Modulation of multidrug resistance by flavonoids.

Inhibitors of glutathione conjugation and MRP-mediated transport.

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Abstract

Modulation of multidrug resistance by flavonoids.
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In this thesis, the use of flavonoids for inhibition of two important players in the glutathione related biotransformation system involved in multidrug resistance was investigated using several *in vitro* model systems. The enzymes of interest included the phase II glutathione S-transferase enzyme GSTP1-1, able to detoxify anticancer agents through conjugation with glutathione and the two multidrug resistance proteins MRP1 and MRP2 involved in glutathione mediated cellular efflux of, amongst others, anticancer drugs.
The studies presented in this thesis reveal that the major site for flavonoid mediated interaction with GSH-dependent multidrug resistance processes are the GS-X pumps MRP1 and MRP2 rather than the conjugating GSTP1-1 activity. Whereas flavonoids are unlikely to be efficient cellular or *in vivo* GSTP1-1 inhibiting agents useful to reverse this aspect of multidrug resistance, they might be useful as inhibitors of MRP1 and MRP2 activity. A model compound used in this thesis able to inhibit both MRP1 and MRP2 activity, the flavonoid myricetin, was shown to effectively inhibit vincristine efflux by these transporters in MRP1- and MRP2-transfected cells, thereby effectively sensitizing the cells towards the anticancer drug. Moreover, phase II metabolism, occurring to a major extent *in vivo*, of the other model flavonoid used in this thesis, quercetin, resulted in equally potent or even better inhibitors of MRP1 and MRP2. This indicates that phase II metabolism is unlikely to reduce the MRP inhibiting potential of quercetin for use of this flavonoid as an inhibitor to overcome MRP-mediated multidrug resistance. Furthermore, it was shown that the flavonoid myricetin is unlikely to affect MRP-mediated transport of glutathione conjugates to a significant extent, because, in general, glutathione conjugates such as the glutathione conjugates of the endogenous compound prostaglandin A_2, are high affinity substrates of MRP1 and MRP2. These results provide an argument for the possible absence of specific negative side effects on the kinetics and physiology of endogenous MRP substrates, to be expected upon use of these natural MRP inhibitors in the reversal of multidrug resistance. Testing of the *in vitro* outcomes of the present study in clinical settings may start with flavonoids that have already a safe history of use in for example food supplements and requires the confirmation of involvement of the MRPs in specific cases of clinical drug resistance prior to therapeutic use of the flavonoids as MRP inhibitors.

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General Introduction and outline of the thesis

Cellular defence against cytotoxic compounds

Living organisms are under constant threat by endogenous or exogenous toxic compounds. Therefore, a range of cellular defensive mechanisms have evolved to deal with to these toxicants. This cellular defence focuses on biotransformation of these compounds to relatively non-toxic metabolites and their subsequent elimination through transport. Most cells are equipped with a multitude of phase I and phase II biotransformation enzymes. In phase I metabolism hydroxylation, oxidation and reduction reactions take place on relatively hydrophobic xenobiotics. Subsequently, phase II conjugation reactions with, among others, glutathione (GSH), glucuronate or sulphate take place resulting in even more hydrophilic compounds. The resulting products (usually less toxic and more hydrophilic) can be excreted through active/facilitated transport processes across the cellular membranes (phase III). This efflux of xenobiotics and/or their metabolites is carried out by plasma membrane transporter proteins.

Multidrug resistance

During the past five decades, the use of anticancer drugs has become one of the most important ways of controlling malignant diseases. However, the emergence of drug resistance in many cases makes the currently available chemotherapeutic agents ineffective. Multidrug resistance (MDR) is the resistance of a tumour cell population against drugs differing in chemical structure and cellular target. The resistance of malignant cells to these drugs through cellular alterations is considered one of the major causes of failures of chemotherapy [1]. Ineffectiveness of chemotherapy may be provoked by other causes in addition to tumour cell alterations. It can be caused, for example, by non-cellular resistance mechanisms like a decreased blood-flow in tumours preventing the drug from reaching its target cells. The main mechanisms involved in cellular MDR, however, are cellular alterations, as a consequence of upregulation of specific genes involved in biotrans-formation processes, cellular efflux, cell replication or apoptosis [1]. Several MDR mechanisms have been identified, but the discovery of the membrane transporter P-glycoprotein (MDR1) was a breakthrough in understanding the MDR phenotype of cancer cells [2]. Upon the discovery of MDR1 many more enzymes were identified which, upon upregulation, could cause or enhance cellular multidrug resistance. Especially some members of the ATP-binding cassette (ABC) transporters superfamily, involved in cellular efflux of compounds across the membrane, against a concentration gradient, with ATP-hydrolysis as a driving force, have shown to be of particular clinical importance in MDR [3]. Other important enzymes responsible for clinical multidrug resistance are glutathione S-transferases (GSTs), especially of the π class, and enzymes involved in cell regulation (for example topoisomerase I/II) [4]. For many identified forms of MDR it was shown that not the upregulation of one enzyme alone, but rather the combined overexpression of several enzymes / transporters is responsible for the reduced therapeutic effect [5-9]. For many types of chemotherapeutic drugs one or more proteins have been identified that can reduce the therapeutic effect of the drugs (Table 1).

Table 1 Proteins involved in MDR and the anti-cancer drugs affected by their upregulation [10-15].

Name	***Anticancer drugs***
Pgp	*Doxorubicin, daunorubicin, epirubicin, etoposide, paclitaxel, docetaxel, vincristine, vinblastine, rhodamine-123, quinidine, aldosterone*
MRP1	*Vincristine, daunorubicin, doxorubicin, etoposide*
MRP2	*Methotrexate, etoposide, cisplatin, vinca alkaloids*
MRP3	*Etoposide, teniposide, estrogen derivatives, methotrexate, vinca alkaloids*
MRP4	*Purine analogues, estrogen derivatives*
MRP5	*Thiopurines, cyclic nucleotides*
GSTs	*Chloroethylnitrosoureas, cisplatin, thiotepa, anthracyclines, phosphanides, acrolein, melphalan, cyclophosphamide*
Topo II	*Chloroethylnitrosoureas, epipodophyllotoxins, anthracyclines*

One complex system of proteins involved in MDR is the glutathione-related biotransformation system, subject of the current thesis. This system consists of the tripeptide glutathione (GSH) and, among others, γ-glutamylcysteine synthetase (γ-GCS), glutathione S-transferases (GSTs) and glutathione-conjugate transport proteins (GS-X pumps). The next paragraphs give a general introduction on the subjects which are relevant within the context of the present thesis.

Glutathione S-transferases

The glutathione S-transferases (GSTs) comprise a family of dimeric phase II detoxification enzymes that catalyse the conjugation of glutathione to a wide variety of endogenous and exogenous electrophilic compounds. GSTs are divided into two distinct super-families: the membrane-bound microsomal and cytosolic family. Cytosolic GSTs are highly polymorphic and can be divided into six classes which share ~30% sequence identity, and are designated by Greek letters α, μ, ω, π, θ and ζ [16, 17]. In addition, the K-class isoforms exist in mitochondria and are structurally similar to the cytosolic forms. These microsomal GSTs are structurally distinct from the cytosolic in that they homo- and heterotrimerize rather than dimerize to form a single active site [16]. Historically, GSTs were named according to their ability to catalyze the nucleophilic addition or substitution of glutathione (GSH; γ-glutamyl-cysteinyl-glycine) at electrophilic centers in a wide range of xenobiotic electrophilic substrates (Figure 1).

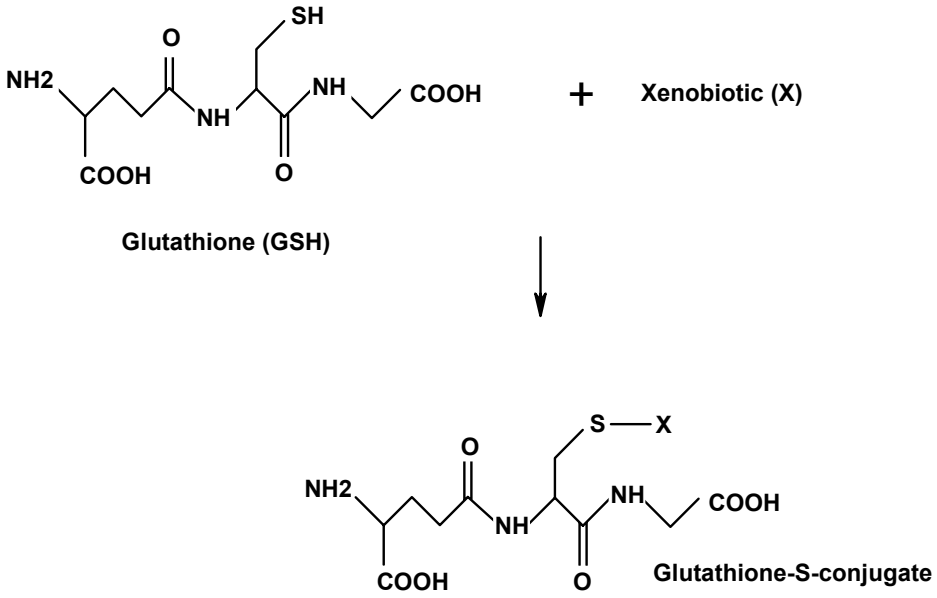

Figure 1 Glutathione conjugation to a xenobiotic (X) via GSTs.

The typical GST-catalyzed reactions include Michael-type addition, nucleophilic aromatic substitution, nucleophilic addition to epoxides, cis-trans double bond isomerization, positional double bond isomerization, and peroxide reduction (reviewed in [18]). Importantly, although many of these reactions are catalyzed by several different GSTs, each isoform exhibits its own substrate selectivity suited to act on functional groups rather than specific compounds (Table 2).

Table 2 Cytosolic GSTs and some of their typical substrates, adapted from [16, 19].

Class	**Genes**	***Typical Substrates***
Alpha (α)	GSTA1-2	*Prostaglandins, lipid peroxidation products*
Mu (μ)	GSTM1-4	*Quinones of dopamine, catecholamines*
Omega (ω)	GSTO1	*Inorganic arsenic*
Pi (π)	GSTP1	*α,β-Unsaturated aldehydes*
Theta (θ)	GSTT1-2	*Molecules with epoxide groups*
Zeta (ζ)	GSTZ1	*Maleylacetoacetate*

GST in Cancer and Multidrug Resistance

Several GSTs can conjugate GSH to anti-cancer DNA alkylating agents like busulfan, melphalan, chlorambucil, thiotepa and other anticancer drugs, thereby detoxifying these drugs [16, 20-26]. The overexpression in tumours of GSTs, especially of GSTP1-1 is,

considered as a possible mechanism of tumour cell drug resistance [27-31]. It is plausible that GSTs serve two distinct roles in the development of drug resistance: (i) via direct detoxification as well as (ii) acting as an inhibitor of the MAP kinase pathway (pi class only). The link between GSTs and the MAP kinase pathway provides a rationale as to why in many cases the drugs used to select for resistance are neither subject to conjugation with GSH, nor substrates for GSTs (reviewed by [16]). The contribution of any GST to drug resistance is likely to vary with cell type and drug, as well as with the expression profile of other enzymes and transporters. However, it is still widely accepted that the GSTs can contribute directly to drug resistance in some cell types via their catalytic activity, so inhibitors of GST catalytic activity are considered as a potential therapeutic tool.

GST inhibition

GST isozymes can accommodate many different substrates in their active site. As a result, many different classes of molecules have been described to competitively or non-competitively inhibit GST-mediated GSH-conjugation [20, 22, 24, 27, 32, 33]. These include endogenous inhibitors like fatty acids [34, 35] and retinoids [36]; and exogenous inhibitors like α,β-unsaturated compounds including for example. ethacrynic acid [37, 38], 1-chloro-2,4-dinitrobenzene (CDNB) [39], quinones [20] and plant polyphenols [40, 41]. The first generation of clinically tested GST inhibitors included ethacrynic acid which is a substrate/inhibitor of several GSTs [38, 42-45]. Although ethacrynic acid effectively increased the sensitivity of cells in model cultures, or even in patients, to melphalan, piriprost, or chlorambucil, its potential toxicity and diuretic effects prevented its development for this therapeutic use. GST inhibitors undergoing extensive clinical testing include glutathione analogues and demonstrate high specificities with few limiting toxicities [22, 46, 47]. Specific inhibitors of GSTP1-1 are rare although some haloenol lactone derivatives have been described as pi-class specific inhibitors [48].

The Multidrug Resistance (Associated) Proteins

Human ABC transporters were originally associated with drug resistance and cystic fibrosis, where upregulation of P-glycoprotein resulted in resistance towards the therapeutics [49]. The identification of novel members of the ABC family revealed that these transporters have important physiological functions indicated by the involvement of transporter dysfunctioning in several human diseases including Tangier disease, Stargardt disease, Dubin-Johnson syndrome, adrenoleucodystrophy and a group of liver disorders known as progressive familial intrahepatic cholestasis [50]. The discovery of the ABC transporter P-glycoprotein (MDR1) marked the beginning in understanding the human drug resistance phenotype [2]. Overexpression of this 170-kDa transmembrane protein confers resistance to a wide spectrum of natural product drugs by an ATP-dependent extrusion of these compounds resulting in decreased intracellular levels (reviewed in [51]). For several years, MDR1 was the only ABC transport protein associated with drug resistance. However, reports describing drug-resistant cell lines without MDR1-overexpression indicated the presence of other MDR-conferring proteins. These observations led to the

identification of a second drug resistance-related ABC transporter, the multidrug resistance protein (MRP) [52-55], further designated as MRP1 (symbol ABCC1). MDR1 and MRP1 confer resistance to a similar, although not identical, group of anticancer drugs. The identification of the MRP1 homologues MRP2 (ABCC2) [56], MRP3 (ABCC3), MRP4 (ABCC4), MRP5 (ABCC5), MRP6 (ABCC6) [57, 58] and more recently MRP7 (ABCC10) [59], MRP8 (ABCC11) [60], MRP9 (ABCC12) [61] and ABCG2/BCRP [62, 63] defined a new subfamily of ABC transporters with potential involvement in drug resistance.

Despite the similarity in the resistance profiles of Pgp, MRP1 and MRP2 (Table 1), the substrate selectivities of these pumps differ markedly, Pgp substrates are neutral or mildly positive lipophilic compounds, whereas MRP1 is able to transport lipophilic anions. MRP1 has a broad substrate specificity including glutathione S-conjugates, glucuronide conjugates, sulphate conjugates, the estrogen glucuronide E217βG, sulphated bile acids, anticancer drugs, heavy metals, organic anions and lipid analogues [55, 64-69]. The ability of MRP1 to transport glutathione conjugates, in combination with its widespread expression in tissues, indicates that it is an ubiquitous GS-X pump [70-72]. The role of MRP1 in clinical drug resistance has been studied extensively [3, 73-78]. MRP2, the major canalicular Multispecific Organic Anion Transporter (cMOAT), is closely related to MRP1 [79, 80]. Nevertheless, the tissue localization of these two transporters differs. Whereas MRP1 is localized in the basolateral membranes of polarized cells and is present in all tissues, MRP2 [56] is found in the apical membranes of polarized cells (Figure 2) and is mainly expressed in the liver, intestine and kidney.

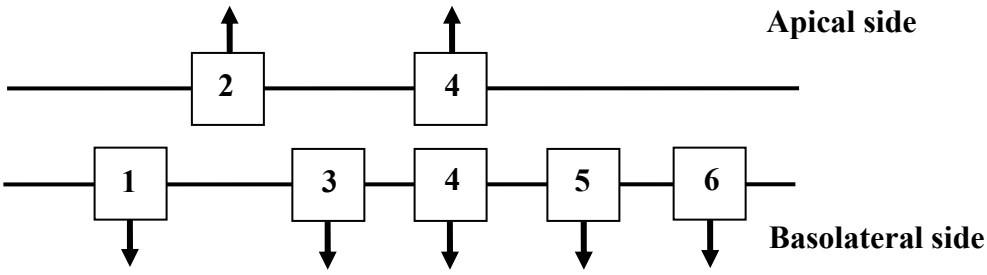

Figure 2. Localization of MRPs in polarized cells. MRP1, MRP3, MRP5 and MRP6 are localized in basolateral membranes [57, 58]. MRP2 is localized in apical membranes [56, 81]. MRP4 is localized in basolateral membranes in human prostatic glandular cells and in apical membranes in rat kidney tubule cells [56]. The localizations of MRP7, MRP8 and MRP9 have not been determined. Pgp and BCRP are apical efflux pumps (not shown) [82].

MRP1 and MRP2 consist of three hydrophobic membrane-spanning domains (MSDs) and two cytoplasmic nucleotide-binding folds (NBFs) (also named nucleotide binding domains NBDs). MRPs require ATP-hydrolysis for their transport function [65, 66, 83, 84]. It has been shown that the ATPase activity of the NBDs from MRP1 and MRP2 provide the

energy for the transport process [85-90]. Topology studies predict a secondary MRP1 structure as: NH2-MSD0-MSD1-NBD1-MSD2-NBD2-COOH.[91]. The extra N-terminal MSD is characteristic for certain members of the MRP family (MRP1, MRP2, MRP3, MRP6 and MRP7) (Figure 3).

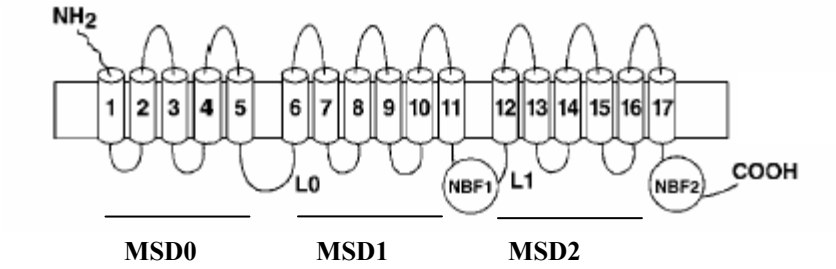

Figure 3. Topological model of MRP1 (which resembles MRP2, MRP3, MRP6 and MRP7). NBF, nucleotide binding fold; MSD, membrane spanning domain. MRP4, MRP5, MRP8 and MRP9 lack the MSD0 domain. MSD domains of the proteins are aligned (described by [59, 82, 92, 93]).

Comparison of human MRP1 and MRP2 shows that both transporters are composed of 1531 and 1545 amino acids, respectively. They exhibit an amino acid identity of 49% with the highest degree of amino acid identity in the carboxyl-terminal domain and in both nucleotide-binding folds [94, 95]. Despite this limited amino acid identity the spectrum of substrates transported by MRP1 and MRP2 overlap to a large extent although MRP1 seems to be less specific [96-98]. MRP1 and MRP2 are the main contributors to GSH-conjugate efflux [99]. The role of glutathione in MRP1 and MRP2 activity is diverse (Figure 4). Besides the efflux of GS-conjugates [100-102] (Figure 4A) agents such as vinca alkaloids and anthracyclines are co-transported with glutathione [66, 103-106] (Figure 4B). Also allosteric interactions of glutathione with the transport of certain anionic conjugates such as estrone-3 sulfate and the glucuronide conjugate of a nitrosamine metabolite, NNAL-O-glucuronide have been described [107-109]. Efflux of these substrates is dependent upon glutathione but does not appear to be associated with co-transport of glutathione, and is therefore considered to be the result of a positive allosteric effect exerted by glutathione (Figure 4C). Additionally, some compounds, such as the Pgp inhibitor verapamil, and certain flavonoids like apigenin, naringenin, genistein, and quercetin, are able to stimulate transport of glutathione by MRP1, but do not appear to be substrates themselves [110, 111]. Hence, these compounds exert an allosteric effect that increases the affinity of the pump for glutathione (Figure 4D). In addition, GSSG, the oxidation product of glutathione, is also a good MRP1 substrate (Figure 4E) [112, 113].

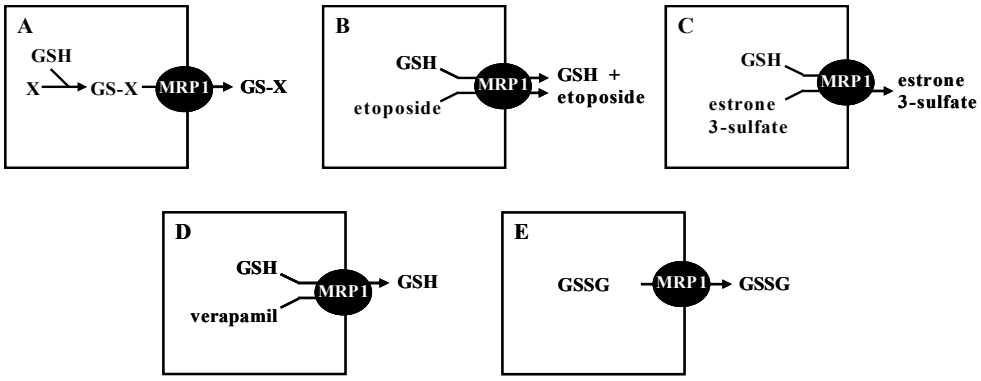

Figure 4. Involvement of glutathione in MRP1-mediated transport, adapted from [82] reflecting: (A) transport of hydrophobic compounds that conjugated to glutathione, (B) co-transport of etoposide and glutathione, (C) transport of estrone 3-sulfate stimulated by glutathione, without co-transport of glutathione representing allosteric regulation by glutathione, (D) transport of glutathione is stimulated by verapamil, without verapamil being transported and (E) transport of oxidized glutathione (GSSG).

MRP1 and MRP2 in Multidrug Resistance

Natural product drugs such as doxorubicin, vincristine and VP-16 were shown to be substrates of MRP1 since transfected and drug-selected cells overexpressing MRP1 became resistant to these cytotoxic agents [14, 80, 114]. Importantly, the ATP-dependent transport of at least some natural product drugs by MRP1 (e.g. vincristine and daunorubicin) requires physiological amounts of GSH (see previous paragraph). In addition to the resistances mentioned in Table 1, MRP1 can confer resistance to short-term exposures to the folate antimetabolite methotrexate [115], the topoisomerase I inhibiting camptothecin derivative, CPT-11 (irinotecan) and its metabolite SN-38 [116]. Conjugates of the alkylating agents including thiotepa [23], cyclophosphamide, chlorambucil, and melphalan are also substrates of MRP1. With the latter drugs, resistance conferred by MRP1 is often enhanced by upregulation of the conjugating enzyme, glutathione S-transferase, or the GSH biosynthetic enzyme, γ-glutamylcysteine synthetase (γ-GCS) [5, 117]. The antiandrogen flutamide, a drug commonly used in the treatment of prostate cancer, and its metabolite, hydroxyflutamide, were reported to be substrates of MRP1 [118] as were the protease inhibitors of the human immunodeficiency virus (HIV), ritonavir and saquinovir [119, 120]. This extensive list, which is not complete, clearly emphasizes the important role of MRP1 in multidrug resistance. MRP2 has many substrates in common with MRP1 although there are some differences. For instance, with respect to anticancer drugs, overexpression of MRP2 in intact tumour cells is associated with cisplatin resistance whereas MRP1 is not. Resistance to other drugs like vincristine, etoposide, doxorubicin and methotrexate can be

the result of both MRP1 and/or MRP2 overexpression [97, 115, 116, 121, 122]. Both proteins transport compounds of remarkably diverse chemical structures.

Inhibitors of MRP1 and MRP2

Inhibition of MRP1 and MRP2 activity can be obtained via different routes. One route is depletion of GSH by using buthionine sulfoximine (BSO) to inhibit γ-GCS which is an indirect way of sensitizing MRP1-overexpressing cells to certain cytotoxic agents [84, 103, 104, 123]. However, this is a very crude method not suitable for therapeutic use. Another route of MRP inhibition is through direct interaction of compounds with the transport protein. Several mechanisms in which inhibitors might interact with MRPs are known. Inhibition of MRPs might affect drug binding, ATP binding, ATP hydrolysis, drug transport, and the ADP release. Numerous inhibitors that directly inhibit MRP transport activity have been described, but for many of them, their specificity towards different transport proteins including the MRPs has not been defined. Of those inhibitors for which the specificity has been characterised, four different classes of inhibitors can be distinguished (Table 3) [116]. First, there are inhibitors that modulate the activity of many transporters, including organic anion transporters that do not belong to the ABC superfamily (general inhibitors of organic anion transport). These compounds include probenecid, benzbromanone, sulfinpyrazone and indomethacin [80, 124-127]. The second class of inhibitors are compounds that modulate the transport activity of MRP1 and several distantly related ABC transporters such as Pgp (non-specific inhibitors of MRPs). To this class belong VX-710 (Biricodar/Incel) [128, 129], the polyhydroxylated sterol agosterol A [130], the dihydropyridine PAK-104P [131, 132], verapamil, and cyclosporin A [133] as well as a number of different flavonoids (e.g. genistein, quercetin) [134-138] and steroid derivatives (e.g. RU486, budesonide) [139, 140]. However, it should be noted that most of these compounds have different efficacies towards the different transporters. At present, there is little direct evidence that these inhibitors are actually substrates of MRP1 [110, 111, 116, 141], and their mechanisms of inhibition have not been well characterized, although many are reported to restore drug accumulation. The third class of inhibitors are compounds that are relatively specific to the MRP-related transporters like MK571 [142], ONO-1078 [143], glibenclamide [144]. It should be noted that the leukotriene D_4 receptor antagonist MK571, which is an excellent MRP1 inhibitor in vesicular transport experiments, has only limited effect on MRP1 in intact cells, even at subtoxic dose, thereby limiting the usability of this inhibitor [80, 145]. Also a number of peptidomimetic GSH-conjugate analogues have been described which may have potential as relatively specific *in vivo* MRP1 inhibtors including, for example, adapted GSH conjugates of ethacrynic acid. [46]. The fourth class of MRP inhibitors consists of GSH dependent, highly specific and potent MRP1 inhibitors and contains some tricyclic isoxazoles including LY475776 and LY402913 [145-147].

Typical inhibitors of MRP2 have been described but few, if any, are known to be highly specific for this transporter alone. Indeed, many of them, such as MK571 and cyclosporin A, also inhibit MRP1 and MRP3, although with different potency and efficacy. Other

agents, such as the uricosuric agent sulfinpyrazone, inhibit MRP1 transport activity but stimulate MRP2 transport activity [126].

Table 3. Some inhibitors of MRP1 and related proteins, adapted from [116, 148].

General inhibitors of organic anion transport
probenecid, sulfinpyrazone, indomethacin
Non-specific inhibitors of MRP1
VX-710, agosterol A, PAK-104P, verapamil, cyclosporin A, certain flavonoids, RU486, budesonide
Relatively specific inhibitors of MRP1
MK571, ONO-1078, glibenclamide, some GSH conjugates
GSH-dependent highly specific inhibitors of MRP1
LY475776, LY402913

Finally, an alternative approach to the use of small molecules to inhibit MRP activity is the application of MRP1-specific antisense oligonucleotides and cDNAs as well as ribozymes and small interfering RNAs (RNAi). Rather than inhibiting MRP1 activity, these agents act to decrease levels of MRP1 mRNA and prevent synthesis of the MRP1 protein. Several of these nucleic acid inhibitors have shown considerable mRNA decreasing efficacy in *in vitro* cell culture model systems [149-152]. This method has also been applied to downregulate MRP2 expression with some success [153]. However, up to date downregulation of MRP expression by antisense oligonucleotides or RNAi has not been clinically tested. Another approach, which was shown to effectively reverse MRP2 mediated cisplatin resistance in A2780RCIS ovarium carcinoma cells [154], uses anti-MRP hammerhead ribozymes to silence MRP gene expression. This approach might prove applicable as specific means to overcome MRP-mediated drug resistance in the near future.

Reversal of Multidrug Resistance

Although many different strategies to reduce multidrug resistance are possible [1], the most important cellular mechanism of resistance is the increased detoxification and/or efflux of anticancer drugs by biotransformation enzymes and transport proteins. The glutathione related biotransformation enzymes and transporters are among the key players in these processes. One strategy to overcome transporter mediated drug resistance relies on the identification of inhibitors of these enzymes and transporters. These compounds should be relatively non-cytotoxic allowing high levels to be administered and maintained without encountering toxicity [1]. Many of the known inhibitors are relatively non-specific and might therefore exert unwanted drug-drug interactions or interference with other physiological systems reducing their potential use in clinical settings, as observed for several tested Pgp inhibitors [80, 155]. The quest for relatively non-cytotoxic GST/MRP inhibitors is a strategy which might give promising results. One group of relatively non-cytotoxic inhibitors of GST and/or MRPs are the flavonoids, subject of this thesis.

Flavonoids

Flavonoids (Figure 5) are a large group of polyphenolic antioxidants found in fruits and vegetables. In foods flavonoids appear as β-glycosides as well as aglycones and methoxylated flavonoids. Upon ingestion, flavonoids get metabolized into glucuronide-, sulphated and methoxylated conjugates [156-159]. Flavonoids and flavonoid-rich extracts have been implicated as beneficial agents in a multitude of disease states (reviewed in [160-163]), most commonly cancer [164-166], cardiovascular disease [167-169], and neurodegenerative disorders [170-172]. Interestingly, it has become clear over the last few years that the bioactive forms of flavonoids *in vivo* are not necessarily the natural phytochemical forms, for example the aglycones or their various glycosides, but possibly also conjugates and metabolites arising from these upon absorption (reviewed in [162]).

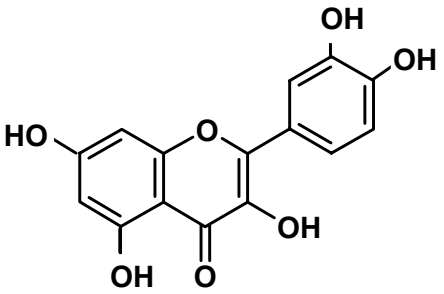

Figure 5. Structural formula of the flavonoid quercetin

The quest for non-toxic GST/MRP inhibitors showed that many natural constituents, including plant polyphenols like flavonoids were promising candidates [41, 134, 173-179]. However, good understanding of the structural parameters necessary for potent GST or MRP inhibition, or their inhibitory mechanism was lacking. The aim of this thesis was to characterise the potential of flavonoids as inhibitors of GSTP1-1, the major GST isozyme involved in MDR, and the transporters MRP1 and MRP2. This thesis describes the structural parameters necessary for potent GST or MRP inhibition using *in vitro* cellular models, and explores the applicability of flavonoids to reverse multidrug resistance *in vitro*.

