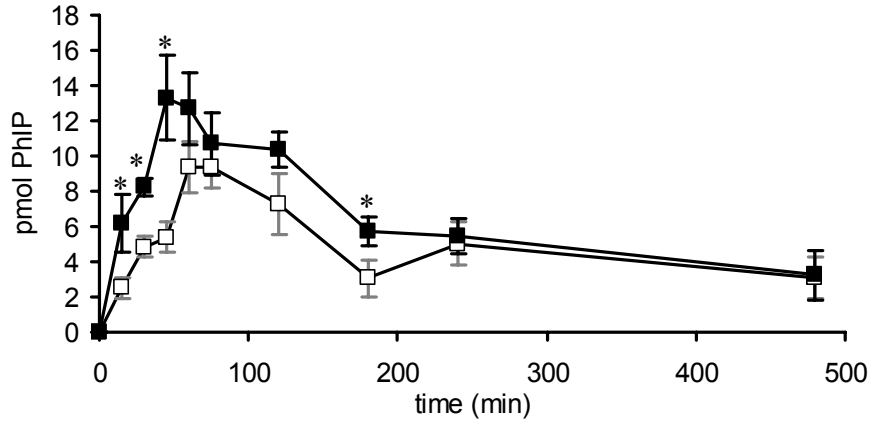

Figure 2 Amount of PhIP in tail vein blood of rats exposed to 0.4 μmol ^{14}C-labeled PhIP in the absence (□) and presence (■) of quercetin at 10 mg/kg bw (30 μmol/kg bw). Data are presented as mean ± SEM for n=8 rats for PhIP and n=9 rats for PhIP in the presence of quercetin.

PhIP concentration in several organs of rats administered with PhIP in the presence and absence of quercetin

Figure 3 depicts the amount of PhIP present in the liver, kidney, small intestine, colon and cecum of rats administered with 1.45 μmol PhIP/kg bw in the absence and presence of 30 μmol/kg bw (20-fold excess) quercetin 8 hours after the administration of the test solutions by oral gavage. The average organ weights were as follows: liver 11.5±0.24 g, kidney 1.2±0.09 g, small intestine 7.23±0.21 g, colon 1.1±0.5 g and cecum 0.46±0.02 g.

The PhIP levels in the cecum were significantly lower in rats co-administered with PhIP and quercetin compared to the rats exposed to PhIP alone, whereas no significant difference in the PhIP levels in the liver, kidney, small intestine and colon was observed between the two treatment groups.

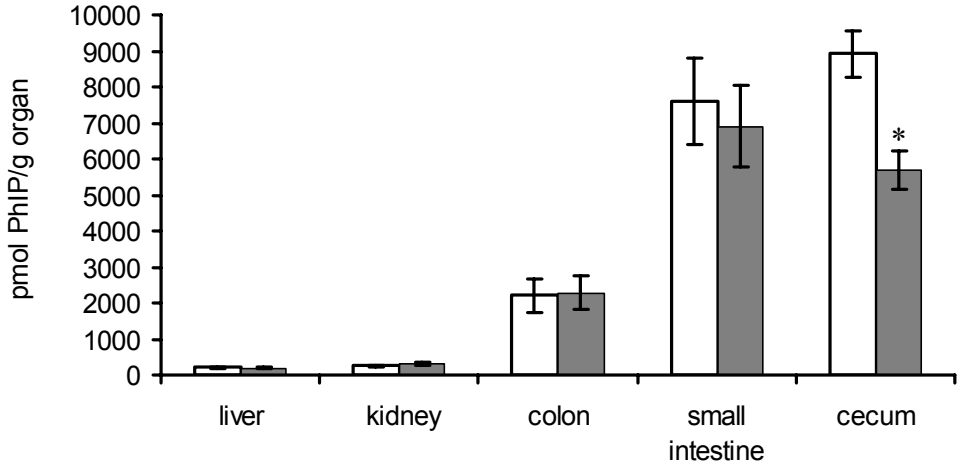

Figure 3 Amount of PhIP in the liver, kidney, small intestine, colon and cecum of rats exposed to 0.4 μmol ^{14}C-labeled PhIP in the absence (□) and presence (■) of quercetin at 10 mg/kg bw (30 μmol/kg bw). Data are presented as mean ± SEM for n=8 rats for PhIP and n=9 rats for PhIP in the presence of quercetin.

The effect of increasing quercetin to PhIP ratios and increasing PhIP and quercetin concentrations on the flux values of PhIP through Caco-2 monolayers

Previous studies using Caco-2 monolayers revealed a flux value for apical to basolateral transport of 5 μM PhIP through Caco-2 monolayers in the presence of 7.5 μM quercetin that amounted to 380% of the flux for apical to basolateral PhIP

transport in the absence of quercetin. Since in the rat model of the present study the systemic PhIP bioavailability in the presence of quercetin amounted to only 131% of the PhIP bioavailability in the absence of quercetin it was investigated whether this difference would be due to the different PhIP and quercetin concentrations and the higher quercetin to PhIP ratio applied in the rat study as compared to the *in vitro* Caco-2 model. The ratio quercetin to PhIP was 1.5 in the *in vitro* Caco-2 study as compared to 20 in the rat model of the present study. Furthermore, assuming only two-fold dilution of the quercetin and PhIP upon dosing by oral gavage and transfer to the intestine, the intestinal concentrations of quercetin and PhIP are estimated to be about 4 mM and 200 μM and thus respectively 533 and 40 fold higher than the concentrations used in the Caco-2 model.

Figure 4a depicts the flux values for the transport of PhIP (at 1.1 and 2.7 μM) through the Caco-2 monolayer in the absence and presence of about 20-fold higher concentrations of quercetin (25 and 40 μM quercetin respectively), the quercetin to PhIP ratio used in the *in vivo* study.

For Caco-2 cells exposed to 1.1 μM PhIP in the presence of 25 μM quercetin the increase in flux value amounted to 367±49% of the flux value for PhIP in the absence of quercetin. The flux value for 2.7 μM PhIP in the presence of 40 μM quercetin amounted to 320±36% of the flux value for PhIP in the absence of quercetin. These values are not much lower than the 380% increase in $Papp_{ab}$ found in the Caco-2 model when using 7.5 μM quercetin and 5 μM PhIP (ratio 1.5). Thus, the reduction in flux when increasing the quercetin to PhIP ratio from 1.5 to 20 does not account for the reduction to the value of 131% bioavailability in the presence of quercetin found in the *in vivo* rat experiment of the present study as compared to 380% observed in the Caco-2 model system. Therefore the lower effect of quercetin on PhIP bioavailability in the *in vivo* study than in the *in vitro* Caco-2 model can not be ascribed to the 20 instead of 1.5 fold excess of quercetin used.

More likely the effect may be caused by the different concentrations of PhIP (1.1 to 5 μM in the Caco-2 experiments and 200 μM estimated to be the intestinal concentration). Because the use of such high concentrations of PhIP and of the corresponding 20-fold excess quercetin concentrations in the Caco-2 model is hampered by the cytotoxic effects at higher PhIP and quercetin concentrations and by the limited solubility of PhIP and quercetin in culture medium, the effect of increasing PhIP and quercetin concentrations on the size of the quercetin mediated effect on PhIP transport was investigated using the previously developed *in silico*

model to predict PhIP transport in the Caco-2 transwell system in the presence of quercetin.

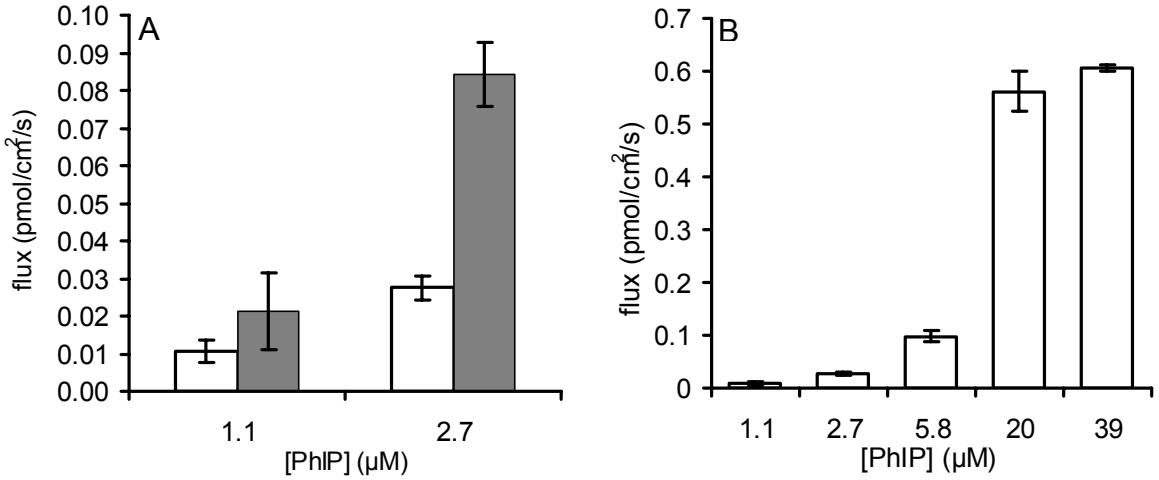

Figure 4 (A) The effect of about 20-fold quercetin to PhIP excess on the flux of PhIP (1.1. and 2.7 µM) transported through Caco-2 monolayers from the apical to the basolateral compartment. Concentrations of quercetin applied were 0 for both controls (open bars) and 25 and 40 µM for the studies with respectively 1.1 and 2.7 µM PhIP (grey bars). (B) The effect of increasing PhIP concentrations on the flux of PhIP transported through the Caco-2 monolayer from the apical to the basolateral compartment. Data are presented as mean ± SEM (n=3)

Kinetic modelling of the effect of 20 fold quercetin excess on PhIP transport at increasing PhIP concentrations

Previously, we described a kinetic model capable of describing the transport of PhIP in the presence or absence of an inhibitor (e.g. quercetin) through Caco-2 monolayers at any given concentration of PhIP and quercetin (*11*). This kinetic model is based on the following assumptions; the transport of PhIP consists of passive diffusion minus active transport by an apparent transporter (see also (*11*)). This apparent transporter is localized in the apical membrane and comprises both the apical and the basolateral located efflux transporters present in the Caco-2 cell. To determine the passive diffusion constant, and the apparent Km and the apparent Vmax values for the active transport of PhIP through the Caco-2 monolayers, the transport of PhIP was measured at increasing concentrations of PhIP in the absence of quercetin. By fitting the data of the uninhibited PhIP flux (Figure 4b) to Equation 2 the passive diffusion constant, the apparent Vmax value and the apparent Km

value of the transport of PhIP for the apparent transporter were determined. For the uninhibited PhIP flux through the Caco-2 monolayer the model predicts a passive diffusion constant of 4.2×10^{-5} cm/s, an apparent Vmax value of 2.9×10^{-6} μmol/cm^2/s and an apparent Km value of 89 μM.