Objective and outline of the thesis

This thesis explores the use of flavonoids for inhibition of two important players in the glutathione related biotransformation system involved in multidrug resistance: GSTP1-1 and the efflux proteins MRP1 and MRP2 using several model systems.

Chapter 1 gives a general introduction on the subjects which are relevant within the context of the present thesis.

To study the potential of the flavonoid quercetin to inhibit GSTP1-1 activity experiments to unravel this specific inhibitory mechanism, including the covalent binding of quercetin quinone methides to specific cysteine residues of GSTP1-1, were performed (*chapter 2*).

The effects of several structurally related flavonoids on GSTP1-1 and GS-X pump (MRP1) activity in a cellular system form the basis for *chapter 3*. Here, the effects of flavonoid structure on GSTP1-1 and GS-X pump inhibitory potency was studied in GSTP1-1 transfected MCF7 breast cancer cells.
In *chapter 4,* a more detailed quantitative structure activity relationship for structurally related flavonoids on MRP1 and MRP2 inhibition was derived using MRP transfected Madin-Darby Canine Kidney (MDCKII) cells.
Chapter 5 describes the use of the flavonoid myricetin to reverse MRP1- or MRP2-mediated vincristine resistance in MRP transfected MDCKII cells, using the chemosensitivity of the cells as measured by cell proliferation as final parameter.
The same transfected MDCKII cells were used for experiments described in *chapter 6* to study the effects of flavonoid mediated MRP inhibition on endogenous PGA_2-glutathione conjugate kinetics and bioactivity.
Finally, the effects of flavonoid metabolism on MRP1 and MRP2 inhibitory potency were studied using inside-out membrane vesicles from MRP transfected *Sf*9 cells (*chapter 7*).
The overall conclusions and the general discussion of this thesis are presented in *chapter 8*.

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2

Inhibition of human glutathione S-transferase P1-1 by the flavonoid quercetin.

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Abstract

In the present study, the inhibition of human Glutathione S-transferase P1-1 (GSTP1-1) by the flavonoid quercetin has been investigated. The results show a time- and concentration dependent inhibition of GSTP1-1 by quercetin. GSTP1-1 activity is completely inhibited upon one hour incubation with 100 μM quercetin or two hours incubation with 25 μM quercetin, whereas 1 and 10 μM quercetin inhibit GSTP1-1 activity to a significant extent reaching a maximum of 25% and 42% inhibition respectively after two hours. Co-incubation with tyrosinase greatly enhances the rate of inactivation, whereas co-incubation with ascorbic acid or glutathione prevents this inhibition. Addition of glutathione upon complete inactivation of GSTP1-1 partially restores the activity. Inhibition studies with the GSTP1-1 mutants C47S, C101S and the double mutant C47S/C101S showed that cysteine 47 is the key residue in the interaction between quercetin and GSTP1-1. HPLC and LC-MS analysis of trypsin-digested GSTP1-1 inhibited by quercetin did not show formation of a covalent bond between Cys47 residue of the peptide fragment 45-54 and quercetin. It was demonstrated that the inability to detect the covalent quercetin-peptide adduct using LC-MS is due to the reversible nature of the adduct-formation in combination with rapid and preferential dimerization of the peptide fragment once liberated from the protein. Nevertheless, the results of the present study indicate that quinone-type oxidation products of quercetin likely act as specific active site inhibitors of GSTP1-1 by binding to cysteine 47.

Introduction

The human pi-class glutathione S-transferase (GSTP1-1) is a valuable target for developing inhibitors that could be used to increase the chemotherapeutic efficiency in patients dealing with multidrug resistance. Together with the tripeptide glutathione (GSH), glutathione S-transferases play an important role in the major defense system of cells against electrophilic compounds and it has been shown several times that if GSH concentrations are low, class pi glutathione S-transferase (GSTP1-1) might serve as a scavenging protein [1, 2]. Overexpression of GSTP1-1 is associated with multi-drug resistance of tumor cells, as shown in numerous studies [3-5]. Therefore, the use of inhibitors to modulate human GSTP1-1 activity during chemotherapy is a promising strategy in the battle against multidrug resistance that could result in enhanced therapeutic efficiency of anticancer compounds. Inhibition of GSTP1-1 can be reversible and irreversible and extensive lists of reversible inhibitors are presented in the literature [6, 7]. Irreversible inhibitors modify the enzyme by covalent binding, resulting in loss of activity. Well-known examples of covalent inhibitors of GST enzymes are quinones [7]. GSTP1-1 is known to be susceptible to covalent modification of cysteine residues of the enzyme by electrophiles, the two most reactive or accesible cysteine residues being located on position 47 and 101 [8]. In particular Cys 47, which is located near the active site, has been observed to be a reactive and accessible moiety for electrophilic compounds [9]. Covalent modification of this cysteine residue results in loss of enzyme activity [1, 10-12]. Based on the fact that quinones can be good candidates for GSTP1-1 inhibition and the recent detection of quinoid-type products formed from quercetin [13, 14], quercetin could prove to be a valuable compound for GSTP1-1 inhibition.

Quercetin (Figure 1) is one of the natural polyphenols, which are important constituents of fruits, vegetables, nuts, red wine and tea. The average daily western diet contains about 1 gram of polyphenols of which quercetin is a major component [15-17]. These compounds are claimed to be responsible, at least partly, for the protective effect of fruits and vegetables against cardiovascular diseases [18, 19] and certain forms of cancer [17]. This property makes the flavonoids good candidates for the use as functional food ingredients. On the other hand quercetin is one of the flavonoids which has been reported to be mutagenic without metabolic activation in microbial but also in several mammalian cell systems [16, 20]. The toxic and mutagenic endpoints have been related to the formation of *o*-quinones and the subsequent isomerisation to *p*-quinone methide type metabolites (Figure 1) [13, 16, 20, 21]. Quercetin has been described in the literature to have an inhibitory effect on GST activity from canine erythrocytes [22], but the nature of this inhibition has not been elucidated yet. Phase I clinical trials of the flavonoid quercetin showed that quercetin can be safely administered by i.v. bolus at a 1400 mg/m^2 (approximately 70 mg/kg) dose, resulting in serum concentrations up to 400 μM immediately after injection and in concentrations above the normal 1 μM serum concentration up to 4 hours after administration [23].

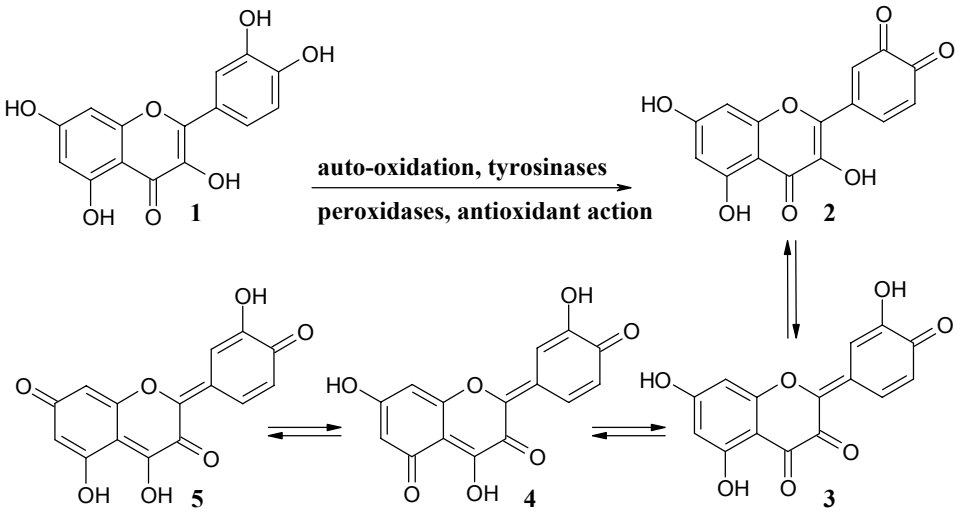

Figure 1. Structural formulas of quercetin (1) and its *ortho*-quinone (2) and *para*-quinone methides (3,4,5) [13]. Official names are: 2-(3,4-dioxo-1,5-cyclohexadienyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one for the *ortho*-quinone (2), 2-(3-hydroxy-4-oxo-2,5-cyclohexadienylidene)-3,4-dihydro- 5,7-dihydroxy-2H-1-benzopyran-3,4-dione (3) and 2-(3-hydroxy-4-oxo-2,5-cyclohexa-dienylidene)-3,5-dihydro-4,7-dihydroxy-2H-1-enzopyran-3,5-dione (4) and 2-(3-hydroxy-4-oxo-2,5-cyclohexadienylidene)-3,7-dihydro- 4,5-dihydroxy-2H-1-benzopyran-3,7-dione (5) for the *para*-quinone methides.

In the present study, we investigated the inhibition of GSTP1-1 by quercetin with special emphasis on a possible role for quercetin quinoid type intermediates and their possible covalent binding to cysteine residues of GSTP1-1. This was done to obtain insight in the possible interactions of B-ring catechol-type flavonoids and their quinoid products with GSTP1-1, in the process of developing new inhibitors that can be used to increase the chemotherapy efficiency in patients with multidrug resistance.

Materials and methods.

Materials

Quercetin, ascorbic acid, 1-chloro-2,4-dinitrobenzene (CDNB), human placental GST (GSTP1-1, EC 2.5.1.18), and tyrosinase were purchased from Sigma (St. Louis, MO USA). Glutathione and trypsin were obtained from Boehringer (Mannheim, Germany). The GSTP1-1 peptide fragment 45-54 (Ala-Ser-Cys-Leu-Tyr-Gly-Gln-Leu-Pro-Lys) was purchased from Sigma Genosys (United Kingdom) at >85 % purity. The construction and characterization of the three mutants of GSTP1-1, C47S, C101S, and C47S/C101S were described previously [24, 25].

Kinetic studies

Incubations of both wild type GSTP1-1 and the GSTP1-1 mutant enzymes were performed as previously described by Van Iersel *et al.* [12]. Incubations (final volume: 250 µl) consisted of 0.2 M potassium phosphate pH 7.4, supplemented with 0.2 mM EDTA, 0.5 µM enzyme, and 1, 10, 25 or 100 µM quercetin in the presence or absence of 6.67 units tyrosinase, 1 mM ascorbic acid or 10 mM GSH (as indicated). The concentration of GSH chosen was 10 mM because this is a relative high but physiologically relevant concentration [26]. The samples were incubated at 25 °C. Quercetin was added as 0.8 % (v/v) of a 125 times concentrated stock solution in methanol. For the time-dependent wildtype GSTP1-1 inhibition experiments at various time intervals during 150 minutes of incubation, GST activity was measured according to Habig *et al*. [27] adapted for a Thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA USA). For the GSTP1-1 mutant enzymes, the remaining activity upon 120 min. incubation with quercetin was measured. A 10 µl sample was added to a well containing 0.2 M potassium phosphate pH 6.5, supplemented with 0.2 mM EDTA, 1 mM GSH (final concentration) and water in a volume of 230 µl. After 2 minutes of incubation at 25 °C, 10 µl substrate, 1-chloro-2,4-dinitrobenzene (CDNB), was added from a 25 times concentrated stock solution to give a final CDNB concentration of 1 mM, and the formation of the CDNB-glutathione conjugate was measured at 340 nm.

Because inactivation of GSTP1-1 by unsaturated carbonyl compounds is known to be reversed by incubating the modified enzyme with an excess of glutathione (retro-Michael cleavage) [12, 28], the reversibility of the inactivation was studied as follows. GSTP1-1 was incubated with 25 µM quercetin for 150 minutes at 25 °C as described resulting in complete loss of activity. Mixtures were centrifuged in micron 10 microconcentrators (Amicon, Beverly, MA, USA) and the volume was adjusted to 125 µl with 0.2 M potassium phosphate pH 7.4, supplemented with 0.2 mM EDTA. GSH was added at a final concentration of 2.5 mM and the mixtures were incubated at 25 °C. GST activity was measured at various time intervals during 140 minutes.

Incubation for LC-MS

To prevent any chance of ionisation suppression by phosphate, the experiments were repeated in 10 mM ammonium carbonate pH 7.6 and the same inhibition curves were found (data not shown). For the measurement with LC-MS, 250 µl incubations were performed, as described above, in 10 mM ammonium carbonate pH 7.6. After 2 hours, mixtures were frozen to -80 °C and freeze-dried overnight. Residues were dissolved in 10 mM ammonium carbonate pH 7.6. Trypsin was added to a final amount of 1% (w/w) of trypsin with respect to GSTP1-1 and the mixture was incubated for 16 hours at 37 °C. During incubation, digestion was checked by electrophoresis on SDS gel [29], using silver staining.

The purchased GSTP1-1 fragment (45-54: Ala-Ser-Cys-Leu-Tyr-Gly-Gln-Leu-Pro-Lys) was further purified up to > 95 % by semi-preparative HPLC (ISCO 2300). To this end 10 mg of sample dissolved in 0.05% (v/v) trifluoracetic acid in water was injected onto a 200*25 mm Delta-Pack C18 column (Waters, Etten-Leur, The Netherlands) equilibrated

with 0.05 % (v/v) trifluoroacetic acid and 5 % (v/v) acetonitril in water at a flow rate of 8 ml/min. A linear gradient to 40 % acetonitril and 0.05 % (v/v) trifluoroacetic acid in water was applied in 55 minutes. UV detection was performed at 214 nm, in order to monitor the separation, using a Separations UVD 170S detector. The purity was checked with an analytical HPLC (Waters M600 pump and a Waters 996 photodiode Array Detector) using the same conditions as described above but with an analytical (150*3.9 mm) Delta Pack C18 column and a flow rate of 1 ml/min. The purified samples were freeze-dried and stored at -20^{o}C until the residues were dissolved in 10 mM ammonium carbonate pH 7.6 for further analysis.

LC-MS

The quercetin incubated tryptic peptide mixture was analyzed on a PepMap C18PM LC Packings column (0.3x150 mm, Amsterdam, The Netherlands) using a ThermoQuest liquid chromatography system (Spectra System, USA). The column was eluted with water containing 0.05% (v/v) trifluoroacetic acid (solvent A) and acetonitrile containing 0.05% (v/v) trifluoroacetic acid (solvent B). The gradient consisted of 5-20% B in 15 min, followed by 20-40% B in 40 min, 40-80% B in 5 min, using a flow rate of 4.6 µl/min. A volume of 1 µl of the samples was injected. The UV detector was equipped with a LC Packings UZ-LI-CAP flow cell and UV detection was performed at 214 nm. The purified purchased peptide fragment was analyzed using the same system as described above but with another gradient. This gradient consisted of 5-25% B in 2 min, followed by 25-32% B in 15 min, 32-80% B in 3 min and 5% B in 1 min, using a flow rate of 4.6 µl/min. All mass spectrometric analyses (Finnigan MAT95, San Jose, CA, USA) were performed in the positive electrospray mode using a spray voltage of 4.5 kV and a capillary temperature of 180 °C with nitrogen as sheath gas.

Results

Inhibition of GSTP1-1 by quercetin

Figure 2 shows the time-dependent inhibition of GSTP1-1 by 25 μM quercetin at 25°C. Incubation of GSTP1-1 with 25 μM of quercetin results in a time-dependent inhibition, which is complete after 2 hours. Addition of ascorbic acid or GSH to these incubations prevents the inactivation to a significant extent, whereas addition of tyrosinase, known to efficiently catalyze the oxidation of quercetin to its quinoid-type products (Figure 1) [13] greatly enhances the inactivation.

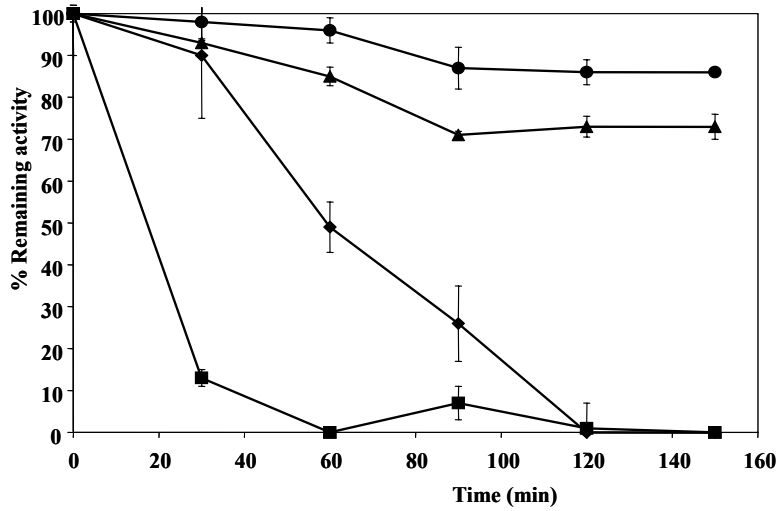

Figure 2. The time dependent effect on GSTP1-1 activity, presented as percentage of control, of 25 μM quercetin (◆), 25 μM quercetin in the presence of 6.67 units tyrosinase (■), 25 μM quercetin in the presence of 1 mM ascorbic acid (▲) and 25 μM quercetin in the presence of 10 mM GSH (●). The control GSTP1-1 activity was 8.6 ± 0.5 μmol DNP-SG/min/mg protein at the start of the experiment for all incubations except for the incubation with 10 mM GSH, for which the higher GSH concentration resulted in an activity of 13.1 ± 0.9 μmol DNP-SG/min/mg protein. All data are the mean values of triplicate measurements ± SD.

Dilution of the incubation mixture does not abolish the inactivation. Incubations with 1, 10 and 100 μM quercetin showed similar results with the maximum level of inhibition reached after two hours, being respectively 25%, 42% and 100% (Figure 3). For 100 μM quercetin this 100% inhibition was already achieved upon one hour of incubation. Figure 3 also presents the data obtained after two hours incubation of GSTP1-1 with the different quercetin concentrations in the presence of ascorbic acid (1 mM), GSH (10 mM) or tyrosinase (6.67 U). Similar to the results obtained with 25 μM quercetin, at all other

quercetin concentrations tested, tyrosinase increased the inhibition efficiency whereas addition of ascorbic acid or GSH protected GSTP1-1 against quercetin-induced inactivation.

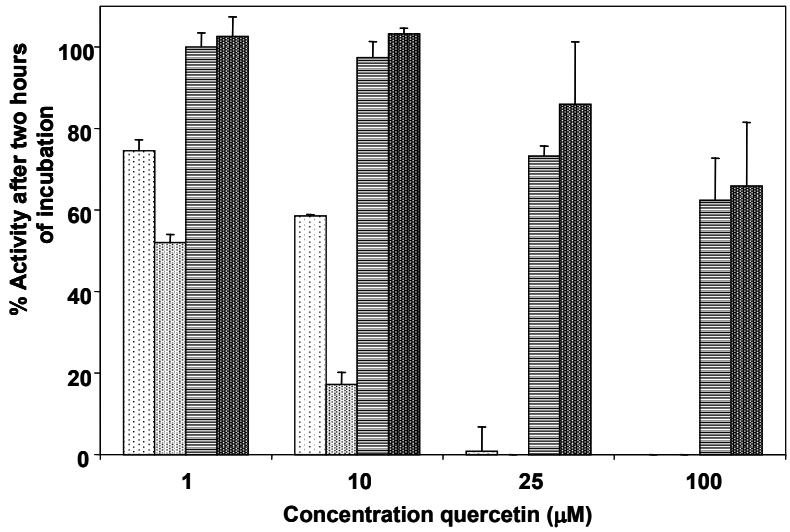

Figure 3. Remaining GSTP1-1 activity after two hours incubation with increasing concentrations of quercetin in the absence (first bar) or presence of 6.67 units tyrosinase (second bar), 1 mM ascorbic acid (third bar) or 10 mM GSH (fourth bar). The control GSTP1-1 activity was 8.6 ± 0.5 μmol DNP-SG/min/mg protein at the start of the experiment for all incubations except for the incubation with 10 mM GSH, for which the higher GSH concentration resulted in an activity of 13.1 ± 0.9 μmol DNP-SG/min/mg protein.

The lag phase seen for the inhibition curves with quercetin alone (Figure 2) might be due to the time required for auto-oxidation of quercetin to form the quinoid-like product, which may be responsible for the inhibition of GSTP1-1. Incubation of the inactivated enzyme with 25 mM glutathione gradually regenerates 30% of the activity over a period of 150 minutes (data not shown).

Inhibition of GSTP1-1 mutants by quercetin

In order to investigate the possible interaction between quercetin and GSTP1-1 cysteine residues, similar inhibition studies were performed with the C47S, C101S and C47S/C101S mutant forms of GSTP1-1. The three mutant enzymes possess similar catalytic properties towards the substrate CDNB as the wild-type enzyme under the testing conditions [24]. Figure 4 presents the percentage activity remaining after 2 hours incubation of the various mutants with quercetin. These results reveal that especially replacement of

cysteine 47 by serine substantially reduces the inhibition of the enzyme by quercetin. Quercetin inhibition of C47S and of C47S/C101S GSTP1-1 is significantly less compared to the wildtype enzyme, whereas the C101S mutant is inhibited to the same extent as the wild-type enzyme. These results clearly demonstrate the involvement of the GSTP1-1 cysteine 47 residue in the interaction between the enzyme and quercetin.

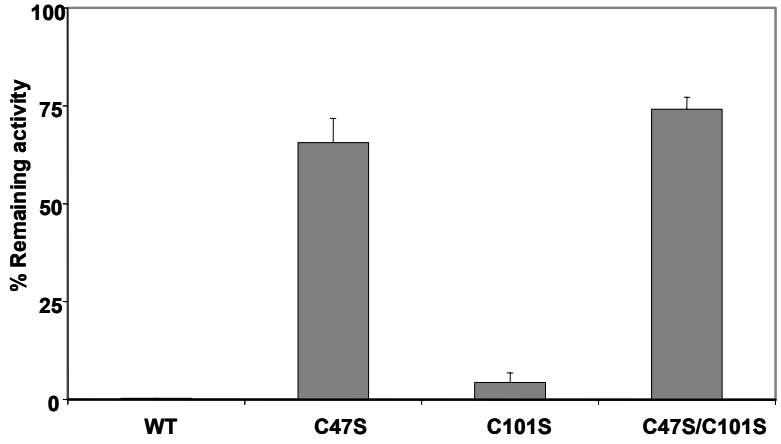

Figure 4. The effect of 25 μM quercetin on the activity of GSTP1-1 wild-type, GSTP1-1 C47S, GSTP1-1 C101S or the double mutant C47S/C101S after 2 hours of incubation. The control GSTP1-1 activity was 8.6 ± 0.5 μmol DNP-SG/min/mg protein at the start of the experiment for the wild-type as well as all the mutant enzymes. All data are the mean values of triplicate measurements ± SD.

HPLC and LC-MS studies

Extra proof for the covalent modification of the cysteine 47 of GSTP1-1 by quercetin had to be provided by mass spectral analysis of a peptic digest of the inhibited enzyme. Therefore the trypsin digested quercetin-incubated sample was compared with a control sample consisting of the trypsin-digested parent GSTP1-1. The GSTP1-1 derived peptide fragment containing Cys 47 has the following sequence: Ala-Ser-Cys-Leu-Tyr-Gly-Gln-Leu-Pro-Lys (amino acids 45-54), with a molecular mass of 1078.6. Upon protonation, the M+1 peptide fragment has a m/z = 1079.6. Quercetin in its reduced state has a molecular mass of 302.0. Covalent binding of quercetin to the 45-54 fragment would give rise to an adduct with a molecular mass of 1378.6 and, upon protonation (M+1), m/z = 1379.6.

Figure 5a shows the LC-MS elution pattern for the trypsin digested, quercetin inactivated, GSTP1-1. Analysis of the results shows that, in spite of the presence of various representative peptide fragments of trypsin digested GSTP1-1, the expected quercetin-peptide adduct of M+1 1379.6 is not observed. Detailed analysis of the elution-pattern for peaks with a M+1 of 1379.6 confirmed the absence of this adduct. Although different incubation times and conditions were tested the calculated adduct was not detected. Figure

5b displays the same data (as shown in Figure 5a) with masses ranging between m/z 1075 and 1082 the range in which at least the unmodified peptide fragment 45-54 may be detected. These results reveal a peak at m/z 1078.7 eluting at RT 46.50 minutes, but also that the peak at m/z = 1079.6 is absent. The exact nature of this peak at m/z 1078.7 was revealed by LC-MS analysis of the purified purchased fragment 45-54.

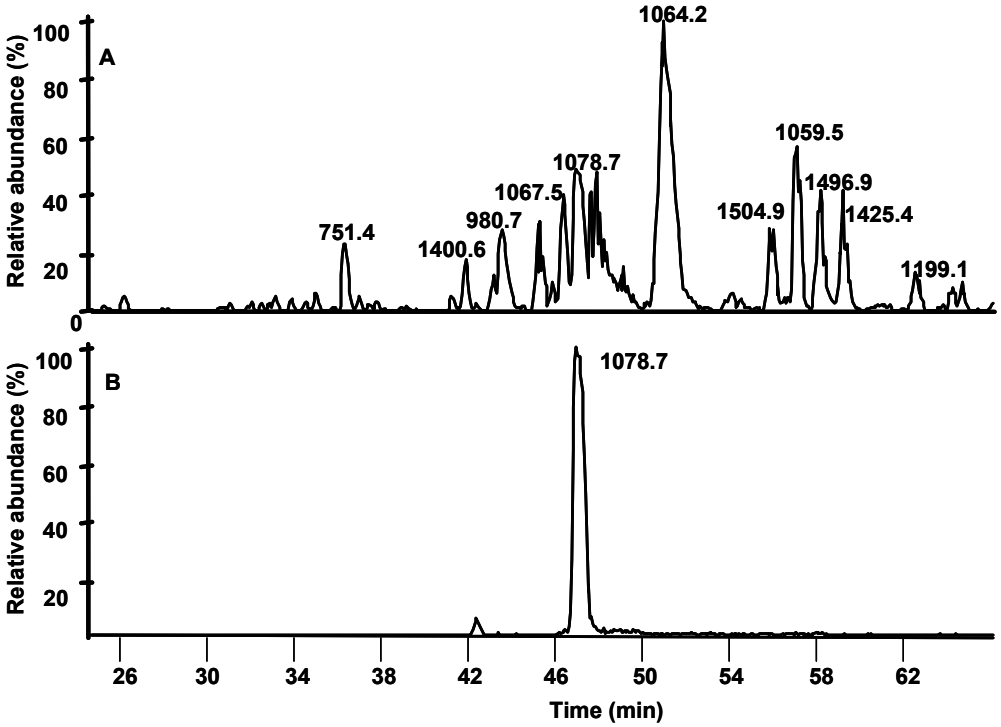

Figure 5. LC-MS analysis of (A) the trypsin digested GSTP1-1 after incubation for two hours with 25 μM quercetin and (B) the same elution representing all peaks with m/z values between 1075-1082. Numbers at each peak represent the major m/z present in this peak.

Figure 6a shows the LC-MS for the purified purchased fragment 45-54 directly after dissolving the freeze-dried purified peptide sample in 10 mM ammonium carbonate pH 7.6. This figure demonstrates two peaks at m/z values of 1079.5 and 1078.9 eluting at consecutive retention times i.e. 9.63 and 10.08 minutes respectively. Figure 6b and 6c show the zoom-scan results for these peaks, indicating the first peak at m/z 1079.5 to be the peptide fragment with a mass of 1079.5 and z=1 (Figure 6b). Zoom-scan analysis of the peak eluting at 10.08 minutes with a m/z 1078.9 (Figure 6c) reveals subsequent peaks which are separated by m/z differences of 0.5 instead of 1.0 showing that this is a peptide fragment has a charge z=2. This implies that the M+2 mass of this fragment must be 2157.8 to give a m/z of 1078.9. From this observation it can be concluded that this peptide fragment, formed from the 45-54 peptide fragment in ammonium carbonate at pH 7.6

represents the oxidized dimer of peptide fragment 45-54. This oxidized dimer of peptide fragment 45-54 was found in both the quercetin-incubated sample and the control sample. The rapid dimerization of this peptide fragment is further illustrated by the fact that upon 30 minutes prolonged incubation of the freshly dissolved freeze-dried 45-54 peptide sample, the peak at m/z 1079.5 (monomer) is fully converted to the dimer (results not shown).