Next, using these kinetic constants, the previously determined apparent Ki value for quercetin of 5.6 μM (*11*) and Equation 3, the PhIP flux in the presence of 20-fold quercetin concentrations was predicted for PhIP concentrations increasing from 1.1 μM up to 400 μM.

Table 1 depicts the results obtained and elucidates the predicted theoretical effect of 20-fold quercetin excess over PhIP on the transport of PhIP through the Caco-2 monolayer at increasing PhIP concentrations. The model predicts an increase in PhIP flux at 1.1 and 2.7 μM PhIP in the presence of 20-fold quercetin excess as compared to the control without quercetin of 361% and 375% respectively. This is in relatively good agreement with the experimentally observed quercetin-mediated increase in PhIP flux of 367% and 320% respectively (Figure 4a).

Table 1 *In silico* predictions of the effect of quercetin on the PhIP flux through the Caco-2 monolayer as predicted by the kinetic model and the resulting quercetin-mediated increase in the PhIP flux.

[PhIP] μM	[quercetin] mM	Predicted flux (pmol/cm^2/s) Solvent control	Predicted flux (pmol/cm^2/s) in the presence of quercetin	% increase
7.73×10^{-4}	0.03	7.28×10^{-6}	2.85×10^{-5}	392
1.1	0.022	0.01	0.04	361
2.7	0.054	0.03	0.11	375
5.0	0.1	0.06	0.20	361
10	0.015	0.13	0.33	263
10	0.2	0.13	0.41	324
20	0.4	0.31	0.83	270
40	0.8	0.78	1.67	214
80	1.6	1.99	3.35	169
100	2.0	2.67	4.19	157
200	4.0	6.39	8.39	131
300	6.0	10.36	12.59	121
400	8.0	14.43	16.79	116

Furthermore, Table 1 shows that the relative increase in the transport of PhIP caused by 20-fold quercetin excess decreases with increasing PhIP concentrations. At 200 μM PhIP and 4 mM quercetin, the values estimated for the intestinal

concentrations of the compounds in our *in vivo* study, the predicted quercetin mediated effect amounts to 131% which is equal to the 131% actually observed *in vivo*.

Discussion

Previously, we showed that quercetin and other flavonoids were capable of significantly increasing the transport of the pro-carcinogen PhIP through Caco-2 monolayers which are a model for intestinal absorption (*11, 12*). In the present study we investigated whether the effect observed in our *in vitro* studies could be confirmed in an *in vivo* bioavailability study in rats.

To the best of our knowledge, the present study shows for the first time that quercetin increases the bioavailability of PhIP in the rat. We showed that 1.45 μmol PhIP/kg bw co-administered with 30 μmol/kg bw (20-fold excess) quercetin significantly increased the blood $AUC_{(0\text{-}8hr)}$ of PhIP in the systemic circulation to 131% compared to the $AUC_{(0\text{-}8hr)}$ of PhIP in the control group. The increase in the systemic bioavailability of PhIP in rats orally dosed with PhIP in the presence of quercetin compared to the control group might be caused by the inhibition of ABC transporters localized at the luminal side of the enterocytes. This hypothesis is supported by the observation that the bioavailability of PhIP is increased in MRP2 deficient rats (*15*), *MRP2* knockout mice (*16*), and Bcrp1(-/-) mice (*14*). Furthermore, Sesink *et al.* (*30*) showed that the Bcrp1 transporter in the small intestine in Wistar Hannover rats transports the intracellular formed quercetin glucuronides back to the intestinal lumen. Thus, the transport of PhIP by the Bcrp1 transporter back to the intestinal lumen may be inhibited by the intracellular formed quercetin glucuronides, ultimately resulting in an increased bioavailability of PhIP.

Other studies showed that flavonoids are capable of increasing the oral bioavailability of other chemicals, including drugs. The bioavailability of diltiazem in Sprague-Dawley (SD) rats increased by 79% when the compound was co-administered with morin (*31*) and by 90% when the rats were pre-treated with naringin (*32*). The flavonoids flavone and quercetin increased the AUC of paclitaxel in SD rats by 80-210% and 76-210% respectively (*33, 34*). Wang and Morris (*35*) showed that the flavonoid chrysin increased the bioavailability of nitrofurantion in Sprague-Dawley rats by 71%. This flavonoid-mediated increase in the bioavailability of the different drugs is probably mediated by transport protein inhibition. The above described studies show that flavonoids capable of inhibiting

ABC transporters *in vitro* are also capable of inhibiting ABC transporters *in vivo*. However, Zhang *et al.* (*36*) showed that chrysin and benzoflavone both potent BCRP inhibitors in human MCF7 MX100 cells had no effect on the pharmacokinetic parameters of topotecan in SD rats and mdr1a/1b(-/-) mice when co-administered with these flavonoids. This apparent inconsistency in the *in vitro* and *in vivo* results was ascribed to the fact that chrysin and benzoflavone are potent human BCRP inhibitors but are not potent inhibitors of mouse Bcrp1 as shown in MDCKII-*Bcrp1* cells (*36*).

Previous studies using Caco-2 monolayers revealed that the apparent permeability constant ($Papp_{ab}$) for apical to basolateral PhIP transport was increased by 380% through Caco-2 monolayers in the presence of quercetin. Since in the present *in vivo* study the PhIP bioavailability was increased by only 131% in the presence of quercetin additional *in vitro* and *in silico* studies were used to investigate whether this difference between the *in vitro* and *in vivo* studies was due to the different quercetin to PhIP ratios and/or the PhIP and quercetin concentrations applied in the *in vitro* Caco-2 model as compared to the *in vivo* rat model. In the Caco-2 model the ratio of quercetin to PhIP was 1.5, and the concentration of PhIP was 5 μM. In the *in vivo* model of the present study the quercetin to PhIP ratio amounts to about 20 and the intestinal PhIP concentration was estimated to amount to 200 μM assuming a 2-fold dilution of the concentration administered by oral gavage upon translocation to the intestine. The additional *in vitro* experiments described in the present study showed that at 20-fold quercetin excess over PhIP a relative increase in the $Papp_{ab}$ value was observed that amounted to 367% and 320% at 1.1 and 2.7 μM PhIP respectively, whereas in the *in vivo* study the systemic bioavailability in the presence of 20-fold excess of quercetin over PhIP amounted to 131% of that in the absence of quercetin. This indicates that the difference in the extent of the quercetin mediated effect on PhIP bioavailability observed in the Caco-2 experiments and the *in vivo* rat experiment can not be ascribed to the different quercetin to PhIP ratio. Furthermore, in the *in vivo* experiment of the present study the PhIP concentration expected in the intestine amounted to 200 μM and that of quercetin being present at a 20 fold excess to 4 mM. Using these concentrations the kinetic model predicts a quercetin mediated increase in PhIP transport to 131% of the transport in the absence of quercetin. This is in good correlation with the actual *in vivo* experimentally observed increase in PhIP bioavailability (131%) in the presence of 20-fold quercetin concentrations compared to the control situation.

These results of the *in silico* kinetic modelling, revealing that at higher PhIP concentrations the relative effect of 20-fold quercetin concentrations on PhIP transport through the intestinal monolayer decreases, might be explained by the fact that at high PhIP concentrations the passive diffusion of PhIP through the intestinal enterocytes into the bloodstream becomes relatively more important. Since this passive diffusion component of the overall transport will not be sensitive to inhibitors the theoretical maximal effect of inhibitors on the overall uptake will be less. Only the active efflux component resulting in PhIP translocation to the apical side and back into the intestinal lumen is sensitive to quercetin inhibition. Because the overall effect of this active transport on the total transport becomes less at higher PhIP concentrations where diffusion becomes dominant, the quercetin mediated effects on the overall process will also be reduced at the tested concentrations.

In line with the observation that at 8 hours post-dosing there was no longer a significant difference in the blood levels of PhIP in the two dose groups, no difference was observed in the PhIP levels in the liver, kidney, small intestine and colon eight hours after the administration of PhIP in the presence or absence of quercetin, although a significant decrease in the PhIP levels in the cecum was observed for rats co-administered with PhIP and quercetin compared to the control group. It is tempting to speculate that this significantly reduced PhIP levels in the cecum of rats exposed to PhIP in the presence of quercetin also reflects the increased systemic uptake and bioavailability of PhIP when administered in the presence of quercetin, resulting in lower residual levels in this part of the large intestine.

Ebeler *et al.* (*37*) showed that PhIP levels in the liver of rats exposed to 140 mg quercetin /kg diet amounting to a dose of approximately 7 mg/kg bw/day quercetin, and administered PhIP by oral gavage were 185% higher than PhIP levels in the liver of control rats receiving control diet without quercetin and a similar dose of PhIP. This discrepancy between the effect of quercetin on PhIP levels in the liver in the present study and the study of Ebeler *et al.* (*37*) might be due to the difference in the administration of quercetin, chronic administration versus one single oral dose. Furthermore, Ebeler *et al.* (*37*) did not observe a difference between the different treatment groups in the plasma of rats 24 hours post dosing. This is in line with our study where blood levels were no longer significantly different already at 4 hours post dosing, but were different at earlier time points after dosing. The

results of our study reveal that earlier time points, e.g. within the first hours after dosing, are required to actually detect the effects on bioavailability.

Dietary heterocyclic amine intake is estimated to be between 2-25 ng/kg/day based on food-questionnaires and the amount of heterocyclic amines formed in the laboratory with different cooking methods (*38, 39*). Assuming a dietary intake of 25 ng PhIP/kg bw, a body weight of 70 kg, a stomach content of one litre, and a ten times dilution upon transfer to the gastrointestinal tract in humans, the PhIP concentration reached in the human intestine was estimated to be approximately 0.77 nM. Using similar assumptions the quercetin concentration that could be reached in the human intestine after intake of a supplement of 100 mg quercetin was estimated to be approximately 30 μM. At these concentrations of PhIP and quercetin the *in silico* kinetic model predicts an increase in the absorption of PhIP to 392% of the value in the presence of quercetin as compared to the situation in the absence of quercetin. Therefore, it is likely that the observed increase in the systemic bioavailability of PhIP in the rat is biologically relevant under normal dietary conditions in humans as well.

In conclusion, we showed that our previous studies in which quercetin increased the PhIP transport through Caco-2 cell monolayers could be confirmed *in vivo* in the rat. *In vitro* and *in silico* modelling of PhIP transport using a Caco-2 transwell system and a previously developed kinetic model for PhIP transport through the Caco-2 cells revealed that the relative increase in PhIP transport induced by quercetin is dependent on the actual concentration of the two compounds. At physiologically relevant concentrations of PhIP and quercetin the effect was predicted to amount to 392%. This increase in the bioavailability of PhIP in rats caused by the flavonoid quercetin may proceed through quercetin-mediated inhibition of transport proteins present in the enterocytes and points at a possible adverse effect of a supposed beneficial food ingredient when present in combination with the heterocyclic amine PhIP.

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6

Summary and general discussion

Summary

The intestinal epithelium is an important factor determining the oral absorption and the bioavailability of ingested food components, pharmaceuticals and xenobiotics. Uptake of compounds in the intestine may be facilitated by transport proteins e.g. SGLT1 and may also be influenced by ATP binding cassette transporters (ABC transporters). ABC transporters are known to efficiently excrete physiological substrates and xenobiotics out of the cell. In the intestinal epithelium the ABC transporters P-gp, MRP2 (ABCC2), and BCRP (*1-5*) are localized in the apical membrane of the enterocyte, catalyzing the efflux of compounds to the intestinal luminal side thereby opposing bioavailability. Localized in the basolateral membrane are the ABC transporters MRP1 (ABCC1), MRP3 (ABCC3) and MRP5 (ABCC5) catalyzing the efflux of compounds to the serosal side and facilitating bioavailability (*6-10*).

One class of compounds for which it is shown that ABC transporters are involved in their intestinal transport are the heterocyclic amines such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Studies using Caco-2 monolayers and typical transport inhibitors have shown that P-gp and MRP2 are likely to be involved in the excretion of PhIP from enterocytes back to the intestinal lumen (*11*). Studies in rodents showed that the bioavailability of PhIP is decreased in the presence of the ABC transporters BCRP (*12*) and MRP2 (*13, 14*).

Research regarding multidrug resistance showed that flavonoids are potent inhibitors of the ABC transporters P-gp, MRP1, MRP2, MRP4, MRP5 and BCRP (*15-20*) (see also Table 2 in the introduction).

Since ABC transporters are involved in the efflux of xenobiotics like PhIP, and since flavonoids are also capable of modulating the ABC transporter activity the aim of this thesis was to investigate the possible effect of flavonoids and mixtures thereof on the transport of PhIP across the intestinal barrier *in vitro* (Caco-2 cell monolayer) and *in vivo* (Wistar Hannover) rats.

Chapter 1 of this thesis describes a literature review of ABC transporters involved in the efflux of drugs, xenobiotics and bioactive compounds from the intestinal cells and the role of flavonoids as important modulators or substrates of intestinal ABC transporters.

In **chapter 2** the effect of myricetin, a flavonoid previously shown to be capable of inhibiting the MRP2 mediated efflux of calcein, on the transport of the pro-carcinogen PhIP through Caco-2 monolayers was investigated. In this study a

concentration dependent increase in the apparent permeability coefficient from the apical to the basolateral compartment ($Papp_{ab}$) was observed for Caco-2 cells exposed to 5 μM PhIP in the presence of 0 to 50 μM myricetin. Furthermore, a concentration dependent decrease in the $Papp_{ba}$ value was observed for Caco-2 monolayers exposed to 5 μM PhIP in the presence of 0 to 50 μM myricetin. It was concluded that myricetin increases apical to basolateral transport of PhIP, in part through the inhibition of the MRP2 mediated excretion of PhIP from the intestinal cells back to the lumen.

In **chapter 3** it was investigated whether in addition to myricetin nine other flavonoids could have a similar effect on the transport of PhIP through Caco-2 monolayers. These flavonoids were selected because of their ability to inhibit one or several ABC transporters including P-gp, MRP1, MRP2 and BCRP. The flavonoids flavone, kaempferol, luteolin, quercetin, myricetin, chrysoeriol and naringenin were capable of significantly increasing the $Papp_{ab}$ value for the transport of PhIP through Caco-2 monolayers whereas the flavonoids morin, robinetin and taxifolin had no significant effect on the $Papp_{ab}$ value of PhIP transport.

In addition to the *in vitro* studies using Caco-2 cell monolayers, chapter 3 also presents an *in silico* kinetic model describing PhIP transport through the Caco-2 monolayer. This model is based on the assumption that PhIP transport consists of passive diffusion into the cell and active transport by an apparent transporter. This apparent transporter describes the activity of all efflux transporters present in the Caco-2 cell in both the apical and basolateral membrane by combining them in one apparent efflux transporter localized in the apical membrane. The modelling of the effects of both the apical and basolateral located transporters in one apparent transporter was possible since the overall effect of the flavonoids on PhIP transport was studied. Using this kinetic model it was possible to quantify apparent inhibition constants (Ki) for inhibition of the apparent transporter by the different flavonoids. For the flavonoids flavone, kaempferol and chrysoeriol the apparent Ki value for inhibition of the active transport to the apical side was estimated to be below 53 μM and for morin, robinetin and taxifolin between 164 and 268 μM. In additional kinetic studies for myricetin, luteolin, naringenin, quercetin and apigenin the apparent Ki values were determined more accurately and were shown to amount to 37.3, 12.2, 11.7, 5.6 and 10.8 μM respectively. Based on these obtained apparent Ki values, it was concluded that the flavonols, at levels present in the Western diet,

are capable of increasing the apical to basolateral transport of PhIP through Caco-2 monolayers.

Whereas in chapter 2 and chapter 3 the effect of individual flavonoids on ABC transporter activity has been studied, in **chapter 4** the effect of mixtures of flavonoids was studied on ABC transporter mediated transport of PhIP since the human diet consists of multiple food constituents and humans are exposed to mixtures of compounds, rather than to single compounds. In **chapter 4** the effect of several binary flavonoid mixtures and one flavonoid mixture containing all five model flavonoids namely quercetin, luteolin, myricetin, naringenin and apigenin on the transport of PhIP was quantified. The binary mixtures of naringenin and quercetin, naringenin and luteolin, naringenin and myricetin and naringenin and apigenin and the mixture containing all five model flavonoids were capable of increasing the apical to basolateral PhIP transport at physiological relevant flavonoid concentrations.

To study the type of interaction of the flavonoids on the PhIP transport the kinetic model developed in chapter 3 was extended in chapter 4 assuming competitive inhibition of the apparent active transporter by the flavonoids and assuming concentration-additivity for the different flavonoids for their inhibiting effect. The model thus obtained appeared to describe accurately the effect of the mixtures of flavonoids on the apical to basolateral transport of PhIP and was thus also suitable to predict the transport of PhIP in the presence of mixtures of flavonoids.