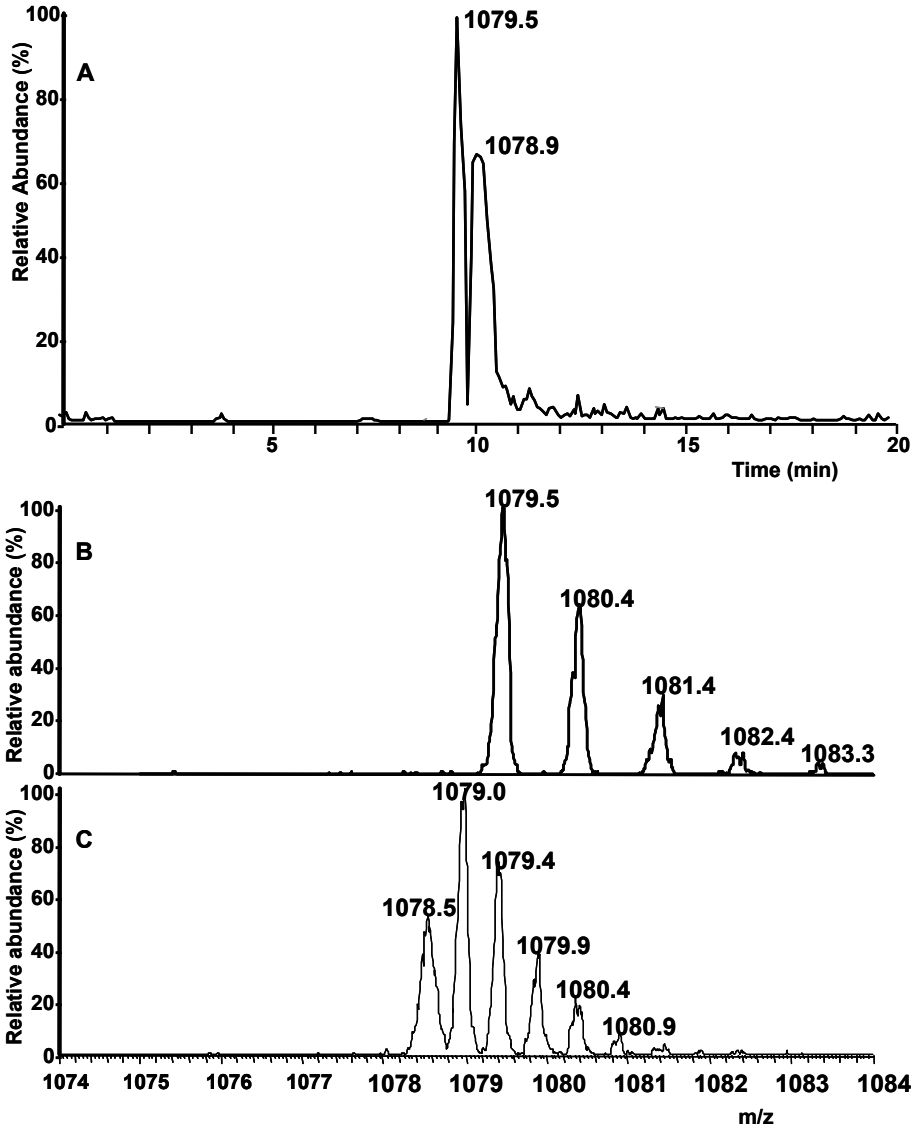

Figure 6. LC-MS analysis of (A) the purified purchased 45-54 peptide fragment injected directly after dissolving the freeze-dried purified peptide in 10 mM ammonium carbonate pH 7.6 and (B+C) the zoom-scans of both peaks. Figure B presents the zoom-scan of the

peak at m/z 1079.5 being the M+1 fragment of the 45-54 peptide and Figure C presents the zoom-scan of the peak at m/z 1078.8 being the peptide fragment dimer (for details see text). Numbers at each peak represent the m/z of the eluted fragment.

Discussion

Glutathione S-transferase P1-1 is a homodimeric enzyme. Each subunit contains one binding site for GSH (G-site) and another for the hydrophobic substrate (H-site), and cysteine residues located at positions 14, 47, 101 and 169 [6]. Glutathione S-transferases play an important role in the major defense system of cells against electrophilic compounds. The observation that a number of human tumors express raised levels of GSTP1-1, which has been associated with multi-drug resistance of tumor cells [3-5], makes GSTP1-1 a valuable target for inhibition studies.

Quercetin has been reported to have an inhibitory effect on GSTs [22, 30] but the nature of this inhibition has not been elucidated yet. Based on the fact that quinones can be good candidates for GSTP1-1 inhibition and the recent detection of quinoid-type products formed from quercetin [13, 14], quercetin could prove to be a valuable compound for GSTP1-1 inhibition. The results of the present study reveal that quercetin is a potent inhibitor of human GSTP1-1 *in vitro*.

Incubation of the wild type enzyme with 25 μM of quercetin, resulted in complete inhibition of the enzyme activity which could be significantly prevented by the addition of ascorbic acid or GSH. The inactivation could be accelerated by the addition of tyrosinase, an enzyme that has been shown to generate the rapid formation of *ortho*-quinone and *para*-quinone methides from quercetin (Figure 1) [13]. Without the addition of tyrosinase a lag phase in the inhibition study occurs, most likely representing the time necessary for the auto-oxidation of quercetin to quinoid type products. Co-incubation of quercetin with 10 mM GSH results in prevention of inhibition similar to co-incubation with 1 mM ascorbic acid. The effects of co-incubation with GSH are probably due to the antioxidant properties of GSH thereby preventing the formation of GSTP1-1 inhibitory *ortho*-quinone and *para*-quinone methides. Furthermore GSH appeared to be able to partially reverse the quercetin mediated GSTP1-1 inhibition. The inhibition of GSTP1-1, obtained upon two hours incubation with 25 μM quercetin, could be partially reversed (30%) by the addition of glutathione. The same GSH dependent rescue of the inactivated enzyme was described upon GSTP1-1 inhibition by the diuretic drug ethacrynic acid [1]. Generally, the inactivation of GSTP1-1 by covalent inhibitors like α,β-unsaturated ketone or carbonyl compounds [2, 12], prostaglandin A_2 [31], chlorambucil [32] or CDNB [11], has been ascribed to modification of Cys 47 and/or other cysteines including especially Cys 101 [8, 33]. The results of the present study show efficient inhibition of the GST-mutant C101S, but not of C47S or C47S/C101S by quercetin. This points at covalent modification of especially cysteine 47 in GSTP1-1 upon incubation with quercetin, mediated by the quinoid type quercetin derivatives. Some quercetin mediated inhibition (~30%) was detected for the C47S mutant and the C47S/C101S GSTP1-1 double mutant. This suggests that in the

absence of Cys47 some less reactive sites in the enzyme could be modified by quercetin. Together, the results of the present study point at a role for the quercetin quinone / quinone methides in the inactivation of GSTP1-1 by quercetin, and the covalent binding of the quercetin quinone or its quinone methides to the Cys47 residue of GSTP1-1.

HPLC-electrospray MS of the quercetin inhibited GSTP1-1 digestion (Figure 5) did not show the expected quercetin-peptide fragment adduct (M+1 at m/z 1379.6), but the dimerized peptide fragment at m/z 1078.8. This rapid dimerization was also observed for the purified purchased 45-54 peptide fragment (Ala-Ser-Cys-Leu-Tyr-Gly-Gln-Leu-Pro-Lys) and is probably due to the highly reactive Cys47. The reversibility of the quercetin-Cys47 adduct formation in combination with the swift dimerization of the 45-54 peptide fragment could explain why no Cys 47-quercetin adducts were detected from the incubation upon the 16 hours trypsin digestion. This reversibility has been reported before with quercetin-glutathione adducts [13] and the two-diastereomeric glutathione adducts of *trans*-4-phenyl-3-buten-2-one [34].

For extrapolation of the present *in vitro* findings to the *in vivo* situation it can be taken into account that human pharmacokinetic studies have demonstrated serum concentrations of quercetin to range from 1 to 400 μM after a non-toxic dose of quercetin. This implies that the *in vitro* incubations of the present study have been carried out at physiologically relevant concentrations [23]. Furthermore it can be foreseen that in most cells the presence of antioxidants like ascorbic acid and GSH might prevent oxidation of quercetin to its quinone (methide) forms, thereby diminishing the GSTP1-1 inhibitory potential of the flavonoid. Nevertheless, in some cases –e.g. chemotherapy- the cellular antioxidants might become depleted thereby allowing quercetin to oxidise into its GSTP1-1 inhibiting quinone methide forms. Additionally, several cell types and tissues contain tyrosinase or peroxidase enzyme activities including myeloperoxidase, lactoperoxidase, eosinophil peroxidase and thyroid peroxidase. Tyrosinase and peroxidase were previously shown to efficiently catalyse the oxidation of quercetin even in the presence of cellular antioxidants, thereby providing possibilities for quercetin quinone-mediated inhibition of GSTP1-1 [13, 21, 35]. Therefore the *in vivo* relevance of the results obtained in the present study can be expected to be dependent on two factors including (1) the antioxidant status of the cells and (2) the cellular presence of tyrosinase and/or peroxidases.

In conclusion: it has been demonstrated that GSTP1-1 is inhibited *in vitro* by quercetin most likely through covalent, but reversible, binding to the GSTP1-1 cysteine residue at position 47. If these *in vitro* results can be used to develop a quercetin based method for the *in vivo* reversal of GSTP1-1 mediated multidrug resistance remains a topic for future research.

Acknowledgements

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Structural requirements for the flavonoid mediated modulation of glutathione S-transferase P1-1 and GS-X pump activity in MCF7 breast cancer cells.

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Abstract

The objective of this study was to investigate the structural requirements necessary for inhibition of Glutathione S-transferase P1-1 (GSTP1-1) and GS-X pump (MRP1 and MRP2) activity by structurally related flavonoids, in GSTP1-1 transfected MCF7 cells (pMTG5). The results reveal that GSTP1-1 activity in MCF7 pMTG5 cells can be inhibited by some flavonoids. Especially galangin was able to inhibit almost all cellular GSTP1-1 activity upon exposure of the cells to a concentration of 25 μM. Other flavonoids like kaempferol, eriodictyol and quercetin showed a moderate GSTP1-1 inhibitory potential. For GSTP1-1 inhibition, no specific structural requirements necessary for potent inhibition could be defined. Most flavonoids appeared to be potent GS-X transport inhibitors with IC50 values ranging between 0.8 and 8 μM. Luteolin and quercetin were the strongest inhibitors with IC50 values of 0.8 and 1.3 μM, respectively. Flavonoids without a C2-C3 double bond like eriodictyol, taxifolin and catechin did not inhibit GS-X pump activity.
The results of this study demonstrate that the structural features necessary for high potency GS-X pump inhibition by flavonoids are (1) the presence of hydroxyl groups, especially two of them generating the 3',4'-catechol moiety; and (2) a planar molecule due to the presence of a C2-C3 double bond. Other factors, like lipophilicity and the total number of hydroxyl groups do not seem to be dominating the flavonoid mediated GS-X pump inhibition. To identify the GS-X pump responsible for the DNP-SG efflux in MCF7 cells, the effects of three characteristic flavonoids quercetin, flavone and taxifolin on MRP1 and MRP2 activity were studied using transfected MDCKII cells. All three flavonoids as well as the typical MRP inhibitor (MK571) affected MRP1-mediated transport activity in a similar way as observed in the MCF7 cells. In addition, the most potent GS-X pump inhibitor in the MCF7 cells, quercetin, did not affect MRP2-mediated transport activity. These observations clearly indicate that the GS-X pump activity in the MCF7 cells is likely to be the result of flavonoid mediated inhibition of MRP1 and not MRP2.
Altogether, the present study reveals that a major site for flavonoid interaction with GSH-dependent toxicokinetics is the GS-X pump MRP1 rather than the conjugating GSTP1-1 activity itself. Of the flavonoids shown to be most active especially quercetin is frequently marketed in functional food supplements. Given the physiological levels expected to be reached upon supplement intake, the IC50 values of the present study point at possible flavonoid-drug and/or flavonoid-xenobiotic interactions especially regarding transport processes involved in toxicokinetics.

Introduction

Drug resistance to chemotherapeutic agents is a major obstacle in human cancer chemotherapy. Among various mechanisms of drug resistance, cellular multidrug resistance is an important form of clinical drug resistance to chemotherapeutic agents. Multidrug resistance is often associated with overexpression of glutathione S-transferases (GSTs) and efflux transporter proteins, such as P-glycoprotein (P-gp) and/or multidrug resistance associated protein (MRP) [1, 2]. Therefore, when a particular drug is a substrate for the isozymes or pumps, the overexpression of these proteins may result in more rapid detoxification and/or excretion, thereby diminishing the effectiveness of the drug. This also holds for the toxicity of reactive electrophilic metabolites known to be metabolized and excreted by GSH-dependent processes. Upon increased activity of the GSTs and/or efflux proteins the toxicity of these electrophiles may be decreased.

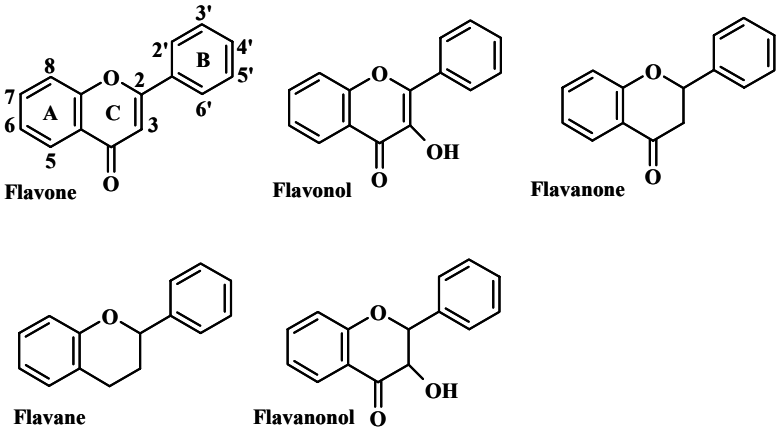
The GSTs are a superfamily of xenobiotic metabolizing enzymes that catalyze the conjugation of various electrophilic compounds with glutathione. The possible role of these enzymes in multidrug resistance has stimulated the search for GST inhibitors [1, 3, 4]. Of all GSTs, GSTP1-1 has been found the most important isozyme in multidrug resistance as derived from overexpression of GSTP1-1 in a large number of solid tumours [3, 5].

The role of transport proteins in multidrug resistance and drug toxicokinetics was first recognized by the discovery of P-gp [6] and later the discovery of MRP1 [7]. MRP1 (ABCC1) and other MRP family members are ATP-dependent membrane associated drug efflux pumps involved in glutathione conjugate (GS-X) transport processes (reviewed in [8, 9]) [9-13].

Since both GSTP1-1 and the GS-X pump may be involved in mechanisms contributing to multidrug resistance, development of GSTP1-1 and/or GS-X pump inhibitors has been considered a promising strategy to increase chemotherapeutic efficiency. The quest for non-toxic GST and GS-X pump inhibitors showed that many natural constituents, including plant polyphenols like flavonoids were promising candidates [14-23]. Studies on the flavonoid mediated inhibition of both GSTP1-1 and the GS-X pump using a cellular model system have not been described before. Insight in the structural requirements necessary for the flavonoid mediated inhibition of GSTP1-1 and GS-X pump activity is also of importance since inhibition of the GST- or GS-X pump mediated processes might result in undesired side effects upon the use of these compounds as functional food ingredients. This, because inhibition of GSH-dependent detoxification and excretion of reactive electrophiles might increase the toxicity of these intermediates.

In order to obtain better insight in the structural features of flavonoids required for significant interference with GSH-dependent toxicokinetics, the present study describes the structural requirements of flavonoids necessary for the modulating potency on both GSTP1-1 conjugating activity and GS-X transport activity in a cellular model system. To identify the GS-X pump involved, the effects of several flavonoids and two model MRP inhibitors, i.e. MK571 and cyclosporin A, on MRP1 and MRP2 activity in MRP1 and MRP2 transfected MDCKII cells has been studied as well. The main structural differences within

the series of the flavonoids tested involve the presence or absence of a C2-C3 double bond, relative lipophilicity and the number and position of the hydroxyl moieties (Figure 1). Altogether, this study aims to provide a more detailed understanding of the way in which structural features influence the GSTP1-1 and GS-X transport inhibiting potential of flavonoids, an important group of presently developed functional food ingredients.



Flavonoid	Class	Hydroxylation Pattern	C2-C3 Double bond
Flavone	flavone	-	+
3',4'dihydroxy-flavone	flavone	3',4'	+
Galangin	flavonol	3,5,7	+
Kaempferol	flavonol	3,5,7,4'	+
Luteolin	flavone	5,7,3',4'	+
Eriodictyol	flavanone	5,7,3',4	-
Morin	flavonol	3,5,7,2',4'	+
Quercetin	flavonol	3,5,7,3',4'	+
Taxifolin	flavanonol	3,5,7,3',4'	-
Myricetin	flavonol	3,5,7,3',4',5'	+
Catechin	flavane	3,5,7,3',4'	-

Figure 1. Structural formulas and classification of the flavonoids used in this study.

Materials and Methods

Chemicals.
Myricetin, N-acetyl-L-cysteine, glutathione (reduced), glutathione (oxidized) and dimethylsulfoxide (DMSO) were obtained from Acros Organics. Morin, galangin, 2-vinylpyridine, flavone and L-proline were purchased from Aldrich. Taxifolin, 3',4'dihydroxyflavone and catechin were obtained from ICN. Luteolin, kaempferol, quercetin, ascorbic acid, sulfosalicylic acid, glutathione reductase, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), NADPH and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma Chemical Co. Glutamine, Hanks Balanced Salt Solution (HBSS) and gentamycine were purchased from Life Technologies. Fetal Calf Serum (FCS) and Minimum Essential Medium (MEM) were purchased from Invitrogen Co. HPLC grade methanol was obtained from Labscan and HPLC grade trifluoro acetic acid (TFA) was obtained from Baker. Eriodictyol was purchased from Extrasynthese. MK571 was obtained from BioMol. Dulbecco's Minimum Essential Medium (DMEM) with GlutaMax, fetal calf serum, penicillin/streptomycin and gentamycin were all from Gibco, (Paisley, Scotland). Cyclosporin A was obtained from Fluka (Zwijndrecht, The Netherlands).

Cell lines.
The transfected breast cancer (MCF7) cell-lines pSV2neo (containing an empty vector) and pMTG-5 stabile transfected with human GSTP1-1, were a generous gift from Dr. A Townsend (Biochemistry Department, Bowman Gray School of Medicine, Winston-Salem, NC USA). Generation and characterization of these transfectants have been described previously by Moscow et al. [24]. Compared to the GSTP1-1 transfected cells the pSV2neo control cells contain negligible GST activity mainly GSTπ and a little GSTμ [25]. The cell lines were grown at 37^{o}C in a humidified 5% CO_2 atmosphere in Minimum Essential Medium (MEM) supplemented with 5% Fetal Calf Serum (FCS), 2 mg/l L-proline, 0.1% gentamycine and 2 mM L-glutamine.
For each experiment 10^6 cells were plated onto a 6-well Costar tissue cluster and cultured for 24 hours before exposure. Cells were exposed for 20 minutes in 2.0 ml HBSS containing different concentrations of the flavonoids. Flavonoid concentrations used were 0, 0.1, 1.0, 2.5, 5, 10, 25 and, 50 μM. Experiments were performed three times in triplicate for each tested flavonoid. Test compounds were freshly prepared and added from a 200 times concentrated stock solutions in DMSO to 0.5% (v/v). At the start of the experiment 100 μl 0.2 mM CDNB was added to give a final concentration of 10 μM CDNB. After 20 minutes incubation at 37^{o}C, 0.2 ml medium was taken and mixed with 5 μl 0.04 M N-acetyl-L-cysteine (to remove unreacted CDNB), vortexed and immediately stored at –20^{o}C until further analysis for the CDNB-GSH (DNP-SG) content. Cells were trypsinised and disrupted by suspension in 1 ml demineralized cold water. Also these cellular fractions were mixed with 5 μl 0.04 M N-acetyl-L-cysteine, vortexed, sonicated and stored at –20^{o}C upon analysis of the DNP-SG content. For the determination of the intracellular GSH concentrations the cellular fractions were resuspended in 50 mM Tris HCl, containing 5

mM EDTA pH 7.5. The Madin-Darby canine kidney (MDCKII) cell lines, stably expressing either human *MRP1* cDNA (hereafter called MRP1 cells) or *MRP2* cDNA (hereafter called MRP2 cells) were kindly provided by Prof. P. Borst (NKI, Amsterdam). The MDCKII cell lines (MRP1 or MRP2 transfected) were cultured in DMEM with GlutaMax (4.5 g glucose per liter), 10% fetal calf serum and 0.01% penicillin/streptomycin, and were grown in a humidified atmosphere of 5% CO_2 at 37°C. For transport experiments 10^5 cells/cm^2 were grown on microporous polycarbonate filters ((0.4 μm pore size, 1.13 cm^2) Costar Corp. Cambridge, MA). It was shown earlier [26] that in these polarized cell lines MRP1 routes to the basolateral plasma membrane, whereas MRP2 localizes to the apical plasma membrane. Culturing MDCKII cells on a filter in transwells provides the opportunity to study both the MRP1- or MRP2-mediated efflux of the parent compound and/or its metabolites to either the apical or basolateral side of intact cells. Medium volumes in the basolateral and apical compartments were 1.8 and 0.5 ml respectively. Cells were cultured to confluency for three days and medium was replaced every 24 h. Confluency of the monolayers was checked by transepithelial electric resistance (TEER) measurement. TEER-values of each monolayer were measured using a Millicell-ERS epithelial volt/ohm meter (Millipore, Bedford). The TEER-value of a confluent monolayer of MDCKII cells ranged between 200-250 $\Omega.cm^2$ as reported before [27]. Cells were exposed for 20 min. to different concentrations of the flavonoids in HBSS containing 0.5 mM acivicin (to prevent degradation of DNPSG by γ-glutamyltranspeptidase), on both the apical and basolateral side. Flavonoid concentrations used were 0, 10, 20, 30, 40 and 50 μM. Experiments were performed two times in duplicate for each tested flavonoid. The flavonoids tested were quercetin, flavone and taxifolin. The leukotriene D4 receptor antagonist MK571 was used as a typical MRP1 inhibitor [28] and cyclosporin A was used as a typical MRP2 inhibitor [29]. Test compounds were freshly prepared and added from a 200 times concentrated stock solutions in DMSO to 0.5% (v/v). At the start of the experiment CDNB was added to give a final concentration of 10 μM CDNB. After 20 minutes incubation at 37°C, 0.2 ml medium from both the apical and the basolateral side were taken and mixed with 5 μl 0.04 M N-acetyl-L-cysteine, vortexed and immediately stored at –20°C until further analysis. The filter membranes containing the cells were washed twice with cold HBSS and removed from the inserts. Cells were sonicated in 1 ml HBSS, and mixed with 5 μl 0.04 M N-acetyl-L-cysteine and stored at –20°C upon analysis of the DNP-SG content.

High-Performance Liquid Chromatography (HPLC) analysis of DNP-SG.

To determine the DNP-SG concentration in medium and cytosolic fractions, reversed-phase HPLC was carried out using a Thermo Finnigan HPLC system equipped with a P200 pump and an AS 3000 autosampler. A volume of 50 μl was injected onto a 150x4.6 mm Alltech Alltima C18 column. The column was eluted at a flow rate of 0.6 ml/min with a linear gradient from 70 % A (0.1% (v/v) trifluoroacetic acid in demineralized water) and 30% B (0.1% (v/v) trifluoroacetic acid in methanol) to 70% B in 13 minutes. Absorbance was

measured at 340 nm using a Thermo Finnigan UV 100 detector, peak areas were compared to those of a standard DNP-SG concentration range.
To investigate the inhibition of the cellular glutathione conjugation activity, the amount of DNP-SG was measured both in the medium (excreted) and in the cytosolic fraction. Comparison of the total (medium + cytosolic) DNP-SG formation in the flavonoid exposed cells to that in the control cells, incubated without the flavonoid, reveals the potency of the flavonoid to inhibit GSTP1-1 in a cellular system. In order to compare the different flavonoids for their DNP-SG excretion inhibitory potency, different concentrations of each flavonoid were tested as indicated. The amount of DNP-SG in the medium was corrected for the change in the total amount of DNP-SG using the ratio: [DNP-SG]excreted / [DNP-SG]total, in order to obtain data for IC50 calculations on inhibition of DNP-SG excretion. The role of MRP1 in these DNP-SG efflux processes was analyzed using the typical MRP1 inhibitor MK571 at 30 μM.

Measurement of intracellular glutathione (GSH).
To determine the effect of flavonoid exposure on the intracellular GSH concentrations, cellular fractions were analyzed using the DTNB-GSSG reductase recycling assay as described by Baker et al. (1990) [30].

High Performance Liquid Chromatography (HPLC) analysis of flavonoid lipophilicity.
To determine the relative lipophilicity of the flavonoids, HPLC was carried out using a Thermo Finnigan HPLC system equipped with a P200 pump and an AS 3000 autosampler. Flavonoids were freshly prepared at a final concentration of 100 μM in DMSO, and 50 μl of this solution were injected onto a 150x4.6 mm Alltech Alltima C18 column. The isocratic mobile phase consisted of 0.1% trifluoroacetic acid and methanol (4.5:5.5 v/v) and elution was carried out at a flow rate of 1 ml/min. Detection was performed by measuring the absorbance at 254 nm using a Thermo Finnigan UV 100 detector. The lipophilicity of the flavonoids was calculated using the capacity factor (K'), calculated by:

$$K' = (t_r - t_o)/t_o$$

In which: K' = capacity factor, t_r = retention time (min) and t_o= retention time of unretained substances (min).

Molecular characteristics of flavonoid structures.
In order to quantify the relative effects of the C2-C3 double bond on the planarity of the flavonoid molecules, the dihedral angle between the B and C ring was calculated, using computational modeling carried out on a Silicon Graphics Indigo workstation using Spartan 5.0 (Wavefunction Inc.). Each molecule was built in Spartan and its geometry was optimized by the semi-empirical PM3 method. After optimization, the C3-C2-C1'-C2' dihedral angles were measured.

Results

GSTP1-1 transfected MCF7 cells as the model system.
In order to investigate the applicability of the GSTP1-1 transfected MCF7 pMTG5 human breast cancer cells as a model system to study GSTP1-1 activity and GS-X transport, DNP-SG formation and efflux in time were measured following exposure to 10 μM CDNB. The results obtained (Figure 2) reveal a time-dependent increase in both intracellular, extracellular as well as total DNP-SG levels. The amount of DNP-SG excreted generally does not exceed 25% of the total amount of DNP-SG formed.

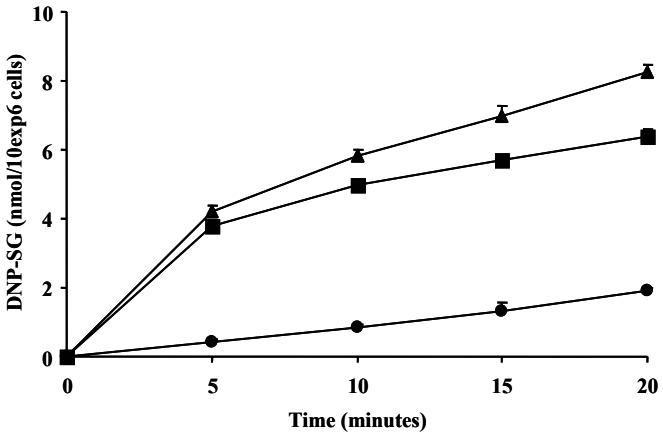


Figure 2. The MCF7 pMTG5 cells as a model system. The formation of DNP-SG after addition of 10 μM CDNB to 10^6 cells. Samples were taken from the medium and cytosolic fraction at each timepoint, over a period of 20 minutes. The results are the means ± SD from triplicate measurements.
(excreted DNP-SG ● , intracellular DNP-SG ■ , total DNP-SG ▲)

To be able to evaluate the effects of the flavonoids on the transfected GSTP1-1 enzyme activity, the formation of DNP-SG in the GSTP1-1 transfected MCF7 pMTG5 cells was corrected for non-specific DNP-SG formation, p.e. CDNB conjugation via chemical conjugation. This was done by comparison of the DNP-SG levels obtained in GSTP1-1 transfected MCF7 pMTG5 cells to the DNP-SG levels observed in MCF7 pSV2neo control cells upon incubation with 10 μM CDNB for 20 minutes. Total DNP-SG formation in the MCF7 pSV2neo cells appeared to be 72 ± 3% of the total DNP-SG formation detected in the GSTP1-1 transfected cells. Both cell lines, the MCF7 pMTG5 and the MCF7 pSV2neo, contained similar intracellular GSH concentrations in the range of 30-35 μmol/10^6 cells. Therefore it can be concluded that 28 ± 3% of the total amount of DNP-SG is formed by GSTP1-1, whereas the major part of DNP-SG is formed by chemical conjugation. In line

with this observation the GSTP1-1 activity data obtained with the GSTP1-1 transfected cells were corrected for non GSTP1-1 catalyzed conjugation.

Inhibition of GSTP1-1 activity in MCF7 pMTG5 cells.
In order to assess the effects of flavonoids on GSTP1-1 activity in the pMTG5 cells the enzymatic formation of DNP-SG upon exposure of the cells to different flavonoids was monitored and compared to the GSTP1-1 mediated DNP-SG formation in the absence of flavonoid. Table 1 shows the amounts of DNP-SG (cytosolic + medium) formed in the GSTP1-1 dependent reaction by the MCF7 pMTG5 human breast cancer cells upon incubation with 10 μM CDNB for 20 minutes in the presence of 25 μM of various flavonoids. These results reveal only galangin to be a potent GSTP1-1 inhibitor while eriodictyol, kaempferol and quercetin, show moderate potency. The other flavonoids tested show hardly any detectable influence on the GSTP1-1 activity. For the flavonoids that could inhibit GSTP1-1 activity by more than 50%, an IC50 was determined (Table 1). The strongest GSTP1-1 inhibitor is galangin with an IC50 of 14.4 μM. IC50's of about 23-26 μM were obtained for eriodictyol, kaempferol and quercetin.

Table 1. The flavonoid mediated inhibition of DNP-SG formation and excretion.

Flavonoid	*a,b* **GST activity: nmol DNP-SG per 10^6cells**	*b* **GSTP1-1 inhibition IC50 (μM)**	*a* **GS-X pump activity: nmol DNP-SG per 10^6cells**	*c* **GS-X pump inhibition IC50 (μM)**
control (0.5% DMSO)	2.1 ± 0.1	-	1.9 ± 0.2	-
Flavone	1.9 ± 0.2	>50	1.7 ± 0.1	>50
3',4'di-hydroxyflavone	2.0 ± 0.1	>50	0.5 ± 0.1	5.6 ± 0.5
Galangin	0.1 ± 0.3*	14.4 ± 2.3	0.4 ± 0.2*	6.2 ± 0.5
Kaempferol	0.9 ± 0.2*	23.1 ± 2.1	<0.2*	4.8 ± 0.2
Luteolin	1.5 ± 0.2*	>50	<0.2*	0.8 ± 0.1
Eriodictyol	0.8 ± 0.3*	22.8 ± 1.6	1.2 ± 0.1*	>50
Morin	2.0 ± 0.1	>50	0.5 ± 0.21*	8.1 ± 0.9
Quercetin	0.9 ± 0.1*	25.9 ± 2.4	<0.2*	1.3 ± 0.3
Taxifolin	2.1 ± 0.3	>50	1.5 ± 0.2	>50
Myricetin	1.6 ± 0.3	>50	<0.2*	5.9 ± 1.0
Catechin	2.1 ± 0.2	>50	1.7 ± 0.1	>50

a Final concentrations for all flavonoids: 25 μM, and for CDNB: 10 μM.
b Amounts are corrected for chemical conjugation
c The IC50 values of flavonoid mediated inhibition of DNP-SG excretion by human breast-cancer cells (MCF7 pMTG5), corrected for the total amount of DNP-SG using: [DNP-SG]excreted / [DNP-SG]total.
All values are the means of triplicate measurements ± SD.
* Statistically significant difference from value for control (P<0.05).