In addition to studying the effect of flavonoids on the transport of PhIP through Caco-2 monolayers **chapter 2** and **3** also present results showing the effect of typical ABC transporter inhibitors used to identify which ABC transporters are involved in this flavonoid-mediated increase in the apical to basolateral PhIP transport. The typical inhibitors PSC833 (a P-gp inhibitor), cyclosporin A (a P-gp and MRP2 inhibitor), GF120918 (a P-gp and BCRP inhibitor) and MK571 (a MRP1 and MRP2 inhibitor) were used to study the involvement of P-gp, MRP1, MRP2 and BCRP in the efflux of PhIP from the Caco-2 monolayer. The presence of the P-gp inhibitor PSC833 had no significant effect on the $Papp_{ab}$ and $Papp_{ba}$ values for PhIP transport compared to the solvent control. This suggests that P-gp was not involved in the transport of PhIP through the Caco-2 monolayer and therefore the flavonoid-mediated increase of apical to basolateral PhIP transport is not likely to be caused by the inhibition of P-gp by the flavonoids. The presence of the MRP2 and P-gp inhibitor cyclosporin A resulted in a significant increase of the $Papp_{ab}$ value for PhIP compared to the uninhibited situation and a significant

decrease of the $Papp_{ba}$ value for PhIP in the presence of cyclosporin A compared to the uninhibited situation. These results suggest the involvement of MRP2 in the efflux of PhIP from the Caco-2 monolayers. The role of MRP2 in the efflux of PhIP was corroborated by the results obtained from experiments using the typical inhibitor MK571 (MRP1 and MRP2 inhibitor). MK571 significantly increased the $Papp_{ab}$ value for apical to basolateral transport of PhIP through the Caco-2 monolayer and significantly decreased the $Papp_{ba}$ value for basolateral to apical transport. Finally, the possible involvement of BCRP was studied using GF120918 (BCRP and P-gp inhibitor) the presence of GF120918 increased the $Papp_{ab}$ value of PhIP transport through the Caco-2 monolayer and decreased the $Papp_{ba}$ value. These results in **chapter 2** and **3** suggest that MRP2 and/or BCRP but not P-gp and MRP1 are involved in the efflux of PhIP.

At present several mechanisms for the inhibition of ABC transporters by flavonoids have been reported. Flavonoids are capable of interacting with the ATPase activity of the ABC transporters. Whereas quercetin is capable of inhibiting the ATPase activity (*26*), the flavonoids kaempferol (*26, 27*) and naringenin (*26*) are capable of stimulating the ATPase activity. In addition to interaction with the ATPase activity of the ABC transporters, flavonoids or their metabolites may act as substrates for ABC transporters and by being transported themselves might cause competitive inhibition towards other substrates (*28, 29*). Walgren *et al.* (*29*) showed that quercetin is transported through the Caco-2 cell monolayer with a preferred transport direction to the apical compartment suggesting the involvement of a transporter located in the apical cell membrane. Sesink *et al.* (*28*) demonstrated that quercetin is transported by *Bcrp1* in MDCKII-Bcrp1 cells.

The comparison of the presence of ABC transporters in the Caco-2 monolayers *in vitro* and the small intestine *in vivo* has been investigated in several studies. Taipalensuu *et al.* (*9*) studied the expression levels of P-gp, several MRPs and BCRP between the jejunum biopsies obtained from healthy volunteers and Caco-2 cell monolayers. They observed a good correlation for MDR1, MDR3, MRP1, MRP2, MRP3, MRP4, MRP5 and MRP6 between Caco-2 monolayers and human jejunum with expression levels differing less than 2.5 fold, whereas the BCRP expression levels in Caco-2 monolayers were 100-fold lower than in a human jejunum biopsy. Prime-Chapman *et al.* (*7*) showed that MRP1, MRP2, MRP3, MRP4, MRP5 and MRP6 are significantly expressed in the human jejunum and ileum and that lower expression levels of the ABC transporters were observed in the duodenum. They also studied the expression of the ABC transporters MRP1

through MRP6 in Caco-2 monolayers grown on filter inserts. All tested transporters were expressed in the Caco-2 monolayers with MRP2 and MRP6 being the most abundant, followed by MRP3 and MRP4 and the expression of MRP1 and MRP5 being the lowest. Thus Caco-2 cell monolayers are a suitable *in vitro* model to study the MRP1 through MRP6 related excretion of compounds. However, using Caco-2 monolayers to study the BCRP mediated excretion of compounds may underestimate the contribution of BCRP to be expected in the human jejunum. Thus the results obtained in this thesis using Caco-2 monolayers and showing a flavonoid-mediated increase of PhIP transport from the apical to the basolateral compartment caused by the inhibition of BCRP point at an effect that may be at least equally relevant in the *in vivo* situation. Since it was important to confirm whether the *in vitro* observed flavonoid-mediated increase in the apical to basolateral PhIP transport could be observed in the *in vivo* situation **chapter 5** describes a rat study in which the effect of quercetin on the bioavailability of PhIP was investigated. For this purpose male Wistar Hannover rats were co-administered with 0.09 mg PhIP (1.45 μmol/kg bw) in the presence of 10 mg/kg bw (30 μmol/kg/bw) quercetin. The $AUC_{(0\text{-}8hr)}$ in blood of rats dosed with PhIP in the presence of quercetin amounted to 3.0±0.3 nmol/min which was 131±14% of the $AUC_{(0\text{-}8hr)}$ for control rats dosed with PhIP alone which amounted to 2.3±0.4 nmol/min. Significantly increased PhIP levels in the systemic blood were detected at 15 minutes (247% of control), 30 minutes (169% of control), 45 minutes (247% of control) and 180 minutes (187% of control). At 4 and 8 hours post-dosing a difference in the PhIP levels in the blood between the two treatment groups was no longer observed. This points at a difference in the initial uptake of PhIP in the presence of quercetin.

These results showed that the *in vitro* observed effect of quercetin on the transport of PhIP through Caco-2 monolayers could be confirmed in the *in vivo* situation. Additional *in vitro* and *in silico* experiments revealed that the relative effect of quercetin on the absorption of PhIP depends on the PhIP and quercetin levels used and the ratio between these two compounds. At higher PhIP levels (>400 μM) the relative effect of quercetin on the absorption of PhIP is less pronounced than at physiological relevant PhIP concentrations (Chapter 5).

In conclusion this thesis shows that flavonoids can increase the apical to basolateral absorption of PhIP through Caco-2 monolayers and the initial bioavailability of PhIP in rats. This effect can be reached at flavonoid levels in the intestine that can easily be met upon supplement intake or upon consumption of food enriched in

flavonoids. It can be concluded that at physiologically relevant flavonoid concentrations, flavonoids or mixtures thereof may inhibit the efflux of compounds by ABC transporters. Thus the flavonoid-mediated increase in the apical to basolateral PhIP transport through Caco-2 monolayers and increased PhIP bioavailability in rats may point at a possible adverse effect of these supposed beneficial food ingredients, when simultaneously present in the luminal side of the intestine.

General discussion

The flavonoid-mediated increase in the bioavailability of the pro-carcinogen PhIP as observed in the *in vitro* and *in vivo* studies described in this thesis suggests that more attention should be paid to the possible adverse effects of flavonoids especially since flavonoid supplements are available in capsules containing 100 and 200 mg of a flavonoid. After consumption of flavonoid supplements, concentrations may be reached of 35 μM and higher in the intestine which is in the same order of magnitude as the apparent Ki values for inhibition of the apparent PhIP transport back to the intestinal lumen by quercetin (5.6 μM), naringenin (11.7 μM), luteolin (12.2 μM), myricetin (37.3 μM) and apigenin (10.8 μM) described in this thesis. It is also in the range of the reported Ki values of *e.g.* 2.4 to 20.8 μM for the competitive inhibition of MRP1 activity mediated by different dietary flavonoids, using LTC_4 as substrate (*26*), or similar Ki values for the flavonoid-mediated inhibition of the efflux of calcein-acetoxymethyl ester (calcein-AM) by MRP1 or MRP2 (*18*). Thus at these physiological relevant concentrations of the flavonoids an interaction with the bioavailability of other compounds including drugs, xenobiotics and food constituents may be expected.

Although flavonoids increase the absorption and bioavailability of PhIP *in vitro* and *in vivo* the overall effect of flavonoids on the potential carcinogenic effect of PhIP has to be studied before a conclusion can be made of the effect of flavonoids on the risk of PhIP exposure.

In addition to studies investigating the effect of flavonoids on the absorption of PhIP or other substrates, several studies have been investigating the effect of antioxidants including flavonoids on the formation of heterocyclic amines in model systems containing the precursors of heterocyclic amines and during the heat processing of meat. Studies using a model system to study the formation of heterocyclic amines showed that the presence of the tea polyphenol EGCG

decreased the formation of IQ-type heterocyclic amines. The authors suggest that EGCG with its antioxidant activity scavenged the radical species formed during the Maillard reaction and by this inhibited the mutagen formation (*30*). The flavonoids studied in the present thesis may have similar effects; although flavonoids might not always be added to the baking process of meat and fish.

Busquets *et al.* (*31*) studied the effect of red wine marinades on the formation of heterocyclic amines in fried chicken breasts. Their findings showed that the effect of the marinade on the heterocyclic amine formation depends on the heterocyclic amine formed, chicken breast marinated in red wine showed a up to 88% reduction in the PhIP levels present whereas the formation of MeIQx and 4,8-DiMeIQx was enhanced by the red wine marinade up to 300%. Thus, the effect of red wine marinade on the formation of heterocyclic amines is inconclusive at present.

Flavonoids may not only have an effect on the formation of heterocyclic amines but may also inhibit the metabolic activation of heterocyclic amines, by inhibiting CYP1A2 or NAT, or stimulating the detoxification enzymes sulfotransferase and glutathione-S-transferase, or scavenging reactive molecules (reviewed in (*32*)). Thus flavonoids may, by inhibiting the metabolic activation of heterocyclic amines, protect against the mutagenic potency of these compounds.