To exclude GSH depletion as a factor influencing the total DNP-SG amount, possible effects of flavonoid exposure on the GSH amount in the cytosolic fractions of all cells were analyzed. GSH concentrations were in the range of 30-35 μmol/10^6 cells. Changes in the intracellular GSH concentration upon flavonoid exposure were not observed

Inhibition of DNP-SG excretion in MCF7 pMTG5 cells.

To assess the effects of flavonoids on GS-X pump activity of the MCF7 pMTG5 cells, the excretion of DNP-SG in the presence of different flavonoids was monitored and compared to DNP-SG efflux from cells incubated in the absence of the flavonoids. Table 1 shows the effects of 25 μM of the model flavonoids on the excreted amount of DNP-SG. Most flavonoids tested inhibit DNP-SG excretion except for eriodictyol, taxifolin, flavone and catechin that showed no inhibitory potential at all. Additional experiments were performed to determine the concentration dependent inhibition of DNP-SG efflux in MCF7 pMTG5 cells by flavonoids. Figure 3 shows an example of the concentration dependent inhibition of DNP-SG efflux by quercetin, kaempferol and taxifolin. Similar graphs were obtained for other flavonoids, enabling calculation of their IC50 values. The IC50 values obtained - expressing the potency of the flavonoids to inhibit the DNP-SG excretion for 50% (e.g. 50% inhibition of GS-X pump activity at 10 μM CDNB)- are presented in Table 1. These results show that the flavonoids tested can be divided into three subgroups with respect to their ability to inhibit GS-X pump activity in the MCF7 pMTG5 cells. The first group consists of luteolin and quercetin being very efficient GS-X pump activity inhibitors with IC50 values of 0.8 and 1.3 μM. The second group of flavonoid-type GS-X pump inhibitors consists of moderate inhibitors with IC50 values between 4.8 and 8.1 μM e.g. kaempferol, 3'4'-dihydroxyflavone, myricetin, galangin and morin. The third group consists of the flavonoids lacking significant inhibitory potential, with IC50 values above 50 μM. This group includes: eriodictyol, taxifolin, flavone and catechin.

Analysis of DNP-SG excretion by the MCF7 pMTG5 cells upon exposure to 30 μM of the typical MRP1 inhibitor MK571 results in complete inhibition of the DNP-SG efflux. In contrast, exposure to 25 μM cyclosporin A hardly reduces DNP-SG efflux (not shown). These experiments suggest an important role for MRP1 in the GS-X efflux by MCF7 cells.

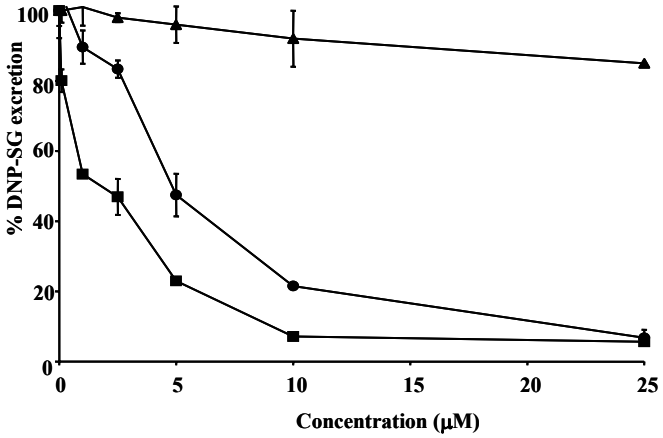


Figure 3. The concentration dependent effect of the flavonoids quercetin (■), kaempferol (●) and taxifolin (▲) on the excretion of DNP-SG in GSTP1-1 transfected pMTG5 cells following 20 minutes exposure to 10 μM CDNB. The results are the means ± SD from triplicate measurements.

Molecular characteristics of flavonoid structures

In order to characterize factors of importance for GSTP1-1 and GS-X pump inhibition, the calculated C3-C2-C1'-C2' dihedral angle between the C and B ring and the relative lipophilicity of the various flavonoids were determined and are presented in Table 2. Regarding GSTP1-1 inhibition, galangin is the only potent inhibitor. The absence of B-ring hydroxyl groups in galangin might result in relatively higher GSTP1-1 inhibition potency, although flavone, another flavonoid without B-ring hydroxyl groups, is not able to inhibit GSTP1-1 activity. With respect to its C3-C2-C1'-C2' dihedral angle and its relative lipophilicity, galangin does not deviate specifically within the range of flavonoids tested. These factors do not seem to be dominant in generating the relative high GSTP1-1 inhibition by galangin.

The results on flavonoid-dependent GS-X pump inhibition reveal a group of flavonoids lacking significant inhibitory potential with IC50 values above 50 μM. The most plausible reasons for this lack of inhibitory potential for the flavonoids catechin, eriodictyol and taxifolin is the effect of C2-C3 saturation on the planarity of the molecule. Table 2 shows the calculated C3-C2-C1'-C2' dihedral angle between the C and B ring of the various flavonoids of the present study and supports that loss of the C2-C3 double bond results in a significant increase in the C3-C2-C1'-C2' dihedral angle to values around 36-42^{o}, reflecting loss of planarity between the B and C ring. Comparison of these data to the IC50 values for GS-X pump inhibition in Table 1 reveals that loss of planarity between the B and C ring upon saturation of the flavonoid C2-C3 results in a loss of the inhibitory potential for the GS-X pump of MCF7 pMTG5 cells.

Table 2 The dihedral angle between B and C ring and the relative lipophilicity of the tested flavonoids.

Flavonoid	***a*** **C3-C2-C1'-C2' Dihedral angle (Degrees)**	***b*** **Relative lipophilicity (K')**
Flavone	5.5	19.7
3',4'-dihydroxy-flavone	4.7	5.5
Galangin	14.5	18.3
Kaempferol	14.4	8.8
Luteolin	7.3	6.1
Eriodictyol	41.9	2.1
Morin	19.3	2.8
Quercetin	14.7	4.4
Taxifolin	36.1	0.7
Myricetin	14.2	2.1
Catechin	38.8	2.9

a The dihedral angle between the C3-C2-C1'-C2' atoms of the flavonoids were measured after PM3 geometry optimization using SPARTAN.
b The relative lipophilicity of the tested flavonoids expressed as the capacity factor K': K'= (tr-to)/to.

Finally, figure 4 shows the relation between the relative lipophilicity (expressed as capacity factor K') of flavonoids that are able to inhibit GS-X pump activity and their IC50 values for GS-X pump inhibition. It is clearly demonstrated that for the present series of flavonoids the relative lipophilicity is not the dominant factor determining GS-X pump inhibitory potency in the MCF7 cells.

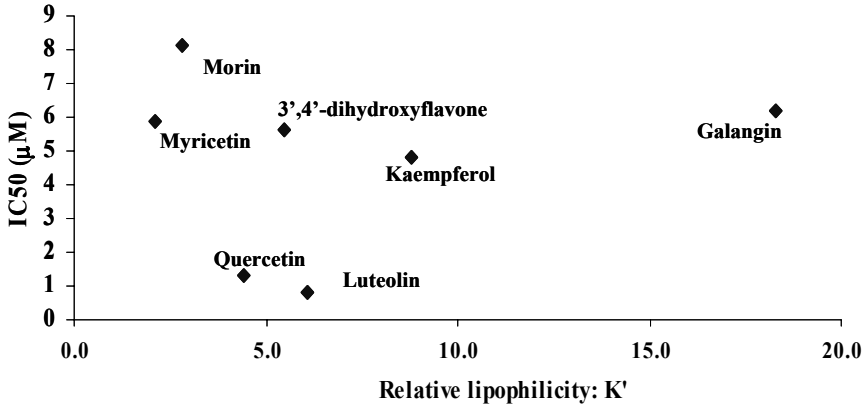


Figure 4. Comparison between the relative lipophilicity (expressed as capacity factor K') of flavonoids which did show GS-X pump inhibition and their IC50 values for this GS-X pump inhibition.

MRP1 and MRP2 inhibition by flavonoids

MRP1 and MRP2 transfected MDCKII cell lines were used to identify the GS-X pump involved in the MCF7 cells and to explore the inhibitory potency of flavonoids on these two major GS-X pumps known to be present in the MCF7 cells. To study whether the most important flavonoid structural characteristics necessary for potent GS-X inhibition in MCF7 cells also hold for inhibition of MRP1 and MRP2, three characteristic flavonoids: quercetin, flavone and taxifolin were tested in the transfected MDCKII cells. Quercetin, shown to be the most potent GS-X pump inhibitor, contains five hydroxyl groups including a B-ring 3',4'-catechol moiety. Flavone contains no hydroxyl groups at all and was shown to be unable to inhibit GS-X pump activity. Taxifolin has the same hydroxylation pattern as quercetin although taxifolin does not have a C2-C3 double bond, which affects the planarity of the molecule. Also taxifolin was unable to inhibit GS-X pump activity in MCF7 cells. The time-dependent efflux of DNP-SG by MDCKII-MRP1 and MRP2 cells has been described before [31]. Figure 5 shows the effects of the tested flavonoids and of the typical MRP inhibitors MK571 or cyclosporin A (CsA) on the formation and distribution of DNP-SG for the MRP1 and/or the MRP2 transfected MDCKII cells. It is shown that of the three tested flavonoids only quercetin (50 µM) was able to inhibit MRP1 mediated DNP-SG efflux to an extent similar to the effects of 50 µM of the typical MRP1 inhibitor MK571. Exposure of the MRP1 cells to 50 µM quercetin results in a 40% decrease of the basolateral efflux with a concomitant increase of the intracellular DNP-SG concentration. The other two tested flavonoids, flavone and taxifolin, do not significantly change DNP-SG formation or distribution. The effects of these flavonoids on MRP1 activity in MDCKII-MRP1 cells is similar to the effects found on GS-X pump activity in the MCF7 cells. In contrast, all three flavonoids did not inhibit MRP2 mediated activity in the MRP2 transfected MDCKII cells. The sole effect found was caused by quercetin on the basolateral efflux of DNP-SG (most likely background canine MRP1 activity). As MRP2 is an apical directed efflux pump, the

results presented indicate that MRP2 is not affected by the flavonoids tested. CsA, a typical MRP2 inhibitor, did affect DNP-SG distribution as expected. These experiments indicate that the GS-X pump activity in the MCF7 cells most likely consists of MRP1 and not MRP2.

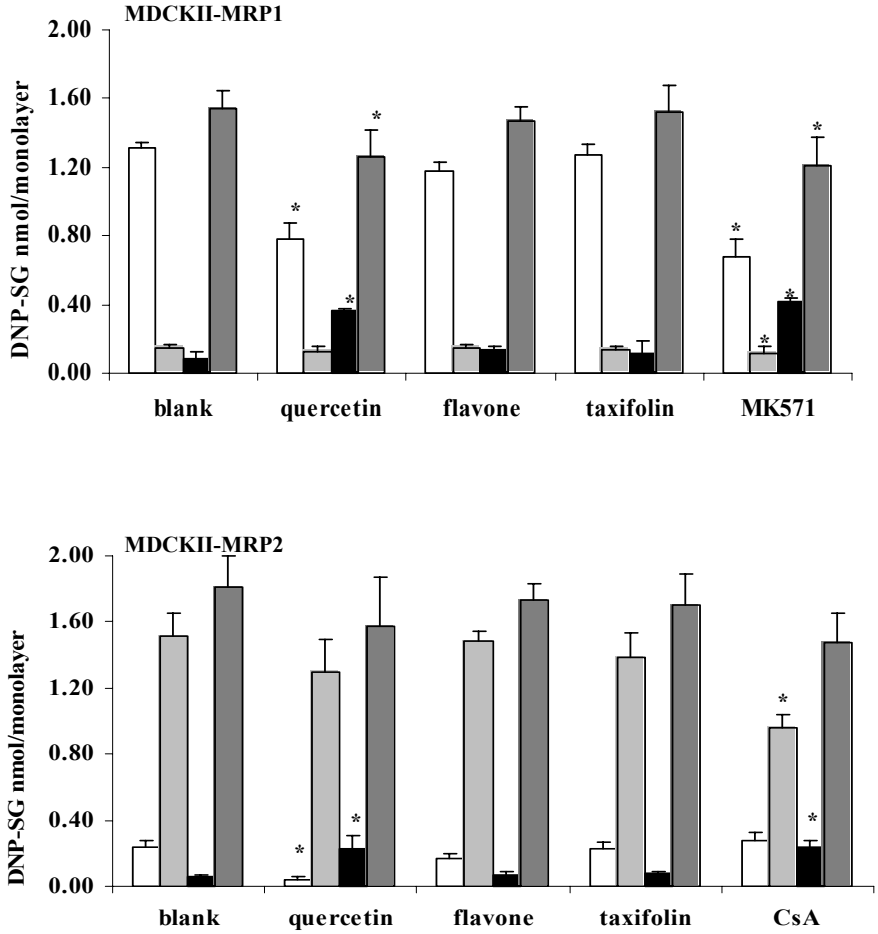


Figure 5. Effect of the flavonoids quercetin, flavone and taxifolin (all 50 µM concentrations) on the formation and distribution of DNP-SG by MDCKII-MRP1 and MDCKII-MRP2 cells. Results present DNP-SG concentrations (nmol/monolayer) in the basolateral compartment (first bar), apical compartment (second bar) and intracellular (third bar). Additionally, the resulting total formation of DNP-SG is presented (fourth bar). For comparison, the effect on DNPSG excretion of the model-inhibitors MK571 (50 µM) and CsA (25 µM) are given. Each bar represents means ± SD of incubations performed in duplicate. Those marked with asterisks differ significantly (ANOVA + Dunnetts' test) from the corresponding value in DMSO-treated cells ($P< 0.05$).

Discussion

The present study investigates structural characteristics of related flavonoids necessary for the modulating potential towards GSTP1-1 conjugating activity and GS-X pump activity in GSTP1-1 transfected MCF7 pMTG5 human breast cancer cells. These cells naturally contain very low levels of GSTs, but have been stabile transfected with human GSTP1-1 [24], [25]. In addition, MCF7 cells have been reported to contain both MRP1 and MRP2, known to be important GS-X transporters [32]. Thus, these MCF7 pMTG5 cells contain both GSTP1-1 and MRPs, two factors supposed to be important in GSH mediated detoxification and excretion of reactive electrophiles including anticancer drugs, the rapid detoxification and excretion of the latter contributing to multidrug resistance. Therefore, the MCF7 pMTG5 cells provide a suitable model to investigate possible effects of modulators of GSTP1-1 and GS-X transport activity in a cellular system. The advantage of cellular systems over purified enzymes or vesicle model systems is that the cellular model is one step closer to the *in vivo* situation taking also into account the process of cellular flavonoid uptake. Because the experimental procedure was relatively short (20 minutes) cellular metabolism of the flavonoids is not a factor of influence using the transfected MCF7 cells of the present study.

The results of the present study demonstrate that GSTP1-1 inhibition in MCF7 pMTG5 cells can be obtained with some flavonoids. Especially galangin appeared to be able to inhibit cellular GSTP1-1 activity with an IC50 value of 14.4 μM. The best comparable flavonoid, flavone, which also does not contain B-ring hydroxyl groups and is also relatively lipophilic cannot inhibit GSTP1-1 activity. Apparently, the absence of hydroxyl moieties in the B-ring, but not in the A-ring, contributes to efficient GSTP1-1 inhibition.

Previously published *in vitro* experiments concerning GSTP1-1 inhibition by the flavonoid quercetin, indicated that quinone-type oxidation products of quercetin are potent inhibitors of GSTP1-1 activity [23]. In contrast, in the present study quercetin shows only moderate GSTP1-1 inhibitory potency. More specific, that previous study pointed at a role for the quercetin quinone / quinone methides in the inactivation of GSTP1-1 by quercetin through covalent binding of the quercetin quinone or its quinone methides to the Cys47 residue of GSTP1-1. Co-incubation with ascorbic acid or glutathione prevented this inhibition, most likely by preventing the formation of these oxidation products of quercetin. In the present study the inhibition of GSTP1-1 was studied in an *in vitro* cellular system. These cells contain reasonable amounts of natural anti-oxidants like vitamin C and GSH. More specific, GSH concentrations in the cytosolic fractions of the MCF7 cells were in the range of 30-35 μmol/10^6 cells. No changes of the intracellular GSH concentration were observed upon exposure to quercetin or other flavonoids. It is therefore likely that the presence of natural antioxidants in the MCF7 cells prevent the formation of quercetin oxidation products thereby preventing covalent GSTP1-1 inhibition by these oxidation products. The moderate GSTP1-1 inhibition by quercetin found in the present study is more likely to be caused by the reduced form of the flavonoid, i.e. quercetin itself.

The most important observation of the present study was that many of the tested flavonoids possess inhibitory potential towards the excretion of DNP-SG by MCF7 pMTG5 cells. Moreover, their IC50 values for transport inhibition varied more than a factor 60 depending on their structural characteristics. Luteolin and quercetin are the most potent inhibitors. Flavonoids executing moderate inhibition are kaempferol, 3'4'-dihydroxyflavone, myricetin, galangin and morin. The presence of hydroxyl groups appear to be necessary for inhibition as flavone cannot inhibit the GS-X pump activity due to the absence of hydroxyl groups. When some hydroxyl groups are present at the A or C rings of the flavone, galangin (3,5,7), or at the B-ring, 3',4'-dihydroxyflavone (3',4'), the inhibitory potency increases remarkably. Quercetin (3,5,7,3',4') which contains the hydroxylation patterns of both galangin (3,5,7) and 3',4'-dihydroxyflavone (3',4') is even a better inhibitor. However the presence of an increasing number of hydroxyl groups does not necessarily result in a higher inhibitory potency. Namely, for flavonoids with increasing number of hydroxyl groups in the order: galangin (3 OH groups) < kaempferol (4 OH groups) < morin (5 OH groups) = quercetin (5 OH groups) < myricetin (6 OH groups) the IC50 values for inhibition of transport activity were 6.2, 4.8, 8.1, 1.3, and 5.9 μM respectively. The presence of a 3',4'-dihydroxy moiety on the B-ring, a structural characteristic of both quercetin and luteolin results in strong inhibition as is shown by comparison of quercetin and luteolin (both containing this 3',4'-dihydroxy moiety in the B-ring) to morin (2',4'-di-OH in B-ring), kaempferol (4'-OH in B-ring) or myricetin (2',3',4'-tri-OH in B-ring). By comparing the inhibitory potency of luteolin (5,7,3',4') to quercetin (3,5,7,3',4'), the presence of a 3-hydroxyl group at the C-ring apparently does not play an important role regarding GS-X pump inhibition. Moreover, planar flavonoids with hydroxyl groups are more potent GS-X pump inhibitors than the non-planar ones like eriodictyol, taxifolin and catechin (dihedral angle B- and C-ring > 15 degrees).

Overall, the results of this study demonstrate that the structural features necessary for high potency GS-X pump inhibition by flavonoids are (1) the presence of hydroxyl groups, especially if two of them generate the 3',4'-catechol moiety; and (2) a planar molecule thanks to the presence of a C2-C3 double bond. To identify the GS-X pump responsible for the DNP-SG efflux in MCF7 cells, the effects of three characteristic flavonoids: quercetin, flavone and taxifolin on MRP1 and MRP2 activity was studied using MRP1 and MRP2 transfected MDCKII cells. The identical modulation of MRP1- and GS-X pump activity by the flavonoids tested, combined with the lack of effects of quercetin, one of the best GS-X pump inhibitors, on MRP2 efflux, clearly show that the GS-X pump activity in the MCF7 cells most likely consists of MRP1 but not MRP2. The important role of MRP1 but not MRP2 in the DNP-SG efflux by MCF7 cells is confirmed by additional experiments reported in this study, showing that the typical MRP1 inhibitor MK571 inhibits DNP-SG efflux by MCF7 cells whereas the typical MRP2 inhibitor cyclosporin A has no significant effect.

Modulation of GS-X/MRP1 transport activity with flavonoids has been reported before, although in different model systems [14-17]. Leslie *et al.* (2001) studied the interaction of flavonoids with MRP1 mediated LTC4 transport and ATPase activity. The best competitive

LTC4 inhibitors (k_i 2.4-21 μM) were found to be, in following order of potency: kaempferol > apigenin > quercetin > myricetin > naringenin. The most important difference between the study of Leslie *et al.* (2001) and the present study is the use of a different model system, namely vesicles instead of cells. The model system of the present study also takes into account the cellular uptake of flavonoids as GS-X pump inhibitors, and this may explain the different order of GS-X transporter inhibition potency for the different flavonoids in the present study as compared to Leslie *et al.* (2001). Another and even more important difference between the study of Leslie *et al.* (2001) and the present study is the use of a different substrate, namely LTC4 and 17β-estradiol-glucuronide instead of DNP-SG. This may be another factor contributing to different inhibition potencies of the flavonoids tested. For MRP2 the absence of inhibitory effects of flavonoids has not been reported before.

Several mechanisms in which inhibitors might interact with the GS-X pump have been proposed. Inhibition of the GS-X pumps might affect: drug binding, ATP binding, ATP hydrolysis, drug transport, and the ADP release. Flavonoids are well known inhibitors of ATPase activity [17, 33-35]. Inhibition of ATP-ase activity might affect the ATP-dependent GS-X pump activity. Structure activity studies for the inhibition of P-glycoprotein (P-gp) ATP-ase activity by flavonoids showed that the presence of a 5-hydroxyl group, the 3-hydroxyl group, and the C2-C3 double bond are required for high potency binding to the C-terminal Nucleotide Binding Domain (NBD) of P-gp [17, 33-35]. Although the necessity of the 3 and 5 hydroxyl group is not demonstrated in the present study, the mode of action of the flavonoids for the GS-X pump inhibition might be by binding to the NBD. However, an inhibitory interaction of the flavonoids with other sites on the GS-X pump than its ATPase site cannot be excluded. An example of such an interaction of flavonoids with a GS-X pump, at a site different than the ATP-binding domain, can be found in studies reported for human colonic carcinoma Caco-2 cells [36, 37].These reports show that flavonoids as well as their glucuronide- and sulphate-conjugates and their glucosylated forms can act as MRP2 substrates and are efficiently transported by this well-known GS-X pump [36, 37]. This points at possibilities for an interaction of flavonoids at the substrate-binding site of the GS-X pump.

For extrapolation of the present *in vitro* findings to the *in vivo* situation it can be taken into account that human pharmacokinetic studies have demonstrated serum concentrations of quercetin to range from 1 to 400 μM after a non-toxic intravenous dose of quercetin [38]. Dietary supplementation with flavonoids might give an increase of the serum concentrations to levels of at most 10 times higher than 1 μM [39, 40]. Relatively high flavonoid concentrations in the intestine can be expected upon supplementation since most quercetin supplements are known to contain 100-300 mg quercetin per serving. This implies that the *in vitro* cellular incubations of the present study have been carried out, and effects have been observed, at concentrations that may be relevant *in vivo*.

Altogether, results of the present study reveal that a major site for flavonoid interaction with GSH dependent toxicokinetics in *in vitro* cell systems is the GS-X pump rather than the conjugating GSTP1-1 activity itself. Of the flavonoids shown to be most active

especially quercetin is frequently marketed in functional food supplements. Given the physiological levels expected to be reached upon supplement intake, the results of the present study point at possible flavonoid-drug and/or flavonoid-xenobiotic interactions affecting the toxicokinetic behavior of these drugs or xenobiotics, especially at the level of some important transport processes.

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4

Quantitative structure activity relationship studies on the flavonoid mediated inhibition of multidrug resistance proteins 1 and 2.

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Abstract

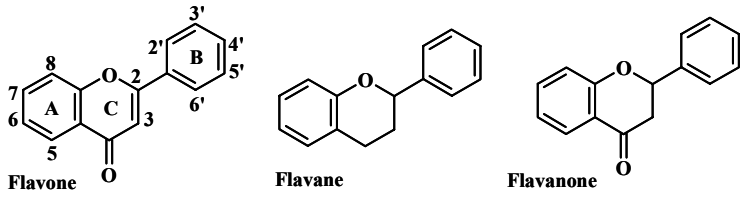
In the present study, the effects of a large series of flavonoids on multidrug resistance proteins (MRPs) were studied in MRP1 and MRP2 transfected MDCKII cells. The results were used to define the structural requirements of flavonoids necessary for potent inhibition of MRP1- and MRP2-mediated calcein transport in a cellular model. Several of the methoxylated flavonoids are among the best MRP1 inhibitors (IC_{50} values, ranging between 2.7 and 14.3 μM) followed by robinetin, myricetin and quercetin (IC_{50} values ranging between 13.6 and 21.8 μM). Regarding inhibition of MRP2 activity especially robinetin and myricetin appeared to be good inhibitors (IC_{50} values of 15.0 μM and 22.2 μM respectively). Kinetic characterization revealed that the two transporters differ marginally in the apparent K_m for the substrate calcein. For one flavonoid, robinetin, the kinetics of inhibition were studied in more detail and revealed competitive inhibition with respect to calcein, with apparent inhibition constants of 5.0 μM for MRP1 and 8.5 μM for MRP2. For inhibition of MRP1 a quantitative structure activity relationship (QSAR) was obtained that indicates three structural characteristics to be of major importance for MRP1 inhibition by flavonoids: the total number of methoxylated moieties, the total number of hydroxyl groups and the dihedral angle between the B- and C-ring. Regarding MRP2-mediated calcein efflux inhibition, only the presence of a flavonol B-ring pyrogallol group seems to be an important structural characteristic. Overall, this study provides insight in the structural characteristics involved in MRP inhibition and explores the differences between inhibitors of these two transporters, MRP1 and MRP2. Ultimately, MRP2 displays higher selectivity for flavonoid type inhibition than MRP1.

Introduction

Membrane proteins belonging to the ATP-binding cassette family of transport proteins play a central role in the defense of organisms against toxic compounds [1, 2]. The multidrug resistance proteins (MRPs) belong to this family, consisting of nine members which differ widely in substrate specificity, tissue distribution and intracellular location [3]. The first cloned member of this family, MRP1 (ABCC1) has a broad substrate specificity including glutathione S-conjugates, glucuronide conjugates, sulphate conjugates, anticancer drugs, heavy metals, organic anions and lipid analogues [4-6]. MRP1 is considered a prototype GS-X pump because of the important role of glutathione (GSH) for its transport action. Besides the transport of glutathione S-conjugates, the efflux of many substrates, like the oxyanions arsenite and antimonite and some drugs like vincristine and daunorubicin, are stimulated by or co-transported with glutathione [7-10]. MRP2 (ABCC2), the major canalicular Multispecific Organic Anion Transporter, is closely related to MRP1 [3, 11]. Nevertheless, the tissue localization of these two transporters differs. Whereas MRP1 is localized in the basolateral membranes of polarized cells and is present in all tissues, MRP2 is found in the apical membranes of polarized cells and is mainly expressed in the liver, intestine and kidney. Comparison of both transporters shows that human MRP1 and MRP2 are composed of 1531 and 1545 amino acids, respectively. They exhibit an amino acid identity of 49% with the highest degree of amino acid identity in the carboxyl-terminal domain and in both nucleotide-binding domains [2, 12]. Despite this limited amino acid identity the spectrum of substrates transported by MRP1 and MRP2 overlap to a large extent although MRP1 seems to be less specific [13-15]. It has been shown that over-expression of MRP1, but also MRP2, confers multidrug resistance characterized by resistance to a broad spectrum of anticancer agents (reviewed in [16]). Identification of MRP1- and MRP2-mediated transport as important mechanisms in multidrug resistance during cancer treatment led to the search for agents that could reverse resistance due to the activity of these transporters. One of the possible strategies for reversal of MRP-mediated multidrug resistance is inhibition of the activity of these proteins. Several inhibitors of MRP1 and MRP2 have been described in the literature. These inhibitors are mostly relatively non-specific inhibitors of organic anion transport, like sulfinpyrazone, benzbromarone and probenecid [3, 11]. Many MRP1 inhibitors, like certain tricyclic isoxazoles, do inhibit MRP1 in intact cells at micromolar concentrations but they are much less active against MRP2 [17, 18]. Another important feature is that some inhibitors, like the leukotriene D_4 receptor antagonist MK571, is an excellent MRP1 inhibitor in vesicular transport experiments, but is less efficient regarding MRP1 inhibition in intact cells [17].

The quest for transport inhibitors showed that many natural constituents, including plant polyphenols like flavonoids were promising candidates for possible MRP1 inhibition [19-24]. Flavonoids are a large group of polyphenolic antioxidants found in fruits and vegetables. Although the literature points at possible inhibition of MRP1 activity by flavonoids, the relation between the chemical structure and the MRP1 inhibitory potency

has hardly been described. For MRP2, up to date no studies regarding the effects of flavonoids on its activity have been described.
In the present study, the effects of a large series of flavonoids (Figure 1) on either the MRP1, or MRP2-mediated efflux of calcein in transfected MDCKII cells were examined. The results were used to derive Quantitative Structure Activity Relationships (QSAR) to quantitatively describe the structural requirements of flavonoids necessary for potent MRP1-, and MRP2 inhibition in a cellular model system. These results provide insight in the structural characteristics involved in MRP inhibition and explore the differences between inhibitors of these two transporters, MRP1 and MRP2.



Flavonoid	Class	Hydroxylation Pattern	Methoxylation Pattern
Flavone	Flavone	-	
3-Hydroxyflavone	Flavone	3	
3'-Hydroxyflavone	Flavone	3'	
4'-Hydroxyflavone	Flavone	4'	
Chrysin	Flavone	5,7	
3,3'-Dihydroxyflavone	Flavone	3, 3'	
3',4'-Dihydroxyflavone	Flavone	3', 4'	
Galangin	Flavone	3,5,7	
Baicalein	Flavone	5,6,7	
Apigenin	Flavone	5,7,4'	
Naringenin	Flavanone	5,7,4'	
3,3',4'-Trihydroxyflavone	Flavone	3,3',4'	
Kaempferol	Flavone	3,5,7,4'	
Fisetin	Flavone	3,7,3',4'	
Luteolin	Flavone	5,7,3',4'	
Eriodictyol	Flavanone	5,7,3',4'	
Morin	Flavone	3,5,7,2',4'	
Quercetin	Flavone	3,5,7,3',4'	
Taxifolin	Flavanone	3,5,7,3',4'	
Catechin	Flavane	3,5,7,3',4'	
Robinetin	Flavone	3,7,3',4',5'	
Myricetin	Flavone	3,5,7,3',4',5'	
Acacetin	Flavone	5,7	4'
Kaempferide	Flavone	3,5,7	4'
5,7,3',4'-Tetramethoxyflavone	Flavone		5,7,3',4'
Diosmetin	Flavone	5,7,3'	4'
Chrysoeriol	Flavone	5,7,4'	3'
Tamarixetin	Flavone	3,5,7,3'	4'
Isorhamnetin	Flavone	3,5,7,4'	3'

Figure 1. The model flavonoids used in the present study.