Besides the prevention of heterocyclic amines formation, flavonoids may also help the chemoprotection against the formation of PhIP-DNA adducts. This was shown in a study using HepG2 cells exposed to PhIP in the presence of increasing concentrations quercetin where the presence of 5-20 µM quercetin reduced the amount of PhIP-DNA adducts (*33*). In the same study the effect of the co-treatment of hepatocytes with PhIP and quercetin was studied in hepatocytes of five different subjects. In three out of five subjects the PhIP-DNA adducts decreased in the presence of quercetin, whereas in two subjects no effect was observed. Huber *et al.* (*34*) showed that F344 rats exposed to 50 mg/kg bw PhIP after a 10 day pre-treatment with 2% black tea in their drinking water had only 34% of the PhIP-DNA adducts in the colon compared to the control diet. Whereas the pre-treatment for 10 days with 1% quercetin in the diet led to 148% of PhIP-DNA adducts in the colon compared to the control diet. Lin *et al.* (*35*) showed that the pre-treatment of SD rats administered with 3% green tea in drinking water resulted in a significant decrease in the PhIP-DNA adducts formed in the colon, heart, lung and liver after exposure with 10 mg/kg bw PhIP. Whereas Huber *et al.* (*34*) showed that the pre-treatment of the rats with 2% green tea in their drinking water did not have an effect on the PhIP-DNA adducts present in the colon in F344 rats exposed to 50

mg/kg bw PhIP. The effect of the presence of polyphenols on the formation of PhIP-DNA adducts depends on the test system (*in vitro* vs *in vivo*), the food matrix in which the polyphenols are present and for *in vivo* studies also the route of administration of the polyphenols.

To get an idea of the magnitude of the effect of flavonoids present in the Western diet on the absorption of PhIP present in the Western diet an *in silico* kinetic model described in chapter 3, 4 and 5 was used to predict the flavonoid-mediated increase in PhIP transport. For this, the information of the study of Hertog *et al.* (*36*) was used which describes the intake of several flavonoids in adults in the Netherlands. They estimated that approximately 23 mg flavonols per day is consumed by the Dutch population of which quercetin contributed with 16 mg. Furthermore, the average human heterocyclic amine intake is estimated to be 2-25 ng/kg/day (*37, 38*). Assuming a worst case scenario in which a human of 70 kg is exposed to 25 ng/kg bw PhIP and 16 mg quercetin the levels that may be reached in the intestine are 0.77 nM and 5.3 μM respectively. Using the parameters for the passive diffusion, apparent Vmax and apparent Km (described in **chapter 5**) and the apparent Ki value for quercetin (**chapter 3**) the model predicts that the absorption of PhIP in the presence of 16 mg quercetin is 2-3 fold higher than in the absence of quercetin.

If we extrapolate this to the intake of a quercetin supplement (with 100 mg quercetin level, leading to an intestinal quercetin concentration of 35 μM) the PhIP absorption would be almost four fold higher than in the absence of quercetin (shown in **chapter 5**).

Although the predictions by the *in silico* kinetic model for PhIP transport show that a quercetin-mediated increase in the PhIP absorption can be expected it is not possible to predict the overall effect of quercetin on PhIP induced carcinogenesis. This is because other factors may also influence the overall effect of flavonoids on the outcome and risk of PhIP exposure including for example the effect of flavonoids on the cytochrome P450 enzymes and thus on the bio-activation of PhIP necessary for the formation of the ultimate carcinogen. In addition, the human diet consists of fruits and vegetables containing flavonoids and fried meat containing heterocyclic amines rather than the pure compounds, and food constituents present in the food matrix may have an effect on the absorption of PhIP, whereas in the model and experiments of the present thesis only the effect of pure compounds could be taken into account. Furthermore, the presence of flavonoids (e.g. in the form of an onion) during the frying of meat may reduce the formation of

heterocyclic amines and in the body the presence of flavonoids may inhibit the metabolic activation of heterocyclic amines.
Since the flavonoid-mediated increase in the bioavailability of PhIP as observed in this thesis is caused by the co-administration of quercetin and PhIP in a single dose, it would be of interest to perform an animal experiment investigating the effect of an increasing time interval between administration of the flavonoids and the administration of PhIP, compared to the co-administration of PhIP and flavonoids in a single dose. Such an experiment may shed light on the possibility to reduce the effect of flavonoids on the bioavailability of PhIP, and reduce the potentially increased risk resulting from it, by separating the moment of consumption of fruits and/or vegetables and meat.
The effect of flavonoids on the bioavailability of PhIP was shown to be mediated by inhibition of apical ABC transporters. Since these transporters are involved in transport of other chemicals including drugs it can be concluded that also the effect of food-drug interactions on the ABC transporter system at relevant low level exposure scenarios remains an important subject for further investigations.
In conclusion, the results presented in this thesis show that the flavonoid-mediated inhibition of ABC transporters may affect the bioavailability of food-borne toxic compounds upon oral uptake. It was also demonstrated that flavonoids and their mixtures, which are an important class of bioactive food ingredients, are expected to result in these interactions at physiologically relevant levels of intake. The relevance of this finding is further supported if one takes into consideration the actual flavonoid levels in food products and currently marketed food supplements. This may indicate that intake of selected flavonoids and flavonoids in a mixture might be an important factor in the regulation of the ABC transporter mediated efflux of toxic xenobiotics and thus in the bioavailability of xenobiotics upon their oral intake.

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Samenvatting

Het darmepitheel speelt een belangrijke rol bij de opname en biobeschikbaarheid van oraal ingenomen voedingscomponenten, geneesmiddelen en lichaamsvreemde stoffen. Opname van stoffen in de darm kan worden gefaciliteerd door transporteiwitten zoals SGLT1 en kan ook worden beïnvloed door ATP binding cassette transporters (ABC transporters). ABC transporters transporteren fysiologische substraten en lichaamsvreemde stoffen de cel uit. De ABC transporters P-gp, MRP2 (ABCC2) en BCRP (*1-5*) zijn gelokaliseerd in de apicale membraan van het darmepitheel, en kunnen de uitscheiding van stoffen naar het lumen bevorderen en hierdoor de biobeschikbaarheid verrminderen. In de basolaterale membraan van het darmepitheel zijn de ABC transporters MRP1 (ABCC1), MRP3 (ABCC3) en MRP5 (ABCC5) gelokaliseerd en deze kunnen de excretie van stoffen naar het bloed katalyseren en hierdoor de biobeschikbaarheid verhogen (*6-10*).

Tot de stoffen die getransporteerd worden door de ABC transporters in de darm behoren ook de heterocyclische amines zoals 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Heterocyclische aromatische amines zijn mutagene verbindingen die ontstaan tijdens de verhitting van vlees en in verband worden gebracht met een verhoogd risico op dikke darmkanker in de mens. Studies met Caco-2 cel monolagen waarin remmers van ABC transporters worden gebruikt tonen aan dat P-gp en MRP2 betrokken zijn bij het transport van PhIP van het darmepitheel terug naar het darmlumen (*11*). Dit wordt ondersteund door *in vivo* studies met knaagdieren waarbij werd aangetoond dat de biobeschikbaarheid van PhIP verlaagd is in aanwezigheid van de ABC transporters BCRP (*12*) en MRP2 (*13,14*).

Ook is bekend dat de ABC transporters kunnen worden geremd door flavonoïden, die als voedingscomponent in ruime mate aanwezig zijn in groente en fruit. Uit onderzoek naar multidrugresistentie blijkt dat flavonoïden goede remmers zijn van de ABC transporters P-gp, MRP1, MRP2, MRP4, MRP5 en BCRP (*15-20*) (zie ook Tabel 2 in de introductie).

Aangezien ABC transporters betrokken zijn bij de uitscheiding van lichaamsvreemde stoffen zoals PhIP, en omdat bekend is dat flavonoïden deze zelfde ABC transporteractiviteit kunnen beïnvloeden, is het doel van dit proefschrift om het effect van (mengsels van) flavonoïden te bestuderen op het

transport van PhIP door het darmepitheel. Dit onderzoek vindt plaats zowel *in vitro* (Caco-2 cel monolagen) als *in vivo* (Wistar Hannover ratten).

Hoofdstuk 1 van dit proefschrift geeft een literatuuroverzicht van de betrokkenheid van ABC transporters bij de uitscheiding van geneesmiddelen, lichaamsvreemde stoffen en bioactieve stoffen uit het darmepitheel. Het hoofdstuk geeft ook een overzicht van het effect van de flavonoïden, die de activiteit van ABC transporters kunnen veranderen of als substraat van ABC transporters kunnen dienen, op het transport van geneesmiddelen, lichaamsvreemde stoffen en bioactieve stoffen uit het darmepitheel.

In **hoofdstuk 2** werd *in vitro* het effect van myricetin bestudeerd op het transport van de pro-carcinogeen PhIP. Myricetin is een flavonoïd waarvan eerder is aangetoond dat het de MRP2 gerelateerde efflux van calceïne kan remmen. In deze studie werd een concentratieafhankelijke toename van het apicale naar basolaterale PhIP transport gemeten in Caco-2 monolagen die werden blootgesteld aan 5 µM PhIP in aanwezigheid van 0 tot 50 µM myricetin. Als maat wordt daarbij gebruikt de zogenaamde "apparent permeability" coëfficiënt (Papp) waarbij in dit geval het transport van het apicale naar het basolaterale compartiment werd bepaald. Daarnaast werd ook een concentratieafhankelijke afname in de $Papp_{ba}$ (van basolateraal naar apicaal) waargenomen. Hieruit werd geconcludeerd dat myricetin het apicale naar basolaterale transport van PhIP verhoogt en dat dit mogelijk wordt veroorzaakt door de remming van de MRP2-afhankelijke uitscheiding van PhIP naar de apicale kant van de cellen. In **hoofdstuk 3** werd onderzocht of, behalve myricetin, negen andere flavonoïden een vergelijkbaar effect op het transport van PhIP door de Caco-2 monolagen hebben. De flavonoïden werden geselecteerd op basis van literatuurgegevens die de remming van verschillende ABC transporters zoals P-gp, MRP1, MRP2 en BCRP door deze flavonoïden rapporteren. De flavonoïden flavone, kaempferol, luteolin, quercetin, myricetin, chrysoeriol en naringenin waren in staat om de $Papp_{ab}$ van PhIP significant te verhogen, terwijl de flavonoïden morin, robinetin en taxifolin geen effect hadden op de $Papp_{ab}$-waarde van het transport van PhIP.