Materials and methods

Materials.
The Madin-Darby Canine Kidney (MDCKII) cell lines, stably expressing either human *MRP1* cDNA (hereafter called MRP1 cells) [25] or *MRP2* cDNA (hereafter called MRP2 cells) [15] were kindly provided by Prof. P. Borst (NKI, Amsterdam).
Dulbecco's Minimum Essential Medium (DMEM) with GlutaMax, fetal calf serum, penicillin/streptomycin and gentamycin were all from Gibco. MK571 was obtained from BioMol; PSC833 was a kind gift from Novartis Pharma AG. Cyclosporin A was from Fluka. Calcein acetoxymethyl ester (Calcein AM) was obtained from Molecular Probes. Morin, 3-hydroxyflavone, galangin, flavone and apigenin were purchased from Aldrich. Taxifolin, chrysin, naringenin, acacetin, 3'-hydroxyflavone, 4'-hydroxyflavone, 3,3'-dihydroxyflavone, 3,3',4'-trihydroxyflavone, robinetin, isorhamnetin and catechin were obtained from Indofine. Luteolin, myricetin and quercetin were purchased from Sigma Chemical Co.. HPLC grade methanol was obtained from Labscan and HPLC grade trifluoro acetic acid (TFA) was obtained from Baker. Eriodictyol, kaempferol, baicalein, kaempferide, 5,7,3',4'-tetramethoxyluteolin, diosmetin, chrysoeriol, tamarixetin were purchased from Extrasynthese.

MDCKII cell culture.
The Madin Darby Canine Kidney cell lines (control and MRP1 or MRP2 transfected) were cultured in Dulbecco's Minimum Essential Medium (DMEM) with GlutaMax (4.5 g glucose per liter), 10% fetal calf serum and 0.01% penicillin/streptomycin, and were grown in a humidified atmosphere of 5% CO_2 at 37°C.
For transport experiments $4*10^5$ cells/cm^2 were grown on microporous polycarbonate filters ((0.4 μm pore size, 4.7 cm^2) Costar Corp.). It was shown earlier that in these polarized cell lines MRP1 routes to the basolateral plasma membrane, whereas MRP2 routes to the apical plasma membrane. Culturing MDCKII cells on a filter in transwells provides the opportunity to study both the MRP1- or MRP2-mediated efflux of the parent compound and/or its metabolites to either the apical or basolateral side of intact cells. The volume of media in the basolateral and apical compartments was 1.8 and 0.5 ml, respectively. Cells were cultured to confluency for three days and medium was replaced every 24 h. Confluency of the monolayers was checked by transepithelial electric resistance (TEER) measurement. TEER-values of each monolayer were measured using a Millicell-ERS epithelial volt/ohm meter (Millipore). The TEER-value of a confluent monolayer of MDCKII cells ranged between 200-250 $\Omega.cm^2$ as reported before [26]. The leukotriene D4 receptor antagonist MK-571 was used as a typical MRP1 inhibitor [27] and cyclosporin A was used as a typical MRP2 inhibitor [13].

Efflux of calcein in MDCKII cells.
The efflux of calcein, which is a good substrate for MRP1 and MRP2, was determined using confluent monolayers of control, MRP1 and MRP2 cells. First, cells were loaded with

the calcein-acetoxymethyl ester (calcein-AM) at a final concentration of 1 μM in DMEM without phenol red for 2 hours at 7°C. Calcein-AM uptake and intracellular conversion to calcein in these MDCKII cell lines has been described before [28]. For the kinetic characterization of calcein efflux inhibition by flavonoids, several calcein-AM concentrations were used: 1, 0.5, 0.1 and 0.05 μM. At these calcein-AM concentrations the efflux of calcein by MRPs is not saturated since the calcein efflux was a linear function of the calcein-AM concentration. The calcein-AM concentration used for the QSAR studies (1 μM) was based upon previous studies by our group where 1 μM calcein-AM appeared to be a very suitable concentration for inhibition studies [28]. Essodaïgui *et al* (1998) described that calcein-AM equilibrates very rapidly over the cellular plasma membrane, resulting in similar in- and outside concentrations of calcein-AM [29]. Once inside the cells, cleavage of this non-fluorescent calcein-AM ester by intracellular esterases leads to formation of the fluorescent derivative calcein. The non-fluorescent calcein-AM, is a good substrate for both P-glycoprotein (Pgp) and MRP1 [30]. To diminish the MRP-dependent efflux of calcein-AM - and because it was preferred to use no MRP inhibitors during loading time - cells were loaded with calcein-AM at a temperature of 7°C. In addition, PSC833 (0.1 μM) was added as Pgp inhibitor. After the 3 hours loading, the cells were washed three times with DMEM without phenol red (37ºC) during approximately 10 minutes. The efflux experiments were started by exposing the cells to fresh medium (37ºC) containing 0.1 μM PSC833 and different concentrations of flavonoids (1, 10, 20, 30, 40 and 50 μM) or 50 μM MK571 (as a typical MRP1 inhibitor), or 30 μM cyclosporin A (as a typical MRP2 inhibitor) in both, apical and basolateral, compartments. Figure 1 lists the various flavonoids tested. Cells receiving vehicle only (0.5% DMSO v/v) served as control. The highest flavonoid concentrations tested were 50 μM, because some flavonoids are either cytotoxic or poorly soluble at concentrations above 50 μM. Efflux of calcein was measured in media samples from both the apical and basolateral compartment at t=0, 25 and 45 min and the level of calcein in intracellular compartment before and after the efflux experiments. Fluorescence of the samples was determined using a Varian Cary Eclipse (Varian) with excitation at 485 nm and emission at 530 nm. The fluorescence of the samples was corrected for the minor changes in background fluorescence caused by the flavonoids. Analysis of the calcein concentrations in the apical, basolateral and intracellular compartments at t=0 and t=45 min of the efflux experiments showed that during the efflux experiments no significant increase in total calcein amounts was observed (data not shown). Apparently, all calcein-AM taken up in the cells during loading is converted into calcein during the loading period and/or the period for washing of the cells before the efflux experiments start. IC_{50} values were obtained via curve fitting using the Microsoft Excel data analysis V1.1 toolpack.

High Performance Liquid Chromatography (HPLC) analysis of flavonoid lipophilicity.
To determine the relative lipophilicity of the flavonoids, HPLC was carried out using a Thermo Finnigan HPLC system equipped with a P200 pump and an AS 3000 autosampler. Flavonoids were freshly prepared at a final concentration of 100 μM in DMEM without

phenol red, and 50 µl of this solution were injected onto a 150x4.6 mm Alltech Alltima C18 column. The isocratic mobile phase consisted of 0.1% trifluoroacetic acid and methanol (4.5:5.5 v/v) and elution was carried out at a flow rate of 1 ml/min. Detection was performed by measuring the absorbance at 254 nm using a Thermo Finnigan UV 100 detector. The lipophilicity of the flavonoids was calculated using the capacity factor (K'), calculated by: K'= (tr-to)/to, in which: K' = capacity factor, tr = retention time of the flavonoid (min) and to = retention time of unretained substances (min).

Molecular characteristics of flavonoid structures.
To quantify the relative effects of the C2-C3 double bond, hydroxyl and methoxylated moieties on the planarity of the flavonoid molecules the dihedral angle between the B and C ring was calculated using computational modeling carried out on a Silicon Graphics Indigo workstation using Spartan 5.0 (Wavefunction Inc.). Each molecule was built in Spartan and its geometry was optimized by the semi-empirical PM3 method. After optimization, the C3-C2-C1'-C2' dihedral angles were measured. Other descriptors evaluated besides the dihedral angle were: lipophilicity (determined experimentally as K'), total number of hydroxyl groups, the number of hydroxyl groups on the A, B or C-ring of the flavonoid, the presence of catechol moieties (two adjacent hydroxyl groups) or pyrogallol moieties (three adjacent hydroxyl groups) and the number of methoxylated groups on the flavonoid.

Data analysis.
Descriptive and inferential statistical analyses were performed. The hypothesis of normality was evaluated by the Shapiro-Wilks test. Correlation analysis was evaluated by Spearman's non-parametric correlation analysis. Stepwise multiple regression analysis was used to describe the relation between the percentage MRP inhibition and the main important descriptors in a regression model (QSAR). Models obtained were statistically tested by variance analysis using ANOVA ($P<0.05$). Least square regression analysis was used to determine the correlation between the measured data and the expected (calculated) data from the model (adapted from [31]). All analyses have been performed using SPSS 10.1.0 software from SPSS Inc.

Results

MRP1 and MRP2 efflux characteristics and inhibition
The inhibition of MRP1- and MRP2 activity was studied using the fluorescent calcein as a model substrate. After loading the cells with the non-fluorescent calcein-AM that is converted to the fluorescent MRP-substrate calcein by intracellular esterases, the efflux of calcein was measured in the absence or presence of flavonoids. Figure 2 shows the typical time-dependent efflux patterns of calcein by MDCKII-MRP1 and MDCKII-MRP2 cells. In MRP1 cells, calcein is predominantly excreted to the basolateral side (8 times higher than apical efflux), whereas in MRP2 cells the efflux of calcein is predominantly to the apical

side (11 times higher than basolateral efflux). The presence of the Pgp inhibitor PSC833 did not affect the efflux of calcein by MRP1 and MRP2 (data not shown).

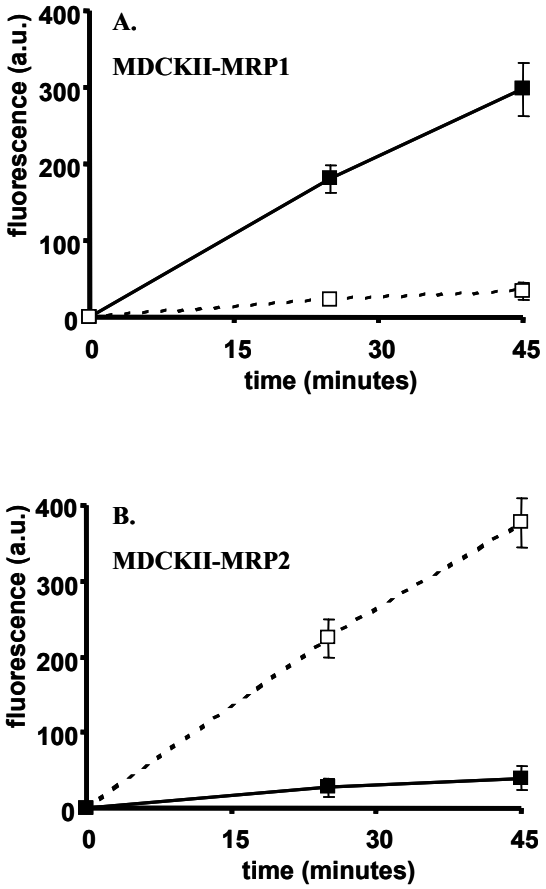


Figure 2. Typical time-dependent efflux patterns of calcein by MDCKII-MRP1 (A) and MDCKII-MRP2 cells (B). Open symbols (□) represent efflux to the apical compartment, closed symbols (■) represent efflux to the basolateral compartment. Each point represents the average ± SD from triplicate measurements.

The percentage inhibition upon exposure to 25 μM flavonoid was determined in both cell lines (t=45 minutes), and is presented in Table 1. This reveals that most flavonoids are able to inhibit MRP1 activity with varying relative inhibitory potencies. Strikingly, the methoxylated flavonoids 5,7,3',4'-tetramethoxyflavone, diosmetin, chrysoeriol, tamarixetin and isorhamnetin are among the best MRP1 inhibitors, except for kaempferide and acacetin which are less potent inhibitors than the other methoxylated flavonoids. Other flavonoids able to inhibit more than 50% of the MRP1 activity at 25 μM concentrations, were 3',4'-

dihydroxyflavone, luteolin, quercetin, robinetin and myricetin. Some of the flavonoids tested inhibited MRP1 activity less than 20%. This group consists of 3-hydroxyflavone, 3'-hydroxyflavone, 4'-hydroxyflavone, chrysin, 3,3'-dihydroxyflavone, naringenin, fisetin, taxifolin and catechin. In contrast to the wide variety of MRP1 inhibiting flavonoids, only a few of the tested flavonoids inhibited MRP2-mediated calcein efflux at 25 μM concentrations. Most profound effects were found for robinetin and myricetin, which inhibited MRP2 activity more than 50% at 25 μM concentrations.

Table 1. The percentage inhibition after exposure to 25 μM of the tested flavonoids and determined IC_{50} values for both MRP1 and MRP2. When no IC_{50} values could be obtained using concentrations up to 50 μM this is indicated by >50. Values were obtained by curve fitting analysis using data measured *in triplo*.

	MRP1		MRP2	
	% inhibition at 25 μM	IC50 (μM)	% inhibition at 25 μM	IC50 (μM)
Flavone	36%	>50	5%	>50
3-Hydroxyflavone	3%	>50	2%	>50
3'-Hydroxyflavone	13%	>50	0%	>50
4'-Hydroxyflavone	15%	>50	1%	>50
Chrysin	10%	>50	2%	>50
3,3'-Dihydroxyflavone	4%	>50	1%	>50
3',4'-Dihydroxyflavone	57%	24.4 ± 4.1	16%	>50
Galangin	43%	35.3 ± 7.3	0%	>50
Baicalein	48%	30.9 ± 4.4	28%	>50
Apigenin	47%	35.1 ± 9.6	2%	>50
Naringenin	2%	>50	0%	>50
3,3',4'-Trihydroxyflavone	26%	>50	17%	>50
Kaempferol	72%	19.4 ± 3.6	2%	>50
Fisetin	2%	>50	1%	>50
Luteolin	53%	22.4 ± 4.8	17%	>50
Eriodictyol	31%	>50	13%	>50
Morin	30%	49.0 ± 7.6	8%	>50
Quercetin	63%	21.8 ± 3.5	5%	>50
Taxifolin	8%	>50	3%	>50
Catechin	15%	>50	0%	>50
Robinetin	75%	13.6 ± 3.9	76%	15.0 ± 3.5
Myricetin	63%	20.2 ± 4.3	68%	22.2 ± 3.9
Acacetin	18%	>50	1%	>50
Kaempferide	40%	>50	2%	>50
5,7,3',4'-Tetramethoxyflavone	76%	7.9 ± 1.5	20%	>50
Diosmetin	84%	2.7 ± 0.6	17%	>50
Chrysoeriol	85%	4.0 ± 0.7	31%	>50
Tamarixetin	68%	7.4 ± 3.4	8%	>50
Isorhamnetin	60%	14.3 ± 2.8	10%	>50

Figure 3 shows inhibition curves for the MRP1-mediated basolateral calcein efflux by two flavonoids: robinetin and taxifolin. Robinetin shows a typical concentration dependent inhibition of calcein efflux, whereas taxifolin does not inhibit MRP1 activity. From these and similar curves obtained for all other flavonoids, IC_{50} values for the MRP1- and MRP2 activity were determined using flavonoid concentrations up to 50 μM (Table 1). In some cases for MRP1, and almost all for MRP2, it was not possible to derive an IC_{50} due to limited inhibition. Again, the methoxylated flavonoids are among the best MRP1 inhibitors with IC_{50} values between 2.7 (diosmetin) and 14.3 μM (isorhamnetin). Other potent MRP1 inhibitors were robinetin and myricetin (IC_{50} values of 13.6 and 20.2 μM), kaempferol (IC_{50} of 19.4 μM) as well as quercetin, luteolin and 3',4'-dihydroxyflavone (IC_{50} of 21.8, 22.4, 24.4 μM, respectively). Especially the flavonoids with only a few or no hydroxyl groups, and the flavonoids lacking a C2-C3 double bond are the least potent MRP1 inhibitors. For MRP2, only robinetin and myricetin were able to inhibit the activity by more than 50% with IC_{50} values of 15.0 and 22.2 μM. All other flavonoids did not reach 50% MRP2 inhibition using concentrations up to 50 μM. Clearly, the presence of the flavonol B-ring pyrogallol group results in potent MRP2 inhibition as seen for robinetin and myricetin. Also the presence of an A-ring pyrogallol group, as seen in baicalein, results in minor inhibition (28% at 25 μM).

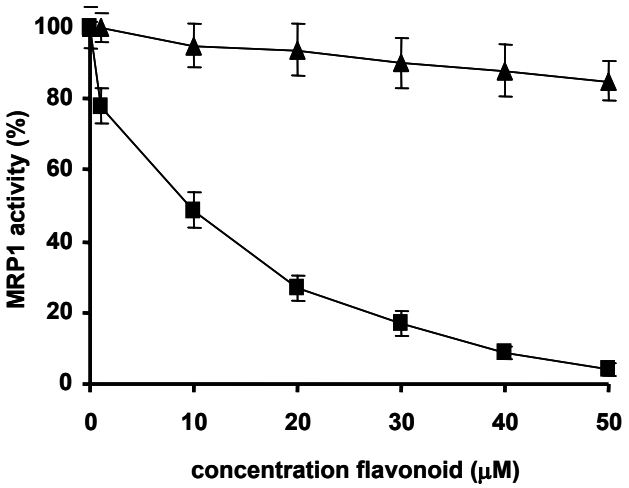


Figure 3. Inhibition of calcein efflux by MDCKII-MRP1 cells after 45 minutes exposure to robinetin (■) and taxifolin (▲) as examples of the effects of flavonoids on MRP1 activity. Cells were loaded with calcein-AM at a final concentration of 1 μM for 2 hours at 7°C after which calcein efflux was measured at 37°C. The results are the means ± SD from triplicate measurements.

MRP1 and MRP2 efflux kinetics

To gain more insight in the mechanism of inhibition, the kinetic parameters (apparent K_m and apparent K_i) for inhibition of calcein efflux by robinetin were determined using Lineweaver-Burk plots based on calcein-AM concentrations used during loading of the cells. Robinetin was taken as a model inhibitor because it appeared to be the flavonoid that most effectively inhibits both MRP1 and MRP2. Figure 4 shows the Lineweaver-Burk plots for calcein efflux at six different robinetin concentrations in MDCKII-MRP1 (A) and MDCKII-MRP2 (B) cells. These plots reveal a typical competitive inhibition pattern. Using these plots the apparent $K_{m\ calcein}$ and apparent $K_{i\ robinetin}$ for both transporter proteins were calculated. For MRP1, the apparent $K_{m\ calcein}$ was 0.13 ± 0.1 μM. The apparent $K_{m\ calcein}$ of MRP2 was 0.40 ± 0.2 μM. The calculated inhibition constants for robinetin, apparent K_i, were 5.0 ± 1.0 μM for MRP1 and 8.5 ± 1.3 μM for MRP2.

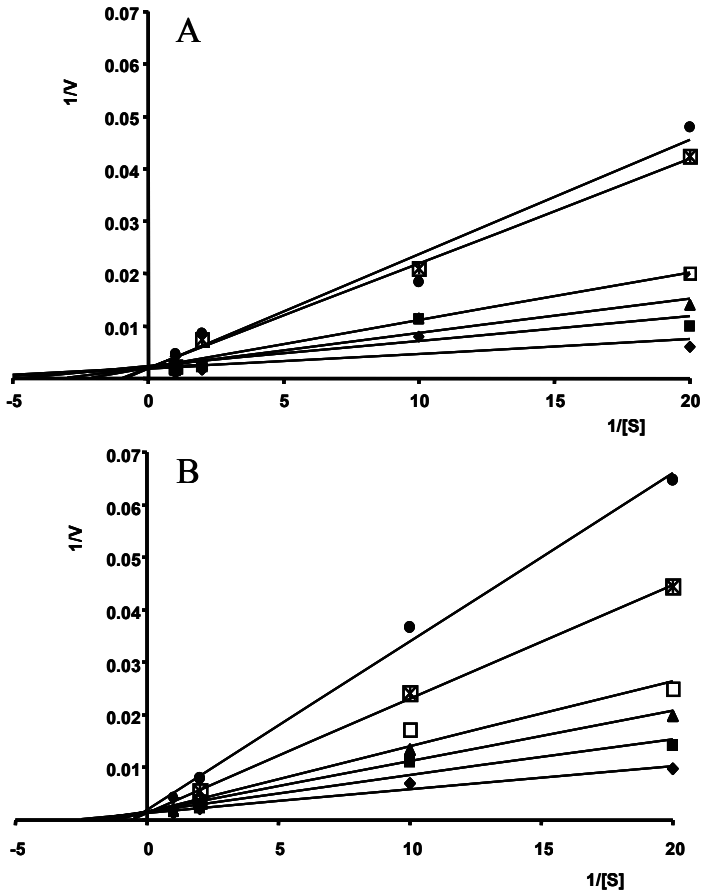


Figure 4. Lineweaver-Burk plots for MRP-mediated transport activity in the presence of six different robinetin concentrations in MDCKII-MRP1 (A) and MDCKII-MRP2 (B) cells. The reciprocal assumed calcein concentration (μM) is plotted on the x-axis, the reciprocal

velocity (in A.U. of fluorescence/min/monolayer in the medium) is plotted on the y-axis. The results are the means from duplicate measurements.

Molecular characteristics of flavonoid structures

To elucidate the structural characteristics of flavonoids necessary for potent inhibition of MRP1 and MRP2 various physical and chemical parameters were quantified. The characteristics of interest were: lipophilicity, dihedral angle between the B- and C-ring, total number of hydroxyl groups, the number of hydroxyl groups on the A, B or C-ring of the flavonoid, the presence of catechol moieties (two adjacent hydroxyl groups) or pyrogallol moieties (three adjacent hydroxyl groups) and the number of methoxylated groups on the flavonoid. Figure 1 summarizes several of these parameters for the different flavonoids. Table 2 lists two other parameters for all flavonoids tested: the dihedral angle between the B- and C ring and the lipophilicity reflected by K', derived from HPLC elution profiles. The dihedral angle between the B- and C ring of a flavonoid quantifies the planarity of the flavonoid molecules. These data confirm that saturation of the C2-C3 double bond results in a major change in the dihedral angle, as seen for eriodictyol, taxifolin, catechin and naringenin.

Table 2. The measured lipophilicity (K') and calculated dihedral angle between the B- and C-ring for all flavonoids tested.

	Lipophilicity (K')	Dihedral angle (degrees)
Flavone	19.7	5.5
3-hydroxyflavone	21.3	14.0
3'-hydroxyflavone	33.9	7.8
4'-hydroxyflavone	8.5	3.8
Chrysin	20.9	4.2
3,3'-dihydroxyflavone	9.5	13.1
3',4'-dihydroxyflavone	5.5	4.7
Galangin	18.3	14.5
Baicalein	9.1	5.4
Apigenin	11.0	5.7
Naringenin	3.8	39.9
3,3',4'-trihydroxyflavone	4.5	21.0
Kaempferol	8.8	14.4
Fisetin	2.4	14.4
Luteolin	6.1	7.3
Eriodictyol	2.1	41.9
Morin	2.8	19.3
Quercetin	4.4	14.7
Taxifolin	0.7	36.1
Catechin	2.9	38.8
Robinetin	1.0	15.4
Myricetin	2.1	14.2
Acacetin	28.0	6.7
Kaempferide	32.4	14.0
5,7,3',4'-teramethoxyflavone	20.4	9.1
Diosmetin	12.1	7.1
Chrysoeriol	11.1	2.3
Tamarixetin	7.2	11.9
Isorhamnetin	10.4	13.9

Flavonoid characteristics for potent MRP1 inhibition

To identify and quantify the effects of the different molecular descriptors (structural characteristics) for potent MRP1 inhibition, stepwise multiple regression was performed. The data of Figure 1 and Tables 1 and 2 were used to derive multiple parameter QSAR models. First, a one-parameter model for each descriptor was derived. Table 3 lists the partial correlation coefficients and P-values for each one-parameter model. In this model, MRP1 inhibition was best predicted by the number of methoxylated moieties ($R = 0.427$, $P = 0.021$). Thereafter, two-parameter models were derived based on the best one-parameter model. For the two-parameter model, the second best descriptor appeared to be the total number of hydroxyl groups ($R = 0.586$, $P = 0.040$) (Table 3). Consecutively, three-parameter models were derived based on the best two-parameter model (Table 3). The optimal three-parameter model ($R = 0.766$, $P < 0.001$) describing MRP1 inhibition by flavonoids uses the following descriptors: the number of methoxylated moieties, the number of hydroxyl groups and the dihedral angle between the B- and C-ring and is described by equation 1;

% inhibition = 45.466 + 18.936 (No. of OCH_3 moieties) + 12.474 (No. of OH groups) – 48.246 (Log dihedral angle) (Equation 1)

Other descriptors like lipophilicity and the presence of catechol and pyrogallol moieties did not significantly improve the model. Figure 5 displays the relation between the measured inhibition of MRP1 activity and the inhibition calculated by equation 1 for all flavonoids tested. Least square regression analysis reveals a correlation coefficient of 0.766.

Table 3. Partial correlation coefficients and P-values for the three consecutive multi-parameter models: one-parameter model, two-parameter model and three-parameter model describing MRP1 inhibition by flavonoids.

	Partial correlation coefficient	**P**	**Partial correlation coefficient**	**P**	**Partial correlation coefficient**	**P**
No. of OCH_3 groups	0.427	0.021	1st parameter	-	1st parameter	-
No. of OH groups	0.010	0.960	0.586	0.040	2nd parameter	-
Log dihedral angle	0.296	0.118	0.484	0.234	0.766	<0.001
Log K'	0.233	0.223	0.210	0.047	0.607	0.080
No. of pyrogallol and catechol moieties	0.031	0.872	0.466	0.041	0.586	0.013

The optimal three-parameter model obtained was: % inhibition = 45.466 + 18.936 (No. of OCH_3 moieties) + 12.474 (No. of OH groups) – 48.246 (Log dihedral angle)

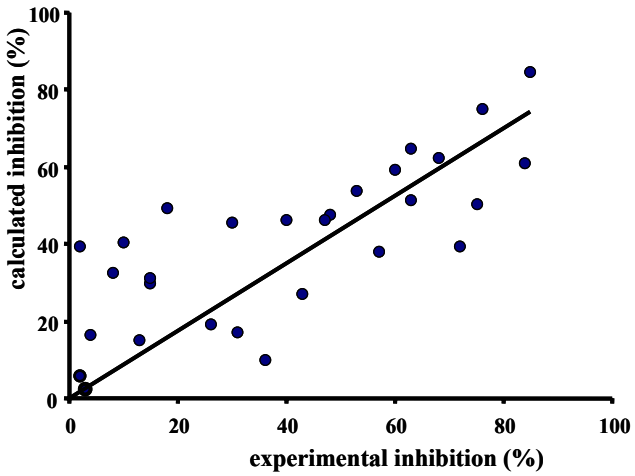


Figure 5. The relation between the measured inhibition and predicted (calculated) inhibition of MRP1 activity for all flavonoids tested (R = 0.766, P < 0.001) using the equation: % inhibition = 45.466 + 18.936 (No. of OCH_3 moieties) + 12.474 (No. of OH groups) – 48.246 (Log dihedral angle).

Flavonoid characteristics for potent MRP2 inhibition

Due to the limited number of flavonoids that were able to inhibit MRP2 activity and the limited inhibition caused by the flavonoids tested, the identification of structural characteristics necessary for potent MRP2 inhibition is straightforward but not complete. The presence of a flavonol B-ring pyrogallol group results in potent MRP2 inhibition as seen for robinetin and myricetin. The presence of an A-ring pyrogallol group, as seen in baicalein, results in minor inhibition (28% at 25 μM). As a consequence, multiple regression analysis resulted in only one significant model: a single-component model (R = 0.408, P =0.028) with the total number of pyrogallol and/or catechol moieties as descriptor (Table 4).

Table 4. Partial correlation coefficients and P-values for the one-parameter model describing MRP2 inhibition by flavonoids.

	Partial correlation coefficient	**P**
No. of OCH_3 groups	0.076	0.697
No. of OH groups	0.364	0.052
Log dihedral angle	0.107	0.579
Log K'	0.281	0.148
No. of pyrogallol and catechol moieties	0.408	0.028

Discussion

The results reported in the present study describe structural characteristics of flavonoids responsible for high potency MRP1 or MRP2 inhibition. MRP1 and MRP2 are well known members of the MRP family, all ATP-binding cassette transporters. Despite the limited amino acid identity, the spectrum of substrates transported by MRP1 and MRP2 overlap to a large extent. The two transporters may differ in affinity towards their substrates. As an example, MRP1 exhibits, in comparison to MRP2, a 10-fold higher K_m to leukotriene C4 (LTC4) and a 5-fold higher K_m to 17β-estradiol-(D-glucuronide) [14]. The apparent K_m of both proteins towards the substrate used in this study, calcein, differs marginally, being 0.13 ± 0.1 μM for MRP1 and 0.40 ± 0.2 μM for MRP2, based on calcein-AM concentrations used during loading of the cells.

The outcomes of this study show that MRP1 is far more susceptible to inhibition by flavonoids than MRP2 (summarized in Table 1). The methoxylated flavonoids 5,7,3',4'-tetramethoxyflavone, diosmetin, chrysoeriol, tamarixetin and isorhamnetin are among the best MRP1 inhibitors as indicated by their low IC_{50} values, ranging between 2.7 and 14.3 μM. Interestingly, these IC50 values are in the same range or slightly above the IC_{50} value of 5 μM of the typical MRP1 inhibitor MK571, measured in the same cellular system [28]. Other flavonoids able to inhibit more than 50% of MRP1 activity were 3',4'-dihydroxyflavone, luteolin, quercetin, robinetin and myricetin with IC_{50} values ranging between 13.6 and 24.4 μM. In contrast to the wide variety of flavonoids able to inhibit MRP1, only a few of the tested flavonoids were able to inhibit MRP2-mediated efflux. Only robinetin and myricetin were able to inhibit MRP2 activity more than 50%, with IC_{50} values of 15.0 and 22.2 μM respectively. Again, these IC_{50} values are in the same range as reported for the typical MRP2 inhibitor, cyclosporine A, for which the IC_{50}, measured under identical experimental conditions, was 10 μM [28]. Apparently, robinetin and myricetin possess comparable MRP2-inhibitory potencies as the typical MRP2 inhibitor cyclosporin A.