Naast de *in vitro* studies waarbij de Caco-2 monolagen werden gebruikt, werd in hoofdstuk 3 ook gebruik gemaakt van een *in silico* kinetisch model waarmee het transport van PhIP door de Caco-2 cel monolaag kan worden bestudeerd. Dit model gaat uit van de aanname dat het totale transport van PhIP kan worden beschreven met een component voor de passieve diffusie, en met een tweede component die het actief gemedieerde transport de cel uit weergeeft. Deze virtuele zogenoemde

“apparent transporter” beschrijft de gezamelijke activiteit van alle efflux transporters in de Caco-2 monolaag zowel aanwezig in de apicale als basolaterale membraan. Deze gezamenlijke apparent efflux transporter is in het model gelokaliseerd in de apicale membraan. Via de modellering van het gezamenlijke effect van alle mogelijke transporters in deze virtuele apparent transporter, wordt het mogelijk het totale effect van de flavonoïden op het transport van PhIP te beschrijven. Bovendien kan met dit kinetische model de apparent inhibitie constante (Ki) worden bepaald voor de remming van de apparent transporter door de verschillende flavonoïden. Voor de flavonoïden flavone, kaempferol en chrysoeriol was de apparent Ki-waarde voor de remming van het actieve transport naar de apicale zijde lager dan 53 μM en voor morin, robinetin en taxifolin lag de waarde tussen 164 en 268 μM. In additionele kinetische studies voor myricetin, luteolin, naringenin, quercetin en apigenin werd de apparent Ki-waarde in meer detail bepaald en de waarden die werden gevonden waren respectievelijk 37,3; 12,2; 11,7; 5,6 en 10,8 μM. Gebaseerd op deze apparent Ki-waarden werd geconcludeerd dat de flavonolen in staat zijn om het apicale naar basolaterale transport van PhIP door de Caco-2 monolaag te verhogen. Dit gebeurt bij concentraties die bereikt kunnen worden met de Westerse voeding. Terwijl in hoofdstuk 2 en hoofdstuk 3 het effect van de individuele flavonoïden op de ABC transporters werd bestudeerd, werd in **hoofdstuk 4** het effect van mengsels van flavonoïden op het ABC transporter gerelateerde transport van PhIP bestudeerd. Immers onze voeding bestaat niet uit een maar uit meerdere voedselbestanddelen die in combinaties voorkomen en de mens wordt in de praktijk eerder blootgesteld aan mengsels van stoffen in plaats van aan een enkele stof. In **hoofdstuk 4** werd het effect van verschillende binaire flavonoïden-mengsels en een mengsel met alle vijf de flavonoïden te weten quercetine, luteoline, myricetin, naringenin en apigenin op het transport van PhIP bestudeerd. Binaire mengsels bestaande uit naringenin en quercetin, naringenin en luteolin, naringenin en myricetin en naringenin en apigenin en het mengsel van alle vijf de flavonoïden waren in staat om het apicale naar basolaterale transport van PhIP bij fysiologische relevante flavonoïd-concentraties te verhogen.

Om het soort interactie van de flavonoïden op het PhIP transport te kunnen bestuderen werd in hoofdstuk 4 het in hoofdstuk 3 beschreven model uitgebreid waarbij werd aangenomen dat alle flavonoïden als competitieve remmers op de apparent transporter aangrijpen. Als extra aanname werd daarbij verondersteld dat het effect van mengsels van flavonoïden met behulp van concentratie-additie

beschreven kan worden. Het verkregen model beschreef accuraat het effect van de mengsels van flavonoïden op het apicale naar basolaterale transport van PhIP. Het model bleek dus in staat te zijn om het transport van PhIP in de aanwezigheid van mengsels van flavonoïden correct te voorspellen.
Naast het effect van flavonoïden op het transport van PhIP door de Caco-2 monolagen wordt in **hoofdstuk 2** en **3** het effect van typische ABC transporter remmers beschreven om op te helderen welke ABC transporters betrokken zijn bij de toename in het PhIP transport, veroorzaakt door de aanwezigheid van de flavonoïden. De typische remmers PSC833 (een P-gp-remmer), cyclosporine A (een P-gp- en MRP2-remmer), GF120918 (een P-gp- en BCRP-remmer) en MK571 (een MRP1- en MRP2-remmer) werden gebruikt om de betrokkenheid van P-gp, MRP1, MRP2 en BCRP in de excretie van PhIP uit de Caco-2 monolaag te bestuderen. De aanwezigheid van de P-gp-remmer PSC833 had geen effect op het transport van PhIP door de Caco-2 monolaag hetgeen erop wijst dat P-gp wellicht niet betrokken is bij de efflux van PhIP uit de Caco-2 monolaag. De aanwezigheid van de P-gp en MRP2-remmer cyclosporine A veroorzaakte een significante toename in het transport van PhIP van het apicale naar het basolaterale compartiment en een significante afname van het transport van de basolaterale naar apicale compartiment, dus waarschijnlijk is MRP2 betrokken bij de uitscheiding van PhIP uit de Caco-2 monolagen. De gevonden resultaten werden bevestigd door de resultaten verkregen met de typische remmer MK571 (een MRP1- en MRP-remmer). De aanwezigheid van MK571 verhoogde significant de $Papp_{ab}$-waarde voor apicaal naar basolateraal PhIP transport door de Caco-2 monolaag en verlaagde significant de $Papp_{ba}$-waarde voor basolateraal naar apicaal PhIP transport. De betrokkenheid van de BCRP transporter in de excretie van PhIP werd bestudeerd door de cellen bloot te stellen aan de P-gp- en de BCRP-remmer GF120918. De aanwezigheid van GF120918 verhoogde significant de $Papp_{ab}$-waarde en verlaagde significant de $Papp_{ba}$-waarde voor PhIP transport door de Caco-2 monolaag. De resultaten in **hoofdstuk 2** en **3** tonen aan dat MRP2 en BCRP wel, maar P-gp en MRP1 niet betrokken zijn bij het transport van PhIP door de Caco-2 monolaag.
In de literatuur zijn verschillende mechanismen beschreven voor de remming van ABC transporters door flavonoïden. Zo is aangetoond dat quercetine in staat is om de ATPase-activiteit te remmen (*26*) terwijl kaempferol (*26,27*) en naringenin (*26*) de ATPase-activiteit kunnen stimuleren. Dit betekent dus dat flavonoïden de ATPase activiteit van de ABC transporters kunnen beïnvloeden. Een andere manier

om de ABC transporter te remmen is gekoppeld aan het feit dat de flavonoïden of de metabolieten van flavonoïden zelf getransporteerd kunnen worden door de ABC transporters en daardoor het transport van andere substraten competitief kunnen remmen (*28, 29*). Walgren *et al.* (*29*) toonden aan dat quercetine getransporteerd wordt door de Caco-2 monolaag en dat er meer quercetine getransporteerd wordt van het basolaterale naar het apicale compartiment dan vice versa. Dit toont waarschijnlijk aan dat een apicale transporter betrokken is bij de uitscheiding van quercetine naar het lumen van de darm. Dit wordt ondersteund door Sesink *et al.* (*28*) die hebben aangetoond dat quercetine door *Bcrp1* getransporteerd wordt in MDCKII-Bcrp1-cellen.

Verschillende studies hebben de aanwezigheid van ABC transporters in de Caco-2 monolaag vergeleken met die in de dunne darm. Taipalensuu *et al*. (*9*) hebben de genexpressieniveaus van P-gp en van verscheidene MRPs en BCRP vergeleken tussen jejenum biopsies uit gezonde vrijwilligers en Caco-2 monolagen. Hierbij was sprake van een goede correlatie tussen de expressie van MDR1, MDR3, MRP1, MRP2, MRP3, MRP4, MRP5 en MRP6 in Caco-2 monolagen en in humane jejunum darmbiopten. De BCRP expressiewaarden waren ongeveer 100 keer lager dan in het humane jejenum biopt. Prime-Chapman *et al.* (*7*) toonden aan dat MRP1 t/m MRP6 significant aanwezig zijn in het humane jejunum en ileum terwijl de expressie van deze ABC transporter niveaus veel lager is in het duodenum. Ook de expressieniveaus van MRP1 t/m MRP6 in Caco-2 monolagen werden bestudeerd. Alle transporters kwamen tot expressie in de Caco-2 cellen waarbij MRP2 en MRP6 het meeste voorkwamen, gevolgd door MRP3 en MRP4 en de expressieniveaus van MRP1 en MRP5 het laagste waren. Uit de resultaten van deze studies blijkt dat de Caco-2 monolagen een geschikt model zijn om de MRP1 t/m MRP6 gerelateerde uitscheiding van stoffen te bestuderen. Echter wanneer de Caco-2 monolagen gebruikt worden om de BCRP-gemedieerde uitscheiding van stoffen te bestuderen en te vertalen naar de mens dan is er hoogstwaarschijnlijk sprake van een onderschatting van de bijdrage van BCRP in het humane jejunum. De resultaten beschreven in dit proefschrift, waarbij een toename van het apicale naar basolaterale transport van PhIP wordt gevonden na blootstelling aan (mengsels van) flavonoïden *in vitro* kan wellicht vertaald worden naar de *in vivo* situatie. Om deze vertaalslag te onderbouwen werd een *in vivo* studie uitgevoerd (beschreven in **hoofdstuk 5**) waarbij het effect van quercetine op de biobeschikbaarheid van PhIP werd bestudeerd in proefdieren. In dit experiment werden mannelijke Wistar Hannover ratten gelijktijdig blootgesteld aan 0.09 mg PhIP (1.45 μmol/kg bw) en

10 mg/kg bw quercetine (30 µmol/kg/bw). De $AUC_{(0-8h)}$, bepaald in het bloed van ratten blootgesteld aan PhIP en quercetine, bedroeg 3.0±0.3 nmol/min. Deze waarde is 131±14% hoger dan de $AUC_{(0-8h)}$ voor controle dieren (2.3±0.4 nmol/min) die alleen werden blootgesteld aan PhIP. De PhIP-gehaltes in het bloed voor ratten, blootgesteld aan PhIP en quercetine, vergeleken met de waarden van de controle-groep ratten waren significant hoger na 15 minuten (247% van de controle), 30 minuten (169% van de controle), 45 minuten (247% van de controle), en 180 minuten (187% van de controle). Vier en acht uur na de toediening van de dosering aan de ratten werd er geen verschil in de PhIP-waarden tussen de twee behandelingen meer waargenomen. Dit wijst er op dat alleen een verschil in de opname van PhIP veroorzaakt door de aanwezigheid van quercetine aan het begin van de blootstelling wordt waargenomen.

Deze resultaten tonen aan dat het *in vitro* waargenomen effect van quercetine op het transport van PhIP bevestigd kon worden in de *in vivo* situatie. Aanvullende *in vitro* en *in silico* experimenten toonden aan dat het relatieve effect van quercetine op de absorptie van PhIP afhankelijk is van de PhIP- en quercetinegehaltes die gebruikt werden en de verhouding tussen deze twee stoffen. Bij hogere PhIP-concentraties (>400 µM) is het relatieve effect van quercetine kleiner dan bij fysiologisch relevante PhIP-concentraties (hoofdstuk 5).

Al met al werd geconcludeerd dat de resultaten in dit proefschrift aantonen dat flavonoïden het apicale naar basolaterale PhIP-transport door de Caco-2 monolaag en de initiële biobeschikbaarheid van PhIP in ratten kunnen verhogen. Dit effect wordt waargenomen bij flavonoïd-concentraties in de darm die bereikt kunnen worden door het innemen van een voedingssupplement of van voedsel verrijkt met flavonoïden. Uit de resultaten in dit proefschrift kan geconcludeerd worden dat bij fysiologisch relevante concentraties, flavonoïden of mengsels van flavonoïden de uitscheiding van stoffen door de ABC transporters kan remmen. De flavonoïd-gemedieerde toename van het apicale naar basolaterale PhIP transport door de Caco-2 monolagen en de verhoogde biobeschikbaarheid van PhIP in de rat duiden op een mogelijk nadelig effect van deze gezondheidsbevorderend geachte voedsel-ingrediënten, wanneer deze gelijktijdig met de pro-carcinogeen PhIP aanwezig zijn in het lumen van de darm.

Algemene discussie

De flavonoïd gemedieerde toename in de biobeschikbaarheid van PhIP zoals waargenomen in de *in vitro* en *in vivo* experimenten van dit proefschrift suggereren

dat meer aandacht besteed zou moeten worden aan de mogelijke nadelige effecten van flavonoïden, te meer ook omdat er op dit moment flavonoïd supplementen vrij verkrijgbaar zijn in capsules die tussen de 100 tot 200 mg aan flavonoïden bevatten. De flavonoïd-concentratie die bereikt kan worden in de darm na inname van een dergelijk supplement is ongeveer 35 µM en dit is in dezelfde orde van grootte als de waarden die in dit proefschrift gevonden zijn voor de apparent Ki-waarden voor quercetine (5.6 µM), naringenin (11.7 µM), luteolin (12.2 µM), myricetin (37.3 µM) en apigenin (10.8 µM). De concentratie komt ook overeen met Ki-waarden voor flavonoïden beschreven in de literatuur van 2.4 µM tot 20.8 µM voor de competitieve remming van de MRP1 transporter, bestudeerd met LTC_4 als substraat (*26*), of de Ki-waarden beschreven voor de flavonoïd gemedieerde remming van de efflux van calceïne-AM door MRP1 en MRP2 (*18*). Bij fysiologisch relevante concentraties van flavonoïden in de darm kan daarom een interactie met de ABC transporters verwacht worden en daarmee een verandering in de biobeschikbaarheid van geneesmiddelen, lichaamsvreemde stoffen en voedsel bestanddelen.

Hoewel flavonoïden de biobeschikbaarheid van PhIP *in vitro* en *in vivo* verhogen moet het algehele effect van flavonoïden op de potentiële kankerverwekkende werking van PhIP bestudeerd worden voordat een uitspraak gedaan kan worden over het effect van flavonoïden op het risico van een blootstelling aan PhIP.

Behalve studies die het effect van flavonoïden op de absorptie van PhIP of andere substraten hebben bestudeerd, hebben andere studies het effect van antioxidanten, waaronder flavonoïden, op de vorming van heterocyclische amines in modelsystemen bestudeerd. Studies die een modelsysteem gebruikten om de vorming van heterocyclische amines te bestuderen, toonden bijvoorbeeld aan dat de aanwezigheid van het polyfenol EGCG, aanwezig in thee, de vorming van IQ-type heterocyclische amines verminderde. De schrijvers suggereren dat de antioxidant-activiteit van ECGC de vrije radicalen die gevormd worden tijdens de Maillardreactie wegvangt en daardoor de vorming van de mutagene heterocyclische amine vermindert (*30*). De flavonoïden die in dit proefschrift werden bestudeerd hebben mogelijk een vergelijkbaar effect, hoewel flavonoïden niet altijd aan het bakproces van vlees en vis worden toegevoegd.

Busquet *et al.* (*31*) hebben het effect van rodewijnmarinades op de vorming van heterocyclische amines in kipfilet bestudeerd. De gevonden resultaten tonen aan dat het effect van de rodewijnmarinade afhankelijk is van de heterocyclische amine die gevormd wordt. Bij in rodewijngemarineerde kipfilet werd er 88% minder PhIP

gevormd terwijl de vorming van MeIQ en 4,8-DiMeIQx toenam door de rodewijnmarinade tot wel 300%. Het mechanisme dat ten grondslag ligt aan de invloed van de rodewijnmarinade op de vorming van heterocyclische amines is op dit moment nog niet duidelijk.

Flavonoïden hebben mogelijk niet alleen effect op de vorming van heterocyclische amines maar kunnen ook de metabole activatie van PhIP remmen door de CYP1A2- of NAT-activiteit te remmen, de detoxificatie-enzymen zoals de sulfotransferases en glutathion-S-transferases te stimuleren of de reactieve moleculen weg te vangen (literatuuroverzicht in (*32*)). Op deze manier zouden de flavonoïden, door het remmen van de metabole activatie van heterocyclische amines, kunnen beschermen tegen de mutagene activiteit van deze stoffen.

Naast het beschermen tegen de vorming van heterocyclische amines kunnen flavonoïden mogelijk ook bescherming geven tegen de vorming van PhIP-DNA-adducten. Dit werd aangetoond in een studie waarbij HepG2-cellen werden blootgesteld aan PhIP in aanwezigheid van een toenemende concentratie quercetine: de aanwezigheid van 5-20 µM quercetine verminderde significant het aantal PhIP-DNA adducten dat werd gevormd (*33*). In dezelfde studie werd het effect van het blootstellen van hepatocyten van vijf verschillende individuen aan PhIP in aan- of afwezigheid van quercetine bestudeerd. In drie van de vijf individuen nam het aantal PhIP-DNA-adducten af terwijl in de andere twee individuen geen effect van quercetine werd waargenomen. Huber *et al.* (*34*) toonden in F344-ratten aan dat ratten die 10 dagen 2% zwarte thee in hun drinkwater hadden gekregen en vervolgens werden blootgesteld aan 50 mg/kg bw PhIP slechts 34% van de PhIP-DNA-adducten in de dikke darm hadden ten opzichte van de controlegroep. Ratten die tien dagen 1% quercetine in hun voeding hadden gehad voordat ze werden blootgesteld aan PhIP hadden 148% van de PhIP-DNA-adducten in de dikke darm ten opzichte van de controlegroep. Lin *et al.* (*35*) toonden aan dat SD ratten die 3% groene thee via hun drinkwater binnenkregen voordat ze werden blootgesteld aan 10 mg/kg bw PhIP significant minder PhIP-DNA-adducten hadden in de dikke darm, hart, longen en lever, terwijl de studie van Huber *et al.* (34) aantoonde dat de behandeling van F344 ratten met 2% groene thee via hun drinkwater, voordat ze werden blootgesteld aan 50 mg/kg bw PhIP, geen effect had op de PhIP-DNA-adducten die werden gevormd in de dikke darm. Het effect van de polyfenolen op de vorming van PhIP-DNA-adducten is dus afhankelijk van het type polyfenol, het gebruikte testsysteem (*in vitro* vs. *in vivo*),