The competitive nature of calcein efflux inhibition by robinetin indicates that robinetin binds to the same binding site on MRP1 and MRP2 as calcein. Competitive inhibition of MRP1 by flavonoids has been reported before for LTC4 transport in reconstituted vesicles [21]. The kinetic studies of MRP1 and MRP2 efflux inhibition by robinetin resulted in calculated apparent inhibition constants $K_{i\ robinetin.}$ These apparent constants were almost similar: 5.0 μM for MRP1 and 8.5 μM for MRP2. Determination of apparent K_i values for flavonoid mediated MRP1 or MRP2 inhibition in a cellular system has not been described before. In a cellular system the apparent K_i values might be influenced by cellular processes including, among others, uptake and metabolism. Nevertheless, K_i values reported by Leslie *et al.* [21] on the competitive flavonoid mediated inhibition of MRP1 activity in reconstituted membrane vesicles, using LTC4 as substrate, were in the same order of magnitude (2.4-21 μM).

Table 5. Schematic overview of the experimental outline and main results from this study and other related studies.

Model system	Pump	Substrate	No. of flavonoids tested	Concen-trations	K_i	Most potent inhibitors	Structural characteristics important for MRP inhibition	Ref.*
MDCKII cells	MRP1	calcein	29	0.1-50 μM	5.0 μM (robinetin)	diosmetin>chrysoeriol>tamarixetin>tetra-methoxyflavone> robinetin > iso-rhamnetin> kaempferol > myricetin > quercetin > luteolin	dihedral angle; number of hydroxyl groups; number of methoxylated moieties	This study
MDCKII cells	MRP2	calcein	29	0.1-50 μM	8.5 μM (robinetin)	robinetin > myricetin	flavonol B-ring pyrogallol moiety	This study
MCF7 cells	GS-X pump	DNP-SG	11	0.1-50 μM	-	luteolin > quercetin > kaempferol > 3',4'-dihydroxyflavone > myricetin	hydroxyl groups (especially two of them generating the 3',4'-catechol moiety); dihedral angle	1
Vesicles	MRP1	LTC4	8	1-100 μM	2.4-21 μM (various)	kaempferol > apigenin > quercetin > myricetin > naringenin	not defined	2
Vesicles	MRP1	17β-estradiol	8	1-100 μM	-	apigenin > kaempferol > naringenin > quercetin > myricetin	lipophilicity	2
Panc-1 cells	MRP1	Dauno-mycin / vinblastin	22	100 μM	-	Morin > kaempferol > quercetin > genistein	not defined	3
Human erythro-cytes	Most likely MRP1	BCPCF	30 (mostly modified)	0-100	-	Euchrestaflavanone A = sophora-flavanone H > other sophoraflavanones	Flavanones with a prenyl, geranyl or lavandulyl group	4

* Used literature: 1 (Zanden van et al., 2004); 2 (Leslie et al., 2001); 3 (Nguyen et al., 2003); 4 (Bobrowska-Hagerstrand et al., 2003)

Multiple regression analysis was used for the identification and quantification of the effects of different structural characteristics regarding potent inhibition of MRP1 and MRP2 activity. For MRP1, an optimal multiple parameter QSAR model was obtained. The resulting QSAR equation (Eq.1) reveals that three structural characteristics are of major importance for MRP1 inhibition: the total number of methoxylated moieties, the total number of hydroxyl groups and the dihedral angle between the B- and C-ring. Using this QSAR equation a correlation ($R = 0.766$) was obtained between the predicted inhibition and the actual measured inhibition ($P<0.001$). Neither the lipophilicity K', nor the total number of catechol and/or pyrogallol moieties significantly influence MRP1 inhibition by flavonoids. Comparison of the flavonoid mediated effects on MRP1 activity from this study with previous studies [21, 23, 24, 32], summarized in Table 5, reveal that both the magnitude and the rank order of MRP1 inhibition by flavonoids varies per study, possibly as a result of the different MRP1 substrates used in the different studies (Table 5). Besides the effect of different substrates, another important difference between the present and the other studies is the use of different *in vitro* model systems.

Regarding MRP2-mediated calcein efflux inhibition, only the presence of a flavonol B-ring pyrogallol group seems to be an important structural characteristic. Ultimately, MRP2 displays a higher selectivity for flavonoid type inhibition than MRP1.

Several mechanisms in which inhibitors might interact with MRPs have been proposed in the literature. Interaction of flavonoids with MRPs might affect: drug binding, ATP binding, ATP hydrolysis, drug transport, and ADP release. Flavonoids are well known inhibitors (but sometimes also stimulators) of ATPase activity [21, 22, 33, 34]. Possibly, more than one interaction/effect might take place simultaneously. Another example of such an interaction of flavonoids with MRPs can be found in studies reported for human colonic carcinoma Caco-2 cells [35, 36]. These reports show that flavonoids as well as their glucuronide- and sulphate-conjugates and their glycosylated forms can act as MRP2 substrates and are efficiently transported by this transporter. This observation suggests an interaction of flavonoids with the substrate binding site of MRP2. The competitive inhibition of MRP1 and MRP2-mediated transport by robinetin demonstrated in the present study corroborates this conclusion of interaction at the substrate binding site.

In contrast to the possible beneficial use of flavonoids as MDR modulators, the increased intake of extreme doses of flavonoids via dietary supplementation might disturb physiological processes. This increased intake of flavonoids might affect the kinetics of other food constituents, pharmaceuticals, xenobiotics or endogenous substrates of MRPs. Especially in the intestine, high flavonoid concentrations can be expected upon supplementation, since quercetin supplements are known to result in daily intakes up to 1 g/day, plasma levels of up to 10 μM and intestine concentrations that are even higher [37, 38]. Comparing these concentrations to the IC_{50} values and K_i values of the present study indicates that the inhibitory effects observed in the present study can be expected to be relevant *in vivo* as well. Some flavonoids are known to become cytotoxic at concentrations above 50 μM [39, 40]. Since the IC_{50} values obtained in the present study are 2-20 times lower, the inhibition of MRPs by flavonoids can be obtained at therapeutic non-toxic

concentrations. In addition, since the present study used calcein concentrations approximately 2.5-8 times higher than the apparent K_m of MRPs for calcein efflux, based on calcein-AM concentrations used during loading of the cells, it can be expected that at lower calcein concentrations the corresponding IC_{50} values for inhibition by flavonoids will be even lower.

In summary, this study describes the inhibitory interaction of flavonoids with MRP1 and MRP2. Moreover, this study also shows that MRP2 displays a higher selectivity for flavonoid type inhibition than MRP1. Molecular characteristics responsible for these inhibitory actions of MRP1 and MRP2 were identified and, for MRP1, a model was developed quantitatively describing the MRP1 inhibitory potency of flavonoids based on their molecular characteristics.

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Reversal of *in vitro* cellular MRP1 and MRP2 mediated vincristine resistance by the flavonoid myricetin.

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Abstract

In the present study, the effects of myricetin on either MRP1-, or MRP2- mediated vincristine resistance in transfected MDCKII cells were examined. The results obtained show that myricetin can inhibit both MRP1- and MRP2- mediated vincristine efflux in a concentration dependent manner. The IC_{50} values for cellular vincristine transport inhibition by myricetin were 30.5 ± 1.7 μM for MRP1- and 24.6 ± 1.3 μM for MRP2 containing MDCKII cells. Cell proliferation analysis showed that the MDCKII control cells are very sensitive towards vincristine toxicity with an IC_{50} value of 1.1 ± 0.1 μM. The MDCKII-MRP1 and MDCKII-MRP2 cells are less sensitive towards vincristine toxicity with IC_{50} values of 33.1 ± 1.9 μM and 22.2 ± 1.4 μM, respectively. In both the MRP1 and MRP2 cells, exposure to 25 μM myricetin enhances the sensitivity of the cells towards vincristine toxicity to IC_{50} values of 7.6 ± 0.5 μM and 5.8 ± 0.5 μM, respectively. The increase of sensitivity represents a reversal of the resistance towards vincristine as a result of MRP1 and MRP2 inhibition. Thus, the present study demonstrates the ability of the flavonoid myricetin to modulate MRP1- and MRP2- mediated resistance to the anticancer drug vincristine in transfected cells, indicating that flavonoids might be a valuable adjunct to chemotherapy to block MRP mediated resistance.

Introduction

Resistance to multiple anticancer drugs is a major obstacle for successful chemotherapy in cancer. This phenomenon, known as multidrug resistance (MDR), can be caused by different mechanisms. One such mechanism of MDR is the overexpression of membrane-bound drug efflux pumps like P-glycoprotein and the multidrug resistance proteins (MRPs), including MRP1 and MRP2 [1-3]. The MRPs belong to the ATP-binding cassette (ABC) transporter family and, at present, nine MRP-subfamily transporters have been identified which differ widely in substrate specificity, tissue distribution and intracellular location [3]. MRP1 (ABCC1) is a 190-kDa protein that transports a number of endogenous and exogenous organic anions and a wide variety of compounds conjugated to glutathione (GSH), glucuronate or sulphate [4]. MRP1 confers resistance to a variety of compounds like anthracyclines, epipodophylotoxins and some vinca alkaloids [3]. Although MRP1 is a typical glutathione-S-conjugate (GS-X) pump, chemotherapeutic agents that are not metabolized to a glutathione conjugate such as daunomycin, methotrexate, fluorouracil, chlorambucil and vinca alkaloids including vincristine are also substrates for MRP1 [5, 6]. MRP2 (ABCC2) was originally identified as the canalicular multispecific organic anion transporter (cMOAT). Despite limited amino acid identity (49%) to MRP1, the spectrum of substrates transported by MRP1 and MRP2 overlap to a large extent. Indeed, like MRP1, MRP2 confers *in vitro* cellular resistance to many anticancer drugs like vincristine [7-9], methotrexate [10] and anthracyclines [7, 8], despite differences in cellular localization and kinetic properties.

In the present study the possible reversal of cellular MRP1- or MRP2- mediated anticancer drug resistance was investigated using vincristine as the model anticancer drug. Vincristine has been demonstrated to be a suitable substrate for MRP1- and MRP2-mediated efflux studies and MRP1 and MRP2 have been reported to confer resistance towards this drug [7-9]. Vincristine is a member of the group of vinca alkaloids and has been used in anticancer chemotherapy since the 1960's [11]. Vincristine is a weak organic base and does not conjugate with GSH. However, GSH is required for vincristine resistance, as depletion of cellular GSH abolished MRP1-mediated resistance against vincristine [12, 13]. Moreover, in vesicular transport experiments, transport of vincristine occurred only in the presence of reduced GSH [14, 15].

One possible strategy for reversal of MRP mediated multidrug resistance is inhibition of the activity of these transport proteins. Several inhibitors of MRP1 and MRP2 have been described in the literature. Compounds such as sulfinpyrazone, benzbromarone and probenecid are relatively non-specific inhibitors of organic anion transporters [3, 16]. Furthermore, some MRP1 inhibitors, like certain tricyclic isoxazoles, do inhibit MRP1 in intact cells at micromolar concentrations but are much less active against MRP2 [17].

The search for MRP inhibitors showed that many natural constituents, including plant polyphenols like flavonoids were promising candidates for both MRP1 and MRP2 inhibition [18-23]. Recently, we described structure activity relationship studies on inhibition of MRP1- and MRP2 mediated calcein efflux by flavonoids in MRP transfected

MDCKII cells [23]. It was demonstrated that many flavonoids, including myricetin, robinetin and quercetin, are able to inhibit MRP1 activity. Regarding inhibition of MRP2 activity, myricetin and robinetin appeared to be particularly good inhibitors [23].
Therefore, in the present study, the effects of myricetin (Figure 1) on either MRP1-, or MRP2 mediated vincristine resistance in transfected MDCKII cells are examined. The possible use of myricetin for reversal of MRP mediated multidrug resistance is discussed.

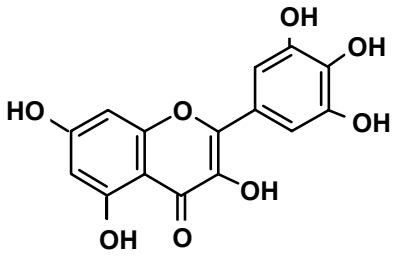

Figure 1. Structural formula of myricetin

Materials and Methods

Materials.
The Madin-Darby Canine Kidney (MDCKII) cell lines, stably expressing either a control vector (hereafter called control cells), human *MRP1* cDNA (hereafter called MRP1 cells) or *MRP2* cDNA (hereafter called MRP2 cells) were kindly provided by Prof. P. Borst (NKI, Amsterdam).
Dulbecco's Minimum Eagle Medium (DMEM) with GlutaMax, fetal bovine serum, penicillin/streptomycin and gentamycin were all purchased from Gibco, (Paisley, Scotland). MK571 was obtained from BioMol (Plymouth Meeting, PA); PSC833 was a kind gift from Novartis Pharma AG (Basel, Switzerland). Cyclosporin A was obtained from Fluka (Zwijndrecht, The Netherlands). [^{3}H]-vincristine sulphate (4.9 Ci/mmol) was obtained from Amersham Biosciences (Buckinghamshire, UK). Flo-Scint scintillation cocktail was purchased from Packard (Groningen, The Netherlands). Myricetin, was purchased from Sigma Chemical Co. (Zwijndrecht, The Netherlands). The cell proliferation ELISA BrdU-kit was purchased from Roche Molecular Biochemicals (Mannheim, Germany). DMSO and HPLC-grade methanol were obtained from Acros Organics (Geel, Belgium).

MDCKII cell culture.
The Madin Darby Canine Kidney cell lines (control and MRP1 or MRP2 transfected) were cultured in Dulbecco's Minimum Essential Medium (DMEM) with GlutaMax (4.5 g glucose per liter), 10% fetal calf serum and 0.01% penicillin/streptomycin, and were grown in a humidified atmosphere in 5% CO_2 at 37°C.

Vincristine transport by MRP1 and MRP2.

For transport experiments $4*10^5$ cells/cm^2 were grown on microporous polycarbonate filters ((0.4 μm pore size, 1 cm^2) Costar Corp. Cambridge, MA). It was shown earlier [24] that in these polarized cells MRP1 routes to the basolateral plasma membrane, whereas MRP2 routes to the apical plasma membrane. Culturing MDCKII cells on a filter in transwells provides the opportunity to study the MRP-mediated efflux of a compound to either the apical or basolateral side of intact cells. Although these cell lines do contain low endogenous MRP-like and/or other efflux transporters, the levels of the introduced MRPs are very profound which makes these cell lines ideal for comparative studies on the role of MRP1 or MRP2 in drug resistance [25, 26]. The volumes of media in the basolateral and apical compartments were 1.8 and 0.5 ml respectively. Cells were cultured to confluency for three days and medium was replaced every 24 h. Confluency of the monolayers was checked by transepithelial electric resistance (TEER) measurement, validated by determination of the paracellular flux of inulin[^{14}C]carboxylic acid (185 kBq/mol, 4.2 μM) [27]. TEER-values of each monolayer were measured using a Millicell-ERS epithelial volt/ohm meter (Millipore, Bedford). The corrected TEER-value of a confluent monolayer of MDCKII cells ranged between 120-140 Ω.cm^2 as reported before [26]. Three days post seeding, the cells were loaded with 0.5 μM [^{3}H]-vincristine (2.8 μCi/well) in DMEM without phenol red containing 0.1 μM PSC833 (to exclude any possible effects of P-glycoprotein), for 2 hours at 37^{o}C. The use of the cyclosporin derivative PSC833 as a Pgp inhibitor was used based on studies by Evers *et al.* (1998) [24]. A relatively low concentration of PSC833 (0.1 μM) was shown to completely inhibit apical efflux of vinblastine from the control cells, whereas the vinblastine efflux by MRP2 in the MRP2 cells appeared not to be affected by this dose [24]. Furthermore, experiments were performed to test this concentration (0.1 μM) of PSC833 on MRP1 and MRP2 vincristine transport by the transfected cells which showed no changes in respectively basolateral or apical efflux characteristics (data not shown). These findings support the use of 0.1 μM PSC833 as a rather specific inhibitor of Pgp that does not affect MRP1 and MRP2 mediated transport in the MRP1 and MRP2 transfected cell lines. Experiments to determine optimal loading conditions revealed that for the MRP transfected cell lines, upon exposure to vincristine for 2 hours at 37^{o}C, the intracellular amount of vincristine reached up to 2.4 % of the total amount of vincristine present. For the control cells the intracellular amount of vincristine reached up to 3.3 % of the total amount of vincristine present. After loading of the cells at 37^{o}C, efflux of vincristine was determined as follows. Loaded cells were washed twice with DMEM without phenol red and exposed to fresh medium (DMEM without serum at 37ºC) containing 0.1 μM PSC833 and different concentrations of myricetin (0 to 50 μM), 30 μM MK571 (as a typical MRP1 inhibitor) or 30 μM cyclosporin A (as a typical MRP2 inhibitor) [28]. Cells receiving vehicle only (0.5% DMSO v/v) served as control (blank). Efflux of vincristine at various time-points was measured in media samples from both the apical and basolateral compartment upon 60 min exposure to myricetin, MK571 or cyclosporin A. After 60 minutes, the efflux medium was removed and cells were washed three times in ice-cold PBS. The filters with cells were placed in KOH

solution containing 20% methanol for 24 hours to disrupt the cells for measurement of the intracellular radioactivity. Radioactivity was counted using a Packard 1600 Liquid Scintilator with Packard Ultima Gold as scintillation cocktail (Packard, Groningen, The Netherlands).

Vincristine detection.
Samples taken from the efflux medium after 20, 40 or 60 minutes were analyzed for vincristine metabolites using reversed-phase HPLC according to a method developed by Tikhomiroff *et al.* (2002) to detect the major indole alkaloids of Catharanthus roseus (e.g. vincristine) and their metabolites [29]. In short, analysis was carried out using a Merck Hitachi HPLC system equipped with a L6200 pump and a L4200 UV-Vis detector combined with a Packard Flo-One on-line radioactivity detector using Flo-Scint as scintillation cocktail. For a typical run, 50 µl of the samples were injected onto a Zorbax Eclipse XDB-C_{18} 250 mm x 4.6 mm column. The column was eluted at a flow rate of 0.8 ml/min by isocratic elution at 95 % A (5 mM Na_2HPO_4 pH 6) and 5 % B (acetonitrile) for 20 minutes followed by a linear gradient to 15 % B in 10 min, and finally a linear gradient to 80 % B in 5 min.

Measurement of intracellular glutathione (GSH).
To determine the effect of myricetin exposure on the intracellular GSH levels the three MDCKII cell lines were treated similar as for the efflux experiments described above. After one hour exposure to 0 µM or 25 µM myricetin the cellular fractions were analyzed using the DTNB-GSSG reductase recycling assay as described by Baker et al. (1990) [30].

Cell proliferation.
The effect of myricetin on the inhibition of cell proliferation by the anticancer drug vincristine was determined for the control and the MRP1 or MRP2 transfected MDCKII cells using the BrdU cell proliferation assay, adapted for transwells, using the Cell Proliferation ELISA, BrdU (colorimetric) kit from Roche Diagnostics (Mannheim, Germany). For the assay $0.5*10^5$ cells/cm^2 were grown on microporous polycarbonate filters ((0.4 µm pore size, 0.33 cm^2) Costar Corp. Cambridge, MA). The volume of media in the basolateral and apical compartments was 0.6 and 0.1 ml respectively. After 24 hours the cells were exposed to a range of vincristine concentrations (0.01-100 µM) in DMEM without phenol red containing 0.1 µM PSC833 (to exclude any possible effects of P-glycoprotein), for 2 hours at 37^{o}C. After this exposure, cells were rinsed twice with DMEM without phenol red and cultured in normal culture medium for 24 hours in the presence or absence of inhibitors. Final concentrations of the inhibitors used were 25 µM myricetin, 30 µM MK571 or 30 µM cyclosporin A. Higher myricetin concentrations showed inhibitory effects on the cell proliferation scores upon 24 hours exposure and could therefore not be tested. After 24 hours culturing, cell proliferation was determined by labeling the cells with BrdU for two hours at 37^{0}C. Absorbance of the converted substrate tetramethyl-benzidine was measured at 370 nm using a Thermomax microplate reader (Molecular Devices Corp.,

Menlo Park, CA USA). Results were expressed as percentage cell proliferation compared to the blank (vehicle only).

Cytotoxicity.
Toxicity of different concentrations myricetin was measured using the LDH-leakage method for cell viability [31] with some minor adaptations for transwell plates. Cells were grown to confluent monolayers as described for the efflux assays. The cells were exposed to different concentrations myricetin in both compartments for 24 hours. Before measurements of the LDH activity in the medium, the samples from the apical (0.6 ml) and basolateral (1.0 ml) compartments were pooled. The filter membranes containing the cells were washed twice with cold PBS and removed from the inserts. Cells were sonicated in 1 ml PBS and all samples were stored at –20^{o}C until analysis of the LDH activity.

Data analysis.
A one-way analysis of variance test was used for all data analysis ($P<0.05$) using SPSS 10.1.0 software from SPSS Inc.

Results

Vincristine transport inhibition in MDCKII MRP1 and MRP2 cells.
To study the efflux of vincristine by MDCKII MRP1 and MDCKII MRP2 cells, the cells were loaded with 0.5 μM [^{3}H]-vincristine (2.8 μCi/well) for 2 hours at 37^{o}C. Table 1 shows the total amounts of intracellular vincristine upon loading. Upon loading, the two MRP transfected cell lines reached comparable total accumulated vincristine quantities that amounted to a maximum of 11.8 ± 0.6 pmol. For the control cell line the total accumulated amount of vincristine reached to a maximum of 16.2 ± 1.0 pmol, a value that was significantly higher ($P<0.05$) than the amount of vincristine reached in the MRP1 or MRP2 cells. After loading, the cells were exposed to fresh efflux medium with or without inhibitor for 60 minutes at 37ºC. After 60 minutes a maximum of ± 20 % (2.2 pmol) of the total amount of vincristine present after loading of the cells appeared to be excreted to the medium (Table 1).

Table 1. Total accumulated vincristine amounts upon two hours loading and the distribution of vincristine after 60 minutes efflux at the apical, basolateral and the intracellular compartment from the vincristine loaded control-, MRP1- or MRP2 cells in the absence or presence of various MRP inhibitors. Concentrations used were 25 μM for myricetin, 30 μM for MK571 and 30 μM for cyclosporin A (CsA). Results represent average ± standard deviation from triplicate measurements.

		Total amount of VCR at t=0[a] (pmol)	Amount of VCR at apical compartment at t=60 (pmol)	Amount of VCR at basolateral compartment at t=60 (pmol)	Amount of VCR at intracellular compartment at t=60 (pmol)	% VCR intra-cellular of total
control	**Blank**	16.2 ± 1.0	0.1 ± 0.1	0.3 ± 0.1	15.8 ± 1.1	98%
	Myricetin		0.1 ± 0.1	0.3 ± 0.1	15.8 ± 0.9	98%
MRP1	**Blank**	11.8 ± 0.6‡	0.2 ± 0.1	2.0 ± 0.2	9.3 ± 0.7	79%
	Myricetin		0.1 ± 0.1	1.0 ± 0.3*	10.2 ± 0.5	87%
	MK571		0.1 ± 0.1	0.2 ± 0.1*	11.5 ± 0.6*	97%
MRP2	**Blank**	10.7 ± 0.7‡	1.1 ± 0.1	0.3 ± 0.1	8.8 ± 0.4	83%
	Myricetin		0.7 ± 0.2*	0.3 ± 0.1	9.7 ± 0.4*	91%
	CsA		0.4 ± 0.2*	0.4 ± 0.1	9.9 ± 0.5*	93%

a the total amount of vincristine was measured after two hours loading time without inhibitors.
‡ Statistically significant different from control cells ($P<0.05$).
* Statistically significant different from corresponding cells exposed to vehicle control (blank) ($P<0.05$).

Figure 2 shows the efflux of vincristine to the basolateral and apical side from vincristine-loaded cells (A) MDCKII control, (B) MDCKII MRP1 and (C) MDCKII MRP2 cells upon exposure to 0 μM or 25 μM myricetin. In the control cells only limited vincristine efflux took place. In MRP1 cells, vincristine was predominantly excreted to the basolateral side (12 times higher than apical efflux), whereas in MRP2 cells the efflux of vincristine was predominantly to the apical side (4 times higher than basolateral efflux). Furthermore, the basolateral efflux of vincristine by MRP1 cells after 60 minutes (2.0 ± 0.2 pmol/monolayer) appeared to be almost twice the apical efflux of vincristine by MRP2 cells after 60 minutes (1.1 ± 0.1 pmol/monolayer). In the presence of 25 μM myricetin the basolateral MRP1 mediated efflux of vincristine was reduced by 52 ± 8% to 1.0 ± 0.3 pmol/monolayer. For MRP2, the presence of 25 μM myricetin reduced the apical MRP2 mediated vincristine efflux by 41 ± 5 % to 0.7 ± 0.2 pmol/monolayer.

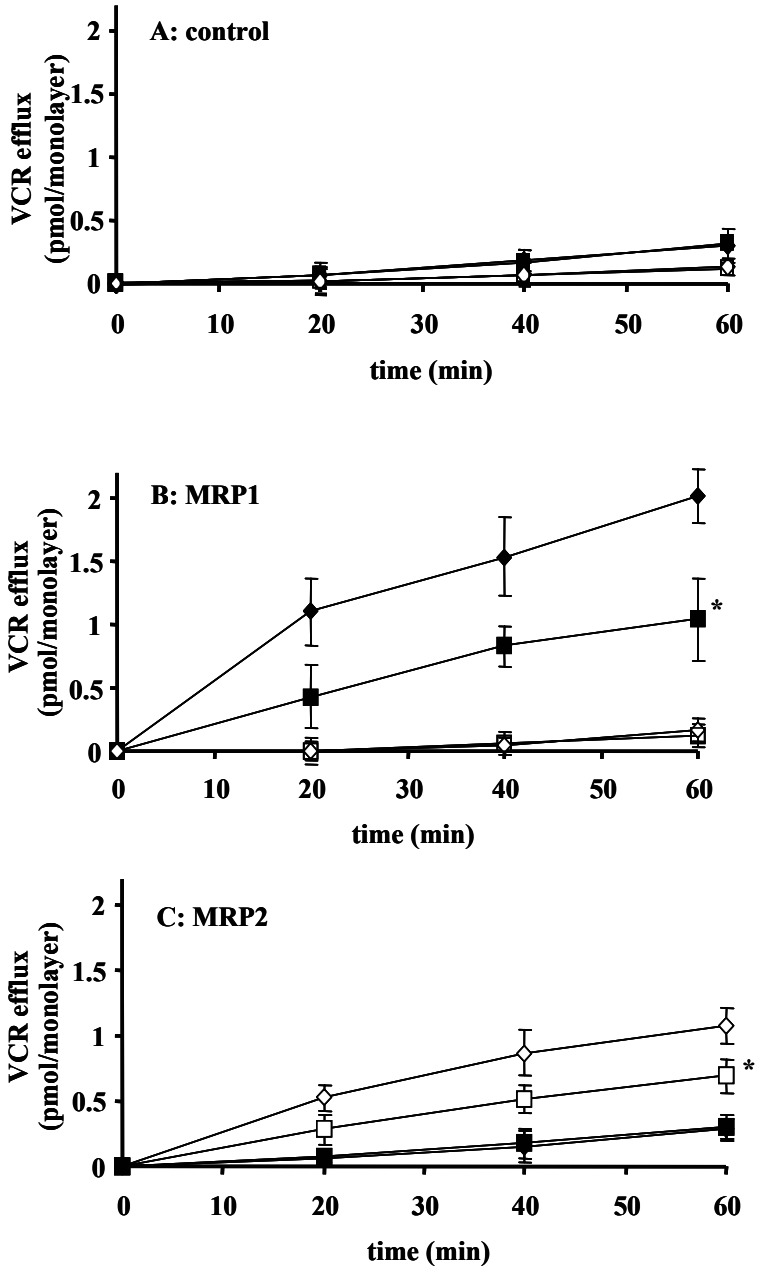

Figure 2. Vincristine efflux to the basolateral (closed symbols) and apical (open symbols) side by (A) MDCKII control, (B) MDCKII MRP1 and (C) MDCKII MRP2 cells upon exposure to 0 μM (◆,◇) or 25 μM (■,□) myricetin for 60 minutes. Data points represent the means ± SD from triplicate measurements Asterisks (*) represent statistically significant differences from the cells exposed to vehicle only (blank) ($P<0.05$).

Intracellular vincristine concentrations

One of the goals that should be reached to reverse MRP mediated multidrug resistance is preservation of the intracellular drug concentration through inhibition of MRP- mediated drug efflux. Table 1 summarizes the effects of 25 μM myricetin and two typical MRP inhibitors, 30 μM MK571 for MRP1 and 30 μM Cyclosporin A for MRP2, on the efflux of vincristine to the apical and basolateral compartments of the three transfected MDCKII cell lines. Furthermore, the accompanying effects of efflux inhibition on the intracellular vincristine concentration in MDCKII control, MRP1 and MRP2 cells are shown in Table 1. From these results it can be derived that inhibition of MRP1 mediated vincristine efflux by 25 μM myricetin at the end of the 1 hr efflux period resulted in a reduced decrease of the intracellular vincristine amount from 79% in the absence, up to 87% of the original quantity after loading of the cells in the presence of myricetin. Exposure to the typical MRP1 inhibitor MK571 results in a residual intracellular vincristine amount of 97% of the original quantity after loading. Thus, the effect of 30 μM MK571 on maintenance of the intracellular vincristine amount in MRP1 cells is almost maximal whereas 25 μM myricetin significantly, but not fully, prevents vincristine efflux from the cells. For MRP2 it can be seen that in the presence of 25 μM myricetin the intracellular vincristine amounts up to 91% of the original quantity after loading of the cells, instead of the 83% observed in the absence of myricetin. Exposure to 30 μM of the typical MRP2 inhibitor cyclosporin A results in intracellular vincristine amounts at the end of the 1 hr efflux that amounts to 93% of the original amount present after loading of the cells. This effect was almost similar to the effect of 25 μM myricetin.