de voedselmatrix waarin de polyfenolen aanwezig zijn en voor de *in vivo* studies ook de manier waarop de polyfenolen worden toegediend.
Om een idee te krijgen van de grootte van het effect van de flavonoïden op de absorptie van PhIP in de concentraties zoals aanwezig in de Westerse voeding werd het *in silico* model, beschreven in hoofdstuk 3, 4 en 5, gebruikt om het effect van de flavonoïden op het PhIP transport te schatten. Om dit te kunnen doen werd de informatie gebruikt uit de studie van Hertog *et al.* (*36*) die de inname van verschillende flavonoïden in volwassenen in Nederland beschrijft. Zij schatten dat een volwassene in Nederland ongeveer 23 mg flavonolen per dag binnenkrijgt waarvan 16 mg quercetine. De gemiddelde humane inname van heterocyclische amines is geschat op 2-25 ng/kg/dag (*37, 38*). Wanneer wordt uitgegaan van een "worst case"-scenario, waarbij een mens van 70 kg 25 ng/kg/dag PhIP en 16 mg quercetine eet, kan worden berekend dat in de darm gehaltes bereikt kunnen worden van 0.77 nM PhIP en 5.3 μM quercetine. Wanneer de parameters voor de passieve diffusie, apparent Vmax en apparent Km uit **hoofdstuk 5** worden gebruikt en de apparent inhibitie constante van quercetine uit **hoofdstuk 3,** voorspelt het *in silico* model een toename in het PhIP-transport in aanwezigheid van quercetine van 2-3 keer vergeleken met de situatie waarbij quercetine niet aanwezig is. Wanneer deze data geëxtrapoleerd worden naar de situatie waarin een quercetinesupplement van 100 mg wordt ingenomen (waarbij de concentratie in de darm geschat wordt op 35 μM) dan wordt de PhIP-opname bijna 4 keer zo hoog in de aanwezigheid van quercetine, vergeleken met de controlesituatie in afwezigheid van quercetine.
Ondanks het feit dat de schattingen van het *in silico* model voor het transport van PhIP in aanwezigheid van quercetine een toename in het transport van PhIP laten zien, is het niet mogelijk om het algehele effect van quercetine op de kankerverwekkende werking van PhIP te voorspellen. Dit komt omdat andere factoren ook het effect van quercetine op het risico van de blootstelling aan PhIP kunnen beïnvloeden, bijvoorbeeld het effect van de flavonoïden op de cytochroom P450-enzymen en dus op de bioactivatie van PhIP, nodig voor de vorming van het uiteindelijke mutageen. Daarnaast bestaat de humane voeding uit groente en fruit die flavonoïden bevatten en gebakken vlees en vis die heterocyclische amines bevatten in plaats van de pure stoffen, en voedselbestanddelen aanwezig in de voedselmatrix kunnen een effect hebben op de absorptie van PhIP, terwijl in het model en de experimenten beschreven in dit proefschrift alleen het effect van de pure stoffen is bestudeerd. Ook kan de aanwezigheid van flavonoïden (bijvoorbeeld in een ui) tijdens het bakken van vlees de vorming van heterocyclische amines

verminderen. Tenslotte kan de aanwezigheid van flavonoïden in het menselijk lichaam de metabole activatie en/of de DNA adductvorming van heterocyclische amines mogelijk remmen.
Aangezien het effect van de flavonoïden op de absorptie van PhIP is waargenomen wanneer PhIP en quercetine gelijktijdig werden toegediend in een enkele dosis, is het interessant om een dierproef uit te voeren waarin het effect van een toenemende periode tussen het toedienen van de flavonoïden en PhIP wordt vergeleken met het gelijktijdig toedienen van PhIP en de flavonoïden. De uitkomsten van dit experiment zouden inzicht kunnen geven in de mogelijkheid om het effect van de flavonoïden op de biobeschikbaarheid van PhIP te verminderen en dus de mogelijke risico's te verminderen, door het moment van inname van groente en fruit en vlees te scheiden.
Dit proefschrift toont aan dat het effect van de flavonoïden op de biobeschikbaarheid van PhIP veroorzaakt werd door de remming van apicale ABC-transporters. Aangezien deze transporters ook betrokken zijn bij het transport van andere chemicaliën, waaronder geneesmiddelen, kunnen we concluderen dat er mogelijk sprake is van een voedsel-geneesmiddelinteractie met de ABC-transporters bij inname van lage concentraties van (mengsels van) flavonoïden. Het is daarom dan ook interessant om aanvullende vervolgstudies uit te voeren waarbij het effect van deze relevante lage concentraties van mengsels van deze teststoffen met geneesmiddelen verder wordt onderzocht.
Al met al kan geconcludeerd worden dat de resultaten in dit proefschrift laten zien dat de aanwezigheid van flavonoïden, door het remmen van de ABC-transporters, de biobeschikbaarheid van een toxische stof die aanwezig is in het voedsel kunnen verhogen. Ook werd aangetoond dat de interacties van de flavonoïden en mengsels van flavonoïden met de ABC- transporters kunnen plaatsvinden bij fysiologisch relevante lage concentraties. De relevantie van de resultaten van dit proefschrift wordt ondersteund door de flavonoïdengehaltes in het voedsel en de voedselsupplementen die op dit moment commercieel verkrijgbaar zijn. Dit wijst er op dat de flavonoïden gebruikt in dit proefschrift en hun mengsels een belangrijke factor kunnen zijn in de regulatie van de ABC-transporter gemedieerde uitscheiding van lichaamsvreemde stoffen en dus in de regulatie van de biobeschikbaarheid van de lichaamsvreemde stoffen nadat deze oraal zijn ingenomen.

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Curriculum Vitae

Maaike Evelien Schutte werd geboren op 24 februari 1978 te Haarlem. In 1997 behaalde zij haar VWO diploma op het Augustinianum te Eindhoven. In datzelfde jaar begon ze met de opleiding Voeding en Gezondheid aan Wageningen Universiteit (WU). Tijdens deze opleiding deed zij een afstudeervak bij de sectie Toxicology (WU) onder begeleiding van dr. ir. Jelmer van Zanden en Prof. dr. ir. Ivonne Rietjens. Vervolgens deed zij een afstudeervak bij de leerstoelgroep levensmiddelenchemie (Wageningen Universiteit) onder begeleiding van Aagje Legger en dr. Jozef Linssen. Ze sloot haar studie af met een onderzoeksstage bij Division of Molecular Pharmacology, Neurotec, Karolinska Institutet, Huddinge (Zweden) onder begeleiding van Dr. Christina Unger en Prof. Dr. Agneta Nordberg. In november 2002 behaalde zij haar doctoraal diploma. Aansluitend begon zij met haar promotie-onderzoek aan Wageningen Universiteit sectie Toxicologie in samenwerking met TNO Kwaliteit van Leven (Zeist), waarvan de resultaten in dit proefschrift beschreven zijn. Tijdens haar promotie-onderzoek heeft zij tevens deelgenomen aan de Postdoctorale Opleiding Toxicologie.
Sinds juni 2007 is zij werkzaam als onderzoeker toxicologie bij LGC Ltd., Teddington (UK).

List of publications

Published articles

Schutte ME., Boersma MG., Verhallen DAM., Groten JP. and Rietjens IMCM. Combined effects of multiple flavonoids on the transport of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) through Caco-2 monolayers: an *in vitro* and a kinetic modelling approach to predict the combined effects on transporter inhibition. *Food and Chemical Toxicology (in press)*

Schutte ME., Freidig AP., van de Sandt JJM., Alink GM., Rietjens IMCM. and Groten JP. (2006) An *in vitro* and *in silico* study on the flavonoid mediated modulation of the transport of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) through Caco-2 monolayers. *Toxicology and Applied Pharmacology* 217: 204-215

Brand W., Schutte ME., Williamson G., van Zanden JJ., Cnubben NHP., Groten JP., van Bladeren PJ. and Rietjens IMCM. (2006) Flavonoid-mediated inhibition of intestinal ABC transporters may affect the oral bioavailability of drugs, food-borne toxic compounds and bioactive ingredients. *Biomedicine and Pharmacotherapy* 60: 508-519

Schutte ME., van de Sandt JJM., Alink GM., Groten JP. and Rietjens IMCM. (2006) Myricetin stimulates the absorption of the pro-carcinogen PhIP. *Cancer Letters* 231: 36-42

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Unger C., Svedberg MM., Schutte M., Bednar I. and Nordberg A. (2005) Effect of memantine on the alpha7 neuronal nicotinic receptors, synaptophysin- and low molecular weight MAP-2 levels in the brain of transgenic mice over-expressing human acetylcholinesterase. *Journal of Neural Transmission* 112: 255-268

Submitted article

Schutte ME., Alink GM., Freidig AP., Vaessen J., van de Sandt JJM., Groten JP. and Rietjens IMCM. Quercetin increases the bioavailability of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in rats *(submitted)*

Training and Supervision Plan

Overview of conferences and courses attended during PhD

Radiation expert 5B (Larenstein)	2003
International Advanced course on Chemistry and Biochemistry of Antioxidants (VLAG)	2003
Food Toxicology and Food Safety (PET)	2003
Ecotoxicology (PET)	2003
European Graduate School of Toxicology symposium	2003
VLAG PhD week (VLAG)	2003
Organising and supervising MSc thesis projects (OWU)	2003
Risk assessment (PET)	2004
Genetic Toxicology and Carcinogenesis (PET)	2004
Toxicology and Law (PET)	2004
PhD symposium (NVT)	2004
Immunotoxicology (PET)	2005
Medical and Forensic Toxicology (PET)	2005
Food toxicants: Threats and opportunities symposium (HEATOX)	2005
Conference on Membrane drug transporters (EUFEPS)	2005
PhD scientific writing (Centa)	2005
Philosophy and Ethics of Food Science and Technology (VLAG)	2006
PhD symposium (NVT)	2006
Talent Day (NWO)	2006
Conference on Membrane drug transporters (EUFEPS)	2006

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