As observed, the effects of 25 μM myricetin on vincristine efflux inhibition and intracellular vincristine quantities are not optimal, since only partial inhibition of the vincristine efflux was observed. To determine whether the inhibition of MRP-mediated vincristine efflux by myricetin was concentration dependent, experiments with myricetin concentrations up to 50 μM myricetin were performed. Figure 3 shows the effects of increasing myricetin concentrations on the relative increase of the residual intracellular vincristine amount after 60 minutes efflux for MDCKII MRP1 and MRP2 cells. The results obtained reveal that myricetin inhibits vincristine efflux, thereby increasing the intracellular vincristine concentration, in a concentration dependent manner. Interestingly, 50 μM myricetin almost completely inhibits vincristine efflux in both cell lines to give an effect identical to the typical MRP inhibitors MK571 and cyclosporin A (Table 1). Based on the data displayed in Figure 3, the estimated IC_{50} values, representing the concentrations at which 50% of the vincristine efflux is inhibited, are 30.5 ± 1.7 μM for MRP1 and 24.6 ± 1.3 μM for MRP2. Additionally, the figure displays the effects of increasing myricetin concentrations on the cell viability of the MDCKII MRP1 and MRP2 cells upon 24 hours incubation. The cell lines showed comparable sensitivity towards myricetin with 50 μM myricetin being slightly cytotoxic to the cells. However, upon incubation of the cells with

increasing concentrations myricetin for only 1 hour, identical to the efflux assay performed in this study, no significant toxicity was observed (data not shown).

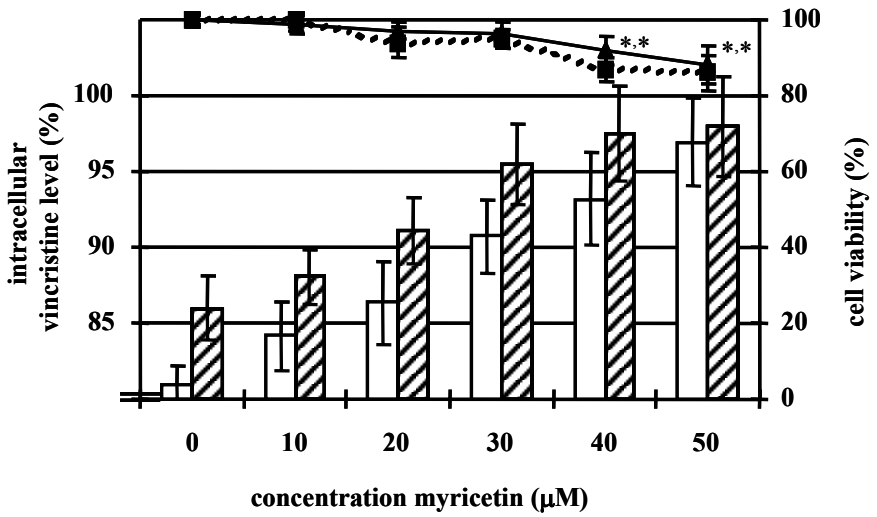

Figure 3. Relative intracellular vincristine levels compared to the vincristine levels present at t=0 in MDCKII MRP1 cells (blank bars) and MDCKII MRP2 cells (striped bars) after 60 minutes efflux in the presence of varying concentrations of myricetin. The y-axis at the right displays the effects of increasing myricetin concentrations on MDCKII MRP1- (▲) or MRP2 (■) cell viability upon 24 hours incubation. Asterisks (*) represent statistically significant differences from the cells exposed to vehicle control (blank) ($P<0.05$). Data points represent the means ± SD from duplicate measurements.

Stability of vincristine

To determine whether vincristine does not become metabolized during the efflux experiments, HPLC radioactivity analysis was performed. The analyses showed that in all samples analyzed, only one major peak (>95% of the total radioactivity) was observed which was identified as vincristine itself. The presence of only one major peak in the HPLC-radioactivity chromatogram confirms that vincristine does not become metabolized during the time course of the efflux experiments.

Effects of myricetin on intracellular GSH levels.
The intracellular GSH levels differs for the three cell lines tested. Whereas the control cells contains 31.3 ± 0.8 nmol/monolayer and the MRP2 cells contain 27.4 ± 2.0 nmol/monolayer, the MRP1 cells contain approximately 10 times less GSH (3.6 ± 0.6 nmol/monolayer). Upon exposure to 25 μM myricetin, neither the GSH levels in the control cells nor in the MRP2 cells are affected. In contrast, in the MRP1 cells the GSH levels decrease with approximately 65% to 1.3 ± 01 nmol/monolayer when exposed to 25 μM myricetin for 1 hour.

Effects of myricetin on vincristine toxicity in transfected MDCKII cells.
The ability of myricetin to sensitize MRP1, or MRP2 transfected MDCKII cells to the cytotoxic effects of vincristine was tested by measuring cell proliferation.
Figure 4 shows the effects of 25 μM myricetin on vincristine sensitivity of control- (A,B) and MRP1 (A) or MRP2 (B) transfected MDCKII cells. Figure 4A reveals that the MDCKII MRP1 cells are less sensitive to vincristine toxicity than the MDCKII control cells, a phenomenon ascribed to the presence of MRP1. When the vincristine loaded MDCKII MRP1 cells are exposed to 25 μM myricetin the curve shifts to the left, demonstrating an increase of the sensitivity towards vincristine toxicity. Figure 4B shows that, upon comparison of the chemosensitivity of the MDCKII control cells and the MDCKII MRP2 cells, the MDCKII MRP2 cells also are less sensitive to vincristine toxicity, an observation ascribed to the presence of MRP2. However, this effect is less profound than for MRP1. When the vincristine loaded MDCKII MRP2 cells are exposed to 25 μM myricetin the curve also shifts to the left, again demonstrating an increase in sensitivity to vincristine toxicity. Addition of myricetin or the typical MRP inhibitors MK571 and cyclosporin A to vincristine loaded MDCKII control cells does not affect the vincristine chemosensitivity of these cells. Table 2 lists the IC_{50} values and the relative resistance factors, calculated as the relative ratios between IC_{50} values, derived from the various curves. These results show that MDCKII cells become 30- fold less sensitive to vincristine due to the presence of MRP1 and 20- fold less sensitive due to the presence of MRP2. Inhibition of MRP mediated vincristine efflux by 25 μM myricetin significantly reduces the resistance factor for both MRP1 and MRP2 to 7- and 5- fold respectively.

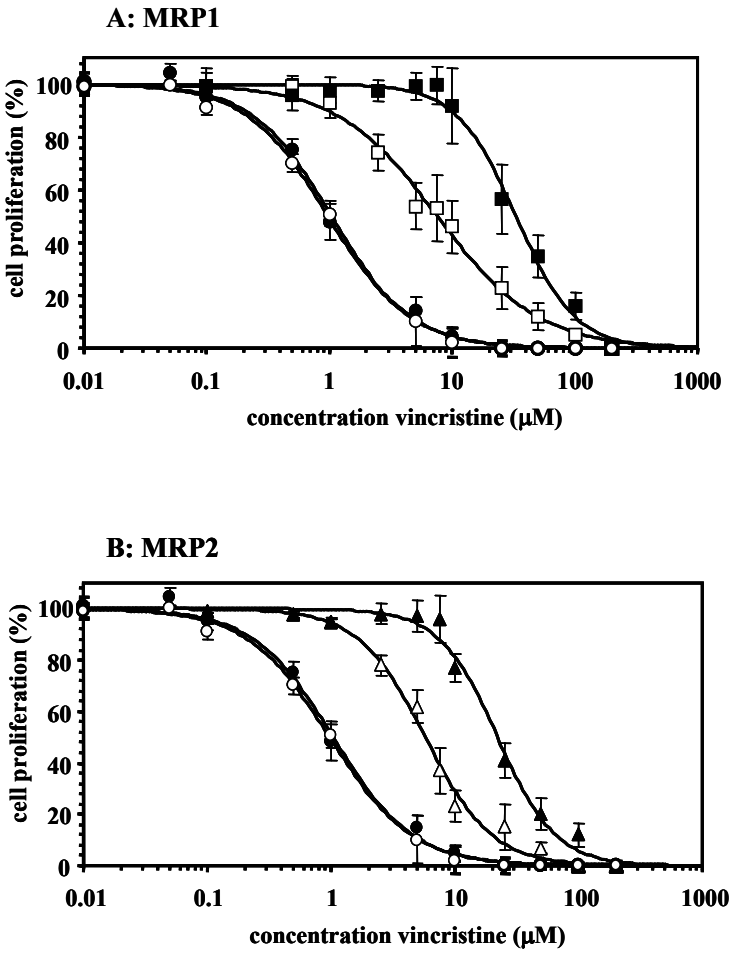

Figure 4. Effects of myricetin on vincristine sensitivity in (A) control and MRP1 or (B) control and MRP2 transfected MDCKII cells. Vector control transfected MDCKII cells (●,○), MRP1 transfected MDCKII cells (■,□) and MRP2 transfected MDCKII cells (▲,Δ) were incubated in the presence (○,□,Δ) or absence (●,■,▲) of 25 μM myricetin. Data points represent the means ± SD from triplicate measurements.

Table 2. IC50 values and relative resistance factors for vincristine toxicity in MDCKII control cells (control vector transfected), MDCKII MRP1 cells and MDCKII MRP2 cells in the absence (blank) or presence of 25 μM myricetin, 30 μM MK571 or 30 μM Cyclosporin A (CsA). Results are means ± SD from triplicate measurements.

		IC_{50} (μM)	relative resistance factor[a]
control	**blank**	1.1 ± 0.1	-
	myricetin	1.0 ± 0.2	0.9
MRP1	**blank**	33.1 ± 1.9	30
	myricetin	7.6 ± 0.5*	7
	MK571	5.1 ± 1.0*	5
MRP2	**blank**	22.2 ± 1.4	20
	myricetin	5.8 ± 0.5*	5
	CsA	5.2 ± 0.8*	5

[a] The relative resistance factor was calculated by dividing the IC_{50} value of cells transfected with MRP1 or MRP2 expression vectors by the IC_{50} values of cells transfected with the control vector (control cells) and exposed to vehicle control (blank)
* Statistically significant difference from IC_{50} value for the control cells exposed to vehicle control (blank) ($P<0.05$).

Discussion

Inhibitors of drug transporters, like MRP1 and MRP2, are potentially useful tools to reverse transporter-mediated cellular resistance to anticancer drugs and, eventually, to enhance the effectiveness of the treatment of patients with drug-resistant cancer. In this study we have tested the ability of the flavonoid myricetin to inhibit the efflux of the anticancer drug vincristine by two drug transporters, MRP1 and MRP2, in transfected MDCKII cells. In previous studies it was shown that myricetin is a suitable inhibitor of MRP1 and MRP2 activity [20, 22, 23]. However, in these studies it was also shown that the magnitude of MRP1 or MRP2 inhibition by myricetin might vary with the type of substrate as well as with the substrate- and inhibitor concentrations and the *in vitro* test system used. Therefore, the *in vivo* effects of myricetin on MRP-mediated vincristine resistance are not easily extrapolated from these *in vitro* studies. The present study examines the use of myricetin to reverse MRP-mediated vincristine resistance in an *in vitro* model. The results obtained show concentration dependent modulation of MRP1- and MRP2 mediated vincristine efflux in MDCKII cells by myricetin, reaching almost complete inhibition of the efflux at 50 μM

concentration. The IC_{50} values, representing the concentrations of myricetin at which 50% of the vincristine efflux is inhibited, are 30.5 ± 1.7 μM for MRP1- and 24.6 ± 1.3 μM for MRP2-MDCKII cells. These values are in the same order of magnitude as the IC_{50} values previously described for the inhibition of MRP1 and MRP2 mediated calcein efflux by myricetin of 20.2 ± 4.3 μM and 22.2 ± 3.9 μM respectively [23]. The mechanism involved in MRP-mediated vincristine efflux inhibition by flavonoids is not known although for calcein it was shown that the flavonoid robinetin inhibits calcein efflux by both MRP1 and MRP2 in a competitive way.

The observed decrease of the intracellular GSH amounts in the MRP1 cells upon exposure to 25 μM myricetin is most likely the result of increased GSH efflux by MRP1 rather than by a decrease of GSH synthesis since GSH levels in the other two cell lines were unaffected by myricetin. Increased efflux of GSH in the MRP1 cells upon exposure to myricetin is in accordance with a study by Leslie *et al.* (2003), who demonstrated that some flavonoids stimulate MRP1-mediated GSH transport by increasing the apparent affinity of the transporter for GSH, although no evidence was found that a co-transport mechanism is involved [32]. However, current opinion suggests that an increase of GSH efflux could be accompanied by an increase, not a decrease, in the efflux of vincristine, since vincristine efflux is under allosteric regulation by GSH [33]. The finding that the MRP1 cells contain approximately 10 times less GSH (3.6 ± 0.6 nmol/monolayer) than the control cells (31.3 ± 0.8 nmol/monolayer) and the MRP2 cells (27.4 ± 2.0 nmol/monolayer) are in accordance to previous findings by Wortelboer *et al*. (2004) for the same cells. The decrease of intracellular GSH levels in the MRP1 cells, upon expoure to myricetin was only observed for the MRP1 cells and not for the MRP2 cells or the control cells. Because the effects of myricetin on MRP1- and MRP2- mediated efflux of vincristine and the corresponding chemosensitizing effects are comparable for both cell lines, it is unlikely that enhanced GSH efflux is an explanatory mechanism for the results obtained. Apparently another, inhibitory mechanism, exists in which flavonoids most likely compete with the substrate at the substrate binding site. The inhibitory effects of flavonoids on the ATPase activity of MRPs might also be one of the mechanisms by which flavonoids inhibit MRP activity [20, 34]. The diverse effects of flavonoids on MRPs are confirmed by a study of Trompier *et al.* in which it was shown that the flavonoid dehydrosilybin and its derivatives interact with multiple binding sites of MRP1, located in both cytosolic and transmembrane domains of MRP1 [35]. From comparison of the vincristine sensitivity of the MDCKII control cells and the MRP1 or MRP2 transfected cells, it is concluded that these MRPs both decrease the cellular sensitivity to vincristine. This decrease in sensitivity represents the MRP mediated resistance towards vincristine. Incubation in the presence of myricetin resulted in an increase in the vincristine sensitivity of the MRP1 and MRP2 transfected MDCKII cells, although vincristine sensitivity is not increased to the level seen for the MDCKII control cells. The myricetin concentration used (25 μM) was shown not to completely inhibit vincristine efflux by MRP1 and MRP2, and this may in part explain the partial instead of full reversal of the vincristine sensitivity. Inhibition of MRP mediated vincristine efflux by 25 μM myricetin significantly reduces the resistance factor for both MRP1 and MRP2 to 7-

and 5- fold respectively. However, these changes in chemosensitivity of the MRP1- and MRP2 cells do not result in a sensitivity similar to that of the control cells. Interestingly, upon exposure to the typical MRP inhibitors MK571 or cyclosporin A that were shown to inhibit vincristine efflux almost to the maximal extent, the reduction of the resistance factor also does not reach the level observed for the control cells, reflecting that also in these MK571 or cyclosporin A exposed MRP cells the vincristine sensitivity does not reach the level of the control cells.

The data presented in Table 1 provide an explanation for this observation. Upon 2 hours loading of the cells, the amount of vincristine accumulated in the control cells appeared to be more than 40% higher than the vincristine levels accumulated in the two MRP transfected cell lines. Since the loading conditions in the cell proliferation assay were identical to the loading conditions used in the efflux assay, it can be concluded that in the cell proliferation assay a comparable loading difference between the control cells and the MRP cells has been present. This higher vincristine loading level in the control cells is likely to be due to the absence of vincristine efflux during the loading period. As a result IC_{50} values in the subsequent cell proliferation assay are lower for the control cells because at lower vincristine concentrations in the medium, higher intracellular vincristine levels are achieved. Since, due to the presence of the MRP protein, loading levels in the MRP cells are lower than in the control cells, intracellular vincristine levels in the MRP cells at a specific medium vincristine concentration will never be as high as those in the control cells. As a result, IC_{50} values obtained for the MRP cells will never be as low as those observed in the control cells, not even in the presence of more efficient inhibitors.

Flavonoids are substances that can interact with several different physiological pathways. Not only are they considered as good antioxidants, they can also exhibit anti-inflammatory, anti-tumor-, anti-thrombogenic- and anti-viral effects [36]. In theory, flavonoids might serve as potent inhibitors of MRP1 and MRP2 for potential clinical use to reverse multidrug resistance since they are generally regarded as safe and relatively non-toxic [37]. It can be argued that flavonoid usage in clinical settings might show negative side effects as a result of the inhibitory effects on other enzymes such as topoisomerases, cytochromes P450, protein kinases and other transporters like P-glycoprotein and ABCG2 (BCRP) [19, 38-40]. However, these possible effects need to be examined *in vivo* in further detail taking into account that increased plasma dosages as a result of supplementation of the diet are considered safe and relatively non-toxic [37]. An important factor that needs to be considered upon extrapolation of the results of the present study to the clinical situation is the fate and concentrations of myricetin in the human body. Elevation of plasma levels to levels approaching the 20-30 μM myricetin, observed in the present study to be active in modulating cellular vincristine sensitivity, might prove difficult if not impossible via oral supplementation due to the low oral bioavailability of flavonoids and the high first pass effect. A more promising option to increase myricetin plasma levels is via intravenous bolus injection. Human pharmacokinetic studies have demonstrated serum concentrations of the related flavonoid quercetin to range from 1 to 400 μM after a non-toxic i.v. dose of

quercetin with a half-life of 1-2 hr [37]; [41]. Therefore repeated i.v. doses or infusion seem to be the preferred way to administer myricetin as an adjunct to chemotherapy.
In conclusion, this study demonstrates the ability of the flavonoid myricetin to modulate MRP1- and MRP2 mediated resistance to the anticancer drug vincristine in transfected cells indicating that this flavonoid might be a valuable adjunct to chemotherapy to decrease MRP mediated resistance.

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6

Interaction of the dietary flavonoid myricetin with PGA_2-SG cellular excretion through inhibition of multidrug resistance proteins 1 and 2.

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submitted

Abstract

In this study the effects of the flavonoid myricetin, a known inhibitor of both MRP1 and MRP2, on the cellular MRP-mediated efflux of the prostaglandin A_2 glutathione conjugate (PGA_2-SG) was investigated. PGA_2 was chosen as a model compound to assess the effects of flavonoids on the cellular formation and excretion of glutathione conjugates of an endogenous compound. Since PGA_2 is known to inhibit cell cycle progression and to induce apoptosis, cell proliferation and apoptosis were used for evaluation of the effects of myricetin-mediated MRP inhibition of cellular efflux of PGA_2-SG. The efflux of PGA_2-SG by MRP1 and MRP2 from MRP1 and MRP2 transfected MDCKII cells was found to be moderately susceptible to inhibition by myricetin resulting in 23% and 13% inhibition respectively, at 25 μM myricetin. The typical MRP inhibitors MK571 and cyclosporin A resulted in a similar moderate inhibition of PGA_2-SG efflux up to 38%. Probably, the affinity of MRP1 and MRP2 towards these PGA_2-SG conjugates is high, thereby limiting potential effects of MRP inhibitors on their efflux. MRP1- and MRP2 transfected cells were less sensitive towards PGA_2 induced apoptosis, supporting an influence of MRP mediated extracellular transport of PGA_2-SG on the PGA_2 mediated cellular effects. Surprisingly, however, in MRP1 and MRP2 transfected cells the MRPs did not influence the PGA_2 mediated inhibition of cell proliferation. Altogether, this study reveals that myricetin supplementation is unlikely to affect MRP-mediated transport of PGA_2-SG conjugates to an extent at which myricetin is likely to influence the physiological effects of PGA_2. This suggests that flavonoid supplementation in general may not significantly affect MRP-mediated transport of endogenous or other GSH conjugates, in such a way that negative health effects, as a result of this inhibition, are to be expected. Together these results provide a first argument for the possible absence of specific negative side effects on the kinetics and physiology of endogenous MRP substrates, to be expected upon use of these natural MRP inhibitors in the reversal of multidrug resistance.

Introduction

An important mechanism in the cellular defence against exogenous and endogenous toxic compounds is the active transport of these chemicals to the extracellular matrix. ATP-binding cassette (ABC) transporters are known to play this central role in the defence of cells. The multidrug resistance proteins (MRPs) belong to this family and are known for their ability to extrude a wide variety of compounds including glutathione-, glucuronide- and sulphate conjugates, anticancer drugs, nucleotide analogues, heavy metals, organic anions and lipid analogues [1, 2]. MRP1 is mainly known as a glutathione conjugate (GS-X) pump, but also extrudes many more substrates. Some substrates of MRP1 like the oxyanions arsenite, antimonite, vincristine and daunorubicin are under allosteric regulation by glutathione [2]. MRP2 (ABCC2), the major canalicular Multispecific Organic Anion Transporter, is closely related to MRP1 [1, 3]. The spectrum of substrates transported by MRP1 and MRP2 overlap to a large extent, despite differences in cellular localization and kinetic properties. Among the conjugated endogenous substrates of MRPs are the glutathione conjugates of prostaglandins. The transport proteins of the MRP family (especially MRP1 and MRP2) are indicated to play a role in the transport of the glutathione conjugates of prostaglandins from cells [4, 5]. Recently, we demonstrated that flavonoids, a group of natural plant polyphenols, can inhibit both MRP1- and MRP2 activity [6-8]. Given the MRP inhibitory potency of flavonoids, showing IC_{50} values in the μM range [6-8], the intake of flavonoids via dietary supplementation might affect the kinetics of other food constituents, pharmaceuticals, xenobiotics or endogenous substrates of MRPs.

In this study, the effects of flavonoid mediated inhibition of MRP1 and MRP2 on the cellular transport of an endogenous model compound, namely the glutathione conjugate of prostaglandin A_2 (PGA_2-SG) were investigated in order to assess the effects of flavonoids on the cellular formation, excretion and cellular effects of glutathione conjugates of an endogenous compound. Myricetin (Figure 1) was used as a model flavonoid since it is a potent inhibitor of both MRP1 and MRP2 [6]. MDCKII cells, transfected with either MRP1 or MRP2 were used as model systems. Since PGA_2 is known to inhibit cell cycle progression and to induce apoptosis, cell proliferation and apoptosis were used for evaluation of the effects of MRP inhibition on PGA_2 mediated cellular effects [9-11]. Ultimately, this study tries to contribute to the evaluation of the possible adverse health effects of MRP inhibition by high doses of dietary flavonoids.

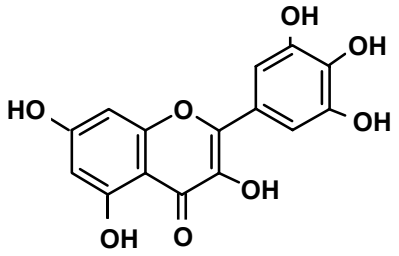

Figure 1. Structural formula of myricetin.

Materials and Methods

Materials.
Dulbecco's Minimum Eagle Medium (DMEM) with GlutaMax, fetal bovine serum, penicillin/streptomycin and gentamycin were all purchased from Gibco, (Paisley, Scotland). MK571 was obtained from BioMol (Plymouth Meeting, PA), PSC833 was a kind gift from Novartis Pharma AG (Basel, Switzerland). Cyclosporin A was obtained from Fluka (Zwijndrecht, The Netherlands). [^{3}H]-prostaglandin E_2 (PGE_2; 185 Ci/mmol) was obtained from Amersham Biosciences (Buckinghamshire, UK). Flo-Scint scintillation cocktail was purchased from Packard (Groningen, The Netherlands). The cell proliferation ELISA BrdU-kit was purchased from Roche Molecular Biochemicals (Mannheim, Germany). DMSO and HPLC-grade methanol were obtained from Acros Organics (Geel, Belgium). The fluorescent caspase 3 substrate Ac-DEVD-AMC (Ac-Asp-Glu-Val-Asp-AMC) and the caspase 3 inhibitor Ac-DEVD-CHO (Ac-Asp-Glu-Val-Asp-CHO) were obtained from Alexis Biochemicals (Breda, The Netherlands). Myricetin and all standard chemical were purchased from Sigma Chemical Co. (Zwijndrecht, The Netherlands) unless stated otherwise.
[^{3}H]-prostaglandin A_2 (PGA_2) was prepared from [^{3}H]-PGE_2 by acid-catalyzed dehydration using the method adapted from [12]. In short: [^{3}H]-PGE_2 was incubated with 1 ml water-acetic acid-85% H_3PO_4 (10:3:2) for 48 hours at room temperature. After the incubation, 10 ml of water was added to the reaction mixture followed by extraction with three 8 ml portions of di-isopropylether. The collected ether extracts were subsequently washed with two 10 ml portions of saturated aqueous sodium chloride and dried over anhydrous Na_2SO_4. Finally, the ether was evaporated under a gentle stream of nitrogen gas and the residue containing [^{3}H]-PGA_2 was dissolved in ethanol. HPLC analysis with on-line radioactivity detection was performed according to Evers *et al.* [4]. To determine the purity of the synthesized [^{3}H]-PGA_2, analysis was carried out using a Merck Hitachi HPLC system equipped with a L6200 pump and a L4200 UV-Vis detector combined with a Packard Flo-One on-line radioactivity detector using Flo-Scint as scintillation cocktail. Of the test sample, 50 µl was injected onto a Zorbax Eclipse XDB-C_{18} 250 mm x 4.6 mm column. The column was eluted isocratically at a flow rate of 1.0 ml/min at 75% 50 mM NH_4Ac pH 3.4 and 25 % acetonitrile for 30 minutes followed by a linear gradient to 50 % acetonitrile in 30 min. The purity of all [^{3}H]-PGA_2 used for further experiments was above 95%.

MDCKII cell culture.
The Madin-Darby Canine Kidney (MDCKII) cell lines, stably expressing either a control vector (hereafter called control cells), human *MRP1* cDNA (hereafter called MRP1 cells) or human *MRP2* cDNA (hereafter called MRP2 cells) were kindly provided by Prof. P. Borst (NKI, Amsterdam).
The cell lines (control and MRP1 or MRP2 transfected) were cultured in Dulbecco's Minimum Essential Medium (DMEM) with GlutaMax (4.5 g glucose/L), 10% fetal calf

serum and 0.01% penicillin/streptomycin, and were grown in a humidified atmosphere in 5% CO_2 at 37°C.

Directional transport assays.

For transport experiments $4*10^5$ cells/cm^2 were grown on microporous polycarbonate filters ((0.4 μm pore size, 4.5 cm^2) Costar Corp. Cambridge, MA). The volume of media in the basolateral and apical compartments was 1.8 and 0.5 ml, respectively. Cells were cultured to confluency for three days and medium was replaced every 24 h. Confluency of the monolayers was checked by transepithelial electric resistance (TEER) measurement, validated by determination of the paracellular flux of inulin[^{14}C]carboxylic acid (185 kBq/mol, 4.2 μM) [13]. TEER-values of each monolayer were measured using a Millicell-ERS epithelial volt/ohm meter (Millipore, Bedford). The TEER-value of a confluent monolayer of MDCKII cells ranged between 120-140 Ω.cm^2 as reported before [14]. After three days, the cells were washed with Hanks' balanced salt solution (HBSS) before exposure. The exposure medium for the donor side, consisted of DMEM, without FCS or phenol red, containing 0.1 μM PSC833 (to exclude any possible effects of P-glycoprotein), 1 μM PGA_2, of which 2 nM (0.09 kBq) [^{3}H]-PGA_2 and the test compound. The tranport medium, for the receiver side, consisted of DMEM, without FCS or phenol red, containing 0.1 μM PSC833 and the test compound. Volumes used were 1 ml for the apical side and 2 ml for the basolateral side. The use of the cyclosporin derivative PSC833 as a Pgp inhibitor was used based on studies by Evers *et al.* (1998) [24]. A relatively low concentration of PSC833 (0.1 μM) was shown to completely inhibits apical efflux of vinblastine from the control cells, whereas the vinblastine efflux by MRP2 in the MRP2 cells appeared not to be affected by this dose [24]. For the MRP1 cells, the exposure medium was added to the apical side (donor side) and the transport medium was added to the basolateral side to study apical to basolateral transport (receiver side). For the MRP2 cells, the exposure medium was added to the basolateral side and the transport medium was added to the apical side in order to study basolateral to apical transport. For the control cells, both apical to basolateral- as well as basolateral to apical transport was studied. Once inside the cells, prostaglandin A_2 can become conjugated to GSH both chemically and enzymatically [15-17]. Efflux of PGA_2-SG to the apical and basolateral side of the MRP-transfected MDCKII cells was monitored. The MRP inhibitors used, added to both apical and basolateral compartments, were 25 μM myricetin, 30 μM MK571 (as a typical MRP1 inhibitor) or 30 μM cyclosporin A (as a typical MRP2 inhibitor) [18, 19]. Cells receiving vehicle only (0.5% DMSO v/v) served as control (blank). Transport of PGA_2-SG by the MDCKII cells was measured by taking 200 μl samples at various time-points (up to 4 hours) from the receiver side of each well. The volume loss was immediately compensated by adding 200 μl fresh transport medium to the wells. The samples were immediately acidified by the addition of 100 μl 4% (v/v) formic acid. Subsequently, samples were extracted twice with 300 μl ethyl acetate to remove radioactivity that is not related to PGA_2-SG and radioactivity in 200 μl of the water phase, containing PGA_2-SG, was determined by liquid scintillation. After 4 hours, medium from both the donor- and receiver side was taken for analysis and

the cells were washed three times in ice-cold PBS. The filters containing the cells were taken out of the inserts and transferred to 1 ml PBS with 2% formic acid. Subsequently, samples were sonicated and 200 µl of the cell samples were extracted twice with 300 µl ethyl acetate and radioactivity in 200 µl of both phases was determined by liquid scintillation. Radioactivity was counted using a Packard 1600 Liquid Scintilator with Packard Ultima Gold as scintillation cocktail (Packard, Groningen, The Netherlands). The efficiency of the ethyl acetate extraction was measured according to the method described Evers *et al.* [4]. The ethyl acetate and water fractions were analysed using the HPLC analysis with on-line radioactivity detection essentially as described above. In the water phase, all radioactivity was accounted for by PGA_2-SG. In the ethyl acetate phase all radioactivity was accounted for by PGA_2 (data not shown).

Cell proliferation.
The effect of MRPs on the inhibition of MDCKII cell proliferation by PGA_2 was determined using the BrdU cell proliferation assay, adapted for transwells using the Cell Proliferation ELISA, BrdU (colorimetric) kit from Roche Diagnostics (Mannheim, Germany). For the assay 10^5 cells/cm^2 were grown on microporous polycarbonate filters ((0.4 µm pore size, 0.33 cm^2) Costar Corp. Cambridge, MA). The volume of media in the basolateral and apical compartments was 0.6 and 0.1 ml respectively. After 24 hours, the cells were exposed to a range of PGA_2 concentrations (0-200 µM) in DMEM without phenol red containing 0.1 µM PSC833, for 24 hours at 37^{o}C. After this exposure, cells were rinsed twice with DMEM without phenol red and cell proliferation was determined by labeling the cells with BrdU for two hours at 37^{o}C. The conversion of the substrate tetramethyl-benzidine was used as the quantitative measurement for cell proliferation. Absorption was measured at 370 nm using a Thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA USA). Results were expressed as percentage cell proliferation compared to the blank (vehicle only).

Apoptosis.
The effect of MRPs on the induction of apoptosis in MDCKII cells by PGA_2 was determined by measuring the activity of caspase 3, an enzyme shown to play a crucial role in apoptosis [20, 21]. For the assay $4*10^5$ cells/cm^2 were grown on microporous polycarbonate filters ((0.4 µm pore size, 1.0 cm^2) Costar Corp. Cambridge, MA). The volume of media in the basolateral and apical compartments was 1.8 and 0.5 ml, respectively. After 24 hours, the cells were exposed to a range of PGA_2 concentrations (0-70 µM) in DMEM without phenol red containing 0.1 µM PSC833, in both compartments for 8 hours at 37^{o}C. Validation and optimization showed that exposure of the three MDCKII cell lines to positive controls, like actinomycin D (4 µM), camptothecin (5 µM), and PGA_2 for 8-10 hours resulted in optimal induction of caspase-3 activity, longer or shorter incubation periods resulted in lower activities (data not shown). To study the effects of MRP inhibition on apoptosis induction by PGA_2, the inhibitors studied were co-administered with the PGA_2. The inhibitors used were 0-70 µM myricetin, 30 µM MK571

or 30 μM cyclosporin A. After the 8 hours exposure, filters containing the cells were rinsed twice with DMEM without phenol red and removed from the inserts and put in 200 μl caspase 3 assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose at pH 7.2). Subsequently samples were sonicated and centrifuged at 13000 rpm for 10 minutes. Of each cell sample two 45 μl samples were incubated with the fluorescent caspase 3 substrate: Ac-DEVD-AMC (f.c. 0.2 mM) for two hours at 37^{o}C. Fluorescence readings were taken every 15 minutes for two hours using a Varian Cary Eclipse (Varian, Bergen op Zoom, The Netherlands) at excitation 380 nm and emission 440 nm. The results obtained were corrected for the amount of cells in a sample by determining the protein content of the sample using Bradford protein analysis [22]. To verify that the measured fluorescence was the result of caspase 3 activity some of the samples were incubated with the caspase 3 substrate in the presence of 20 μM of the caspase 3 inhibitor Ac-DEVD-CHO.

Data analysis.

A one-way analysis of variance test was used for all data analysis ($P<0.05$) using SPSS 10.1.0 software from SPSS Inc.

Results

PGA_2, PGA_2-SG and GSH levels in the model MDCKII cells.
Table 1 presents the levels of cellular PGA_2 in PGA_2 exposed control and MRP1 and MRP2 transfected cells and reveals that MRP transfected PGA_2 exposed cells contain significantly less native PGA_2 than the mock transfected cells (control cells). This might point at higher PGA_2 uptake in the control cells or higher efflux of PGA_2 by MRP1 and MRP2 in the MRP1 and MRP2 cells. Table 1 also presents total PGA_2-SG amounts and reveals that upon PGA_2 exposure the total PGA_2-SG levels were slightly different for the three cell lines tested. The MRP1 cells produced lower total PGA_2-SG amounts (0.16 ± 0.01 nmol) at the end of the experiment (4 hours) than the control cells (0.20 ± 0.01 nmol) or the MRP2 cells (0.21 ± 0.01 nmol). Table 1 also presents the relative activities of GSTs and the GSH levels in the three cell lines tested. Based on these data it can be argued that the somewhat lower total PGA_2-SG amounts in the MRP1 incubations can most likely be ascribed to the significant lower GSH levels in the MRP1 cells compared to the other two cell lines [13].

Table 1. PGA2, GSH and total PGA2-SG amounts and GST activities in the control, MRP1 and MRP2 cells following incubation with [3H]-PGA2 for 4 hours.

	Control cells	**MRP1 cells**	**MRP2 cells**
Intracellular PGA_2 (nmol/monolayer)	18.4 ± 6.5 (*10^{-3})	0.5 ± 0.2* (*10^{-3})	0.4 ± 0.1* (*10^{-3})
GST (cell lysate) [a] (nmol/min/mg protein)	44.3 ± 4.2	32.5 ± 3.1*	22.9 ± 2.5*
GSH (cell lysate) [a] (nmol/mg protein)	29.1 ± 1.3	2.5 ± 0.01*	34.4 ± 0.5*
PGA_2-SG total [b] (nmol)	0.20 ± 0.01	0.16 ± 0.01*	0.21 ± 0.01

a Data taken from Wortelboer *et al.* (2003) to indicate the relative proportions of GSTs and GSH levels in the three cell lines tested. Data are the means ± SD of three incubations of a typical experiment.
b Total amounts consists of intracellular + apical + basolateral PGA2-SG levels per well.
* Differ significantly from the corresponding value in control cells ($P<0.05$).

Directional transport of PGA_2-SG in the three types of MDCKII cells.
To assess the efflux of PGA_2-SG by the MRPs, 4 hours efflux studies were performed, the results of which are presented in Figure 2. The transport of PGA_2-SG was linear in time during the 4 hours sampling time for all three cell lines. For MRP1 cells, higher PGA_2-SG transport rates to the basolateral direction were observed (130.0 ± 9.4 pmol/ 4 h) than in the control cells (99.7 ± 7.3 pmol/ 4 h). In the MRP2 cells, higher PGA_2-SG transport rates to the apical direction were observed (160.3 ± 14.0 pmol/ 4 h) compared to the control cells (62.6 ± 9.3 pmol/ 4 h). The increase in basolateral PGA_2-SG transport in MRP1 cells, compared to the control cells, was accompanied by a reduced PGA_2-SG transport to the

apical side of the MRP1 cells resulting in approximately 4 times more basolateral than apical PGA_2-SG efflux as compared to a ratio of approximately 2 in the control cells. Similar results were observed for the MRP2 cells, resulting in approximately 3 times more apical than basolateral PGA_2-SG efflux. These results confirm the role of MRP1 and MRP2 in PGA_2-SG transport.

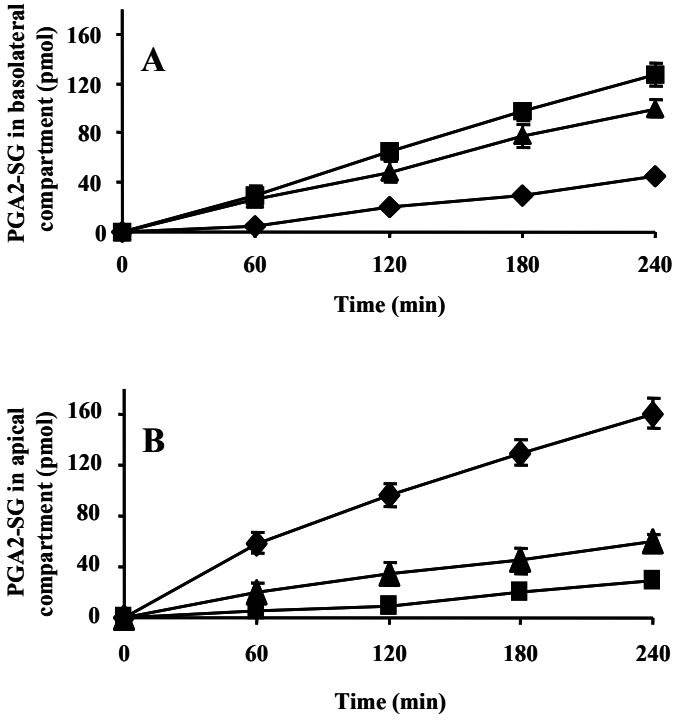

Figure 2. (A) Apical to basolateral transport of PGA_2-SG by MRP1 (■), MRP2 (◆) and control cells (▲); and (B) basolateral to apical transport of PGA_2-SG by MRP1 (■), MRP2 (◆) and control cells (▲). Data points represent the means ± SD from triplicate measurements.

Inhibition of PGA_2-SG transport by myricetin and model inhibitors.

Figure 3 shows the effects of the inhibitors tested, 25 μM myricetin, 30 μM cyclosporin A and 30 μM MK571, on PGA_2-SG formation and compartmental PGA_2-SG distribution in the control cells, MRP1 cells and MRP2 cells upon 4 hours incubation. Figure 3A shows the PGA_2-SG amounts per compartment upon apical [^{3}H]-PGA_2 exposure. Myricetin significantly reduced the MRP1-mediated basolateral PGA_2-SG transport by approximately 23% ($P<0.05$). The typical MRP1 inhibitor MK571 reduced the MRP1 mediated PGA_2-SG transport by 38% ($P<0.05$). Similar results were obtained for the apical directed PGA_2-SG transport by MRP2 (Figure 3B). Here, myricetin reduced the MRP2-mediated apical PGA_2-SG transport slightly, yet significantly, by 13% ($P<0.05$) whereas the typical MRP2 inhibitor cyclosporin A reduced the efflux by 19% ($P<0.05$). From these results it seems that the transport of PGA_2-SG is moderately inhibited by myricetin and the typical MRP inhibitors MK571 (MRP1) and cyclosporin A (MRP2). Furthermore, MRP1 is only a bit more susceptible to transport inhibition by myricetin than MRP2. For all cells, it was observed that the typical MRP inhibitors MK571 and cyclosporin A reduced the PGA_2-SG transport somewhat stronger than 25 μM myricetin, although the inhibitory effects were still moderate. Interestingly, the results obtained for the control cells reveal that these cells contain reasonable amounts of endogenous GS-X pumps, resulting in significant PGA_2-SG efflux at levels that are approximately 1.5 times higher for the basolateral side than for the apical side ($P<0.05$). This basolateral efflux was also moderately susceptible to inhibition by MK571 (Fig. 3A). Apparently, the presence of the MRPs increases the overall PGA_2-SG transport so that, upon intracellular formation, PGA_2-SG is immediately transported to the extracellular compartments resulting in reduced intracellular accumulation and increased levels at the respective extracellular sides. Inhibition of the directional PGA_2-SG transport in the MRP1 cells by the typical MRP inhibitor MK571 resulted in a marginal, yet significant, increase of the intracellular amounts of PGA_2-SG accompanied by a decrease of the total amount of PGA_2-SG formed ($P<0.05$). The intracellular PGA_2 amounts did not change upon incubation of the cells with the inhibitors tested (data not shown).

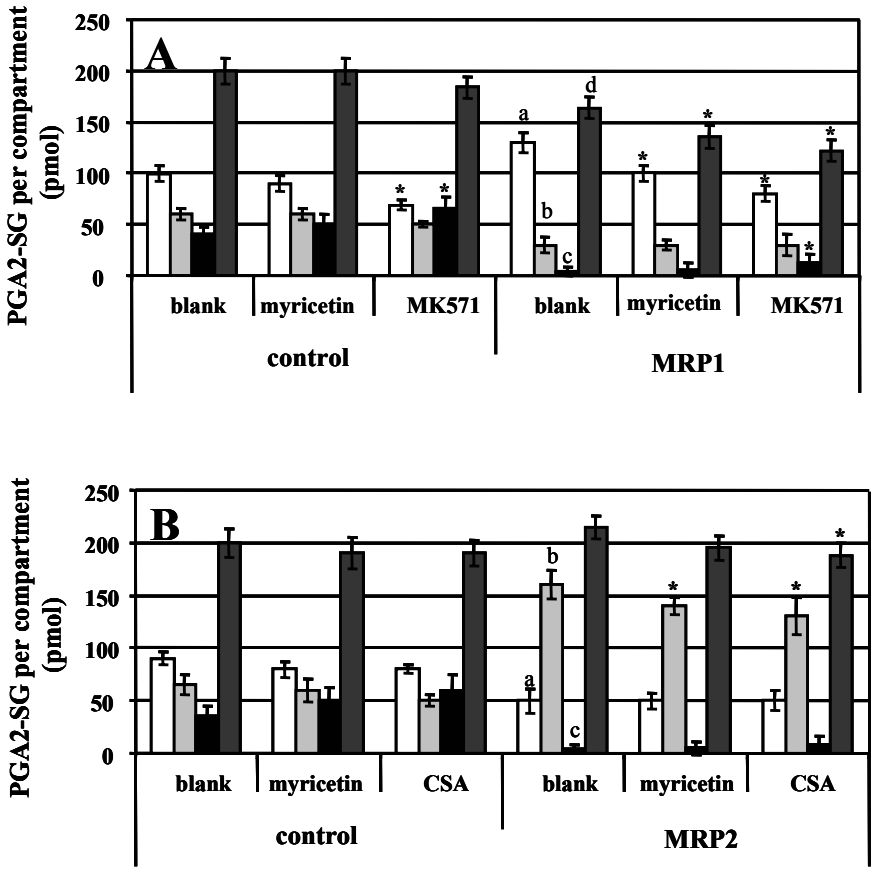

Figure 3. Effects of 25 μM myricetin and of the typical MRP inhibitors MK571 (30 μM) and cyclosporin A (CSA; 30 μM) on the formation and distribution of PGA_2-SG by control, MRP1 and MRP2 cells. Results present PGA_2-SG amounts (pmol/monolayer) in the basolateral compartment (first bar), apical compartment (second bar) and intracellular (third bar). Additionally, the resulting total formation of PGA_2-SG is presented (fourth bar). Figure 3A represents the apical PGA_2 exposed control cells and MRP1 cells. Figure 3B represents the basolateral exposed PGA_2 control cells and MRP2 cells. Each bar represents means ± SD of incubations performed in triplicate. *a,b,c,d* differ significantly from corresponding bar in blank exposed control cells ($P<0.05$),* differs significantly from corresponding bar in blank ($P<0.05$).

Cell proliferation.

To investigate the role of MRPs on the physiological effects of PGA_2 in more detail, the effects of PGA_2 on cell proliferation was determined for all three cell lines. Figure 4 shows the effects of increasing PGA_2 concentrations on the proliferation of the three cell lines, and reveals that the presence of MRP1 or MRP2 does not influence the effects of PGA_2 on cell

proliferation. Apparently, MRP1 and MRP2 do not protect the MDCKII cells against the cytotoxic action of PGA_2 as measured by cell proliferation.

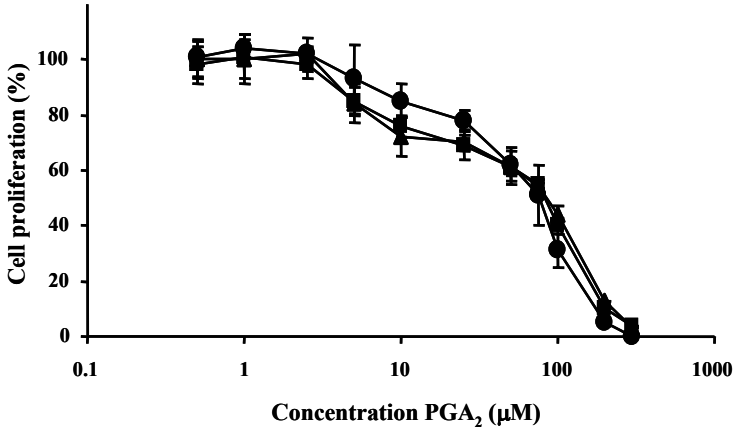

Figure 4. Effects of increasing PGA_2 concentrations on the proliferation of the three cell lines as measured by the BrdU assay: control cells (●), MRP1 cells (■) and MRP2 cells (▲). Data points represent the means ± SD from triplicate measurements.

Apoptosis.

The second physiological assay performed in this study involved the induction of apoptosis by PGA_2. Figure 5 shows the effects of several different PGA_2 concentrations on caspase 3 induction, a key enzyme in apoptosis, for the three cell lines. These results reveal that PGA_2 exposure can result in increased caspase 3 activity. At 50 µM PGA_2 only the control cells, both with and without exposure to 25 µM myricetin, showed an increase in apoptotic activity. Interestingly, also the MRP2 cells showed an increased apoptotic activity upon exposure to 50 µM PGA_2 in combination with myricetin. Control incubations with exposure to only 25 µM myricetin revealed no changes in apoptotic activity for all cell lines (not shown). At 70 µM PGA_2, all cell lines have an increased induction of caspase 3 activity compared to the blank exposure. At this PGA_2 concentration, the caspase 3 activity is significantly more induced in the control cells than in the MRP1 and MRP2 cells. Apparently, MRP1 and MRP2 protect the MDCKII cells against PGA_2 induced apoptosis. Higher PGA_2 concentrations, above 70 µM (100 µM), were cytotoxic to the cells (>10% death) and could therefore not be tested for their effects on apoptosis. For the control cells an effect of myricetin can be observed opposite to what is observed for the MRP2 cells at 50 µM PGA_2. In control cells myricetin addition results in a decrease, rather than an increase, in apoptotic activity. Thus, in these control MDCKII cells, myricetin has a

protective effect regarding apoptosis induction upon high concentration PGA_2 exposure. Like myricetin, neither 30 μM MK571, nor 30 μM cyclosporin A exposure in control incubations without PGA2 resulted in an increase of the apoptotic activity of the MRP1 or MRP2 cells (not shown).

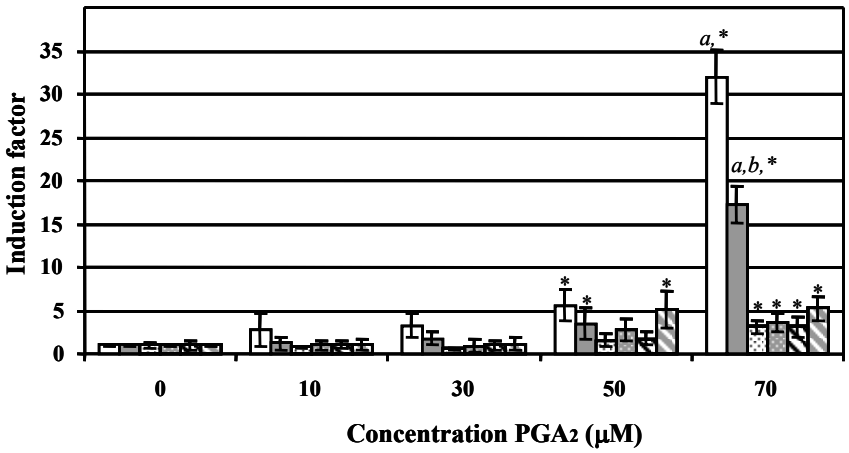

Figure 5. Effects of myricetin on the induction of caspase 3 activity by PGA_2 in control cells (first two bars), MRP1 cells (third and fourth bar) and MRP2 cells (fifth and sixth bar) that were incubated in the absence (first, third and fifth bar) or presence (second, fourth and sixth bar) of 25 μM myricetin. Data points represent the means ± SD from triplicate measurements. * Significantly higher induction factor than corresponding bar in blank exposure ($P<0.05$). *a* Significantly higher induction factor than MRP1 or MRP2 cells at 70 μM PGA_2 ($P<0.05$). *b* Significantly lower induction factor than control cells at 70 μM PGA_2 without exposure to 25 μM myricetin ($P<0.05$)

Discussion

The present study demonstrates that both MRP1 and MRP2 are able to transport glutathione conjugates of prostaglandin A_2 from cells. The directional transport in the MRP cells is approximately 1.5 times higher than in the control cells and maximal 4 times higher than the non-directional efflux. The limited effect of the introduced MRPs is in accordance with Evers *et al.* [4] who found that the directional (basolateral) transport of PGA_2-SG in MDCKII MRP1 cells was approximately 2 times higher than in the control cells and maximal 5 times higher than the non-directional (apical) efflux. As a result of the more efficient PGA_2-SG efflux by the MRP1 and MRP2 cells, the intracellular amounts of both

PGA_2-SG and PGA_2 in these cells are respectively ~10 and ~40 fold lower than in the control cells (Figure 3, Table 1).

The maximal 23% inhibition of PGA_2-SG efflux by myricetin found in this study is very moderate. In previous studies it was shown that in the same MRP-transfected MDCKII cells 25 μM of the flavonoid myricetin inhibits the MRP1 mediated basolateral transport of DNP-SG (10 μM) by approximately 40%, of calcein (1 μM) by 63% and of vincristine (0.5 μM) by 52%, and the MRP2 mediated apical transport of calcein by 68% and of vincristine by 41% [6-8]. Apparently, PGA_2-SG is a high affinity MRP1- and MRP2-substrate which is in line with literature that reports all glutathione-conjugates to be favorable substrates as compared to for example. organic anions, in terms of affinity [23]. Evers *et al.* determined the Km of MRP1 for PGA_2-SG at 1 μM. Since the PGA_2 concentration used in the present study was 1 μM, the conditions used were not saturating and optimal for inhibition studies [4]. Furthermore it was found that the typical MRP inhibitors MK571 and cyclosporin A also only moderately inhibited PGA_2-SG efflux (maximal 38%), again demonstrating that the MRP-mediated efflux of PGA_2-SG is not very susceptible for inhibition. Given the high-affinity of these MRPs for these PGA_2-SG conjugates [4], more drastic inhibition of PGA_2-SG transport is not expected at non-toxic flavonoid concentrations.

The decrease of the intracellular PGA_2-SG and especially the PGA_2 amounts, as a result of the efficient PGA_2-SG transport by MRP1 and MRP2, may have an effect on cells, especially regarding the biological action of PGA_2. In this study, we found that MRP1 and MRP2 have a protective effect regarding the induction of apoptosis by PGA_2, although moderate inhibition of MRP activity did not result in altered sensitivity of the cells towards PGA_2. The protective effect of myricetin regarding induction of caspase 3 activity in the control cells upon high (70 μM) PGA_2 exposure is most likely the result of its antioxidant activity. Moreover, the MRPs did not affect the action of PGA_2 on cell proliferation. It is believed that conjugation of prostaglandins with GSH may result in inhibition of their mode of action [24, 25], although for other glutathione conjugates of oxyeicosanoids (like LTC_4) this is not the case [17]. The results in this study suggest that PGA_2-SG is probably not a biological active compound regarding cell proliferation and induction of apoptosis. Especially for the apoptosis experiments, the main factor of importance seems to be the unconjugated prostaglandin PGA_2. Inhibition of the MRP-mediated PGA_2-SG efflux by the flavonoid myricetin did not affect the chemosensitivity, in terms of cell proliferation and apoptosis, of the MDCKII cells towards PGA_2. Even the typical MRP inhibitors MK571 and cyclosporin A did not affect these parameters tested. This can be explained by the marginal increase of the intracellular PGA_2-SG amounts upon inhibition of MRP activity and the absence of changes in the intracellular native PGA_2 amounts (Figure 3). For another substrate, the anticancer drug vincristine, we have previously demonstrated that inhibition of MRP-mediated transport resulted in a mild increase in the intracellular vincristine amounts, and a significant increase in inhibition of cell proliferation [8].

The increasing intake of extreme doses of flavonoids via dietary supplementation might disturb physiological processes. Especially in the intestine, where transporter proteins including MRP1 and MRP2 play an important role in the uptake and transport of

compounds, high flavonoid concentrations can be expected upon supplementation. However, this study reveals that myricetin supplementation is unlikely to affect MRP-mediated transport of PGA_2-SG conjugates and suggests that flavonoid supplementation in general may not significantly affect MRP-mediated transport of this and other types of endogenous or other SG conjugates, in such a way that negative health effects, as a result of this inhibition, are to be expected. Together these results provide an argument for the possible absence of specific negative side effects on the kinetics and physiology of endogenous MRP substrates, to be expected upon use of these natural MRP inhibitors in the reversal of multidrug resistance.

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The effect of quercetin phase II metabolism on its MRP1 and MRP2 inhibiting potential.

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submitted

Abstract

The present study characterises the effect of phase II metabolism, especially methylation and glucuronidation, of the model flavonoid quercetin on its capacity to inhibit MRP1 and MRP2 activity in model inside-out vesicles. This was done because flavonoids, including quercetin, appeared to be promising agents to revert MRP-mediated multidrug resistance in studies using the flavonoid aglycone, whereas phase II metabolism of the aglycone occurs to a significant extent *in vivo.* The results obtained reveal that methylation of the catechol moiety does not affect the MRP inhibitory potential of quercetin, with the exception of 4'-O-methylation that appeared to reduce the potential to inhibit MRP2. In contrast, glucuronidation in general, and especially glucuronidation at the 7-hydroxylmoiety, resulting in 7-O-glucuronosyl quercetin, significantly increased the potential of quercetin to inhibit MRP1 and MRP2 mediated calcein transport. The potential of the glucuronide metabolites to inhibit MRP1 and MRP2 is in line with the fact that glucuronides are known to be common substrates of MRPs.

Overall, the results of this study reveal that the major phase II metabolites of quercetin are equally potent or even better inhibitors of MRP1 and MRP2**,** indicating that phase II metabolism is unlikely to reduce the potential for use of quercetin as an inhibitor to overcome MRP-mediated multidrug resistance.

Introduction

Multidrug resistance may hamper the efficacy of cytostatic drugs in cancer treatment [1]. One of the mechanisms involved in cellular multidrug resistance is upregulation of efflux proteins like P-glycoprotein and members of the multidrug resistance proteins (MRPs) [2]. MRP1 (ABCC1) has a broad substrate specificity and among its substrates are glutathione S-conjugates, glucuronide conjugates, sulfate conjugates, anticancer drugs and organic anions [3-6]. MRP2, the major canalicular Multispecific Organic Anion Transporter, is closely related to MRP1 and also has a broad substrate specificity [2, 7]. One strategy to overcome transporter-mediated multidrug resistance relies on the identification of compounds that can act as inhibitors of these transporters. Flavonoids are an example of promising agents to revert MRP-mediated multidrug resistance [8-13]. However, these studies were all performed with the flavonoid aglycone under experimental conditions which do not allow extensive evaluation of the effects of human metabolism of flavonoids, expected to occur to a significant extent *in vivo* [14], on their MRP1 and MRP2 inhibitory capacity. Phase II metabolism of flavonoids is a generally recognized determinant of their biological activities and may also influence their interaction with MRPs [14-17]. Naturally occurring flavonoids in plants are glycosylated [18], but may become deconjugated during passage across the small intestine [19] or by bacterial activity in the colon [20]. Studies on the bioavailability of quercetin revealed the metabolism of quercetin to methylated, glucuronidated and/or sulphated conjugates [21-24]. Figure 1 presents an overview of the type of phase II reactions reported to be relevant for the flavonoid quercetin and their regioselectivity. The plasma phase II metabolite pattern is likely to be the result of the interplay of different organs with metabolizing capacity, especially the liver and the small intestine [24].

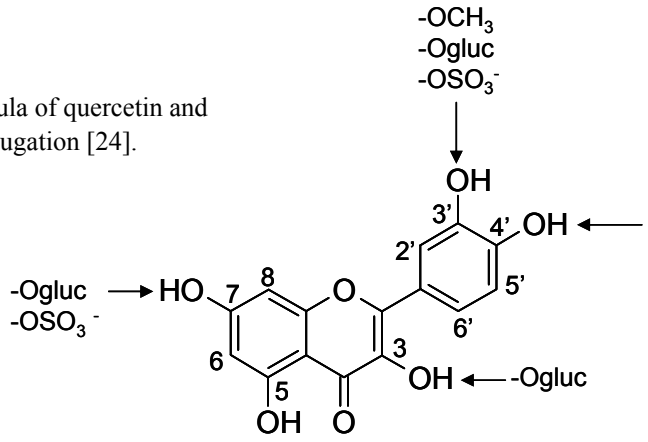

Figure 1. Structural formula of quercetin and the identified sites of conjugation [24].

The objective of the present study was to investigate the effect of phase II metabolism of the model flavonoid quercetin on its capacity to inhibit MRP1 and MRP2. To this end, the effects of phase II metabolism of quercetin on its MRP1 and MRP2 inhibitory potency was studied using inside-out vesicles. Quercetin was used as a model flavonoid since the phase II metabolism of this flavonoid is well described [21-24] and it is a well described inhibitor of MRP1 and MRP2 [12]. The effect of quercetin phase II metabolism on its ability to inhibit MRP1 and MRP2 in inside-out vesicles was studied using model quercetin metabolites as well as characterised quercetin metabolite mixtures produced by incubation of quercetin with specific metabolising cell lines [24]. Together the data reveal how phase II metabolism of quercetin influences its MRP1 and MRP2 inhibiting potential.

Materials and Methods

Materials.

Quercetin was obtained from Acros Organics (New Jersey, USA). 3'-O-methylquercetin (isorhamnetin) and 4'-O-methylquercetin (tamarixetin) were purchased from Extrasynthese (Genay Cedex, France). Rutin (quercetin-3-O-rhamnosylglucoside) and isoquercitrin (quercetin-3-O-ß-glucoside) were obtained from Indofine (Somerville, USA). Dimethylsulfoxide (DMSO) was obtained from Sigma (St. Louis, MO, USA). HPLC-grade acetonitril was purchased from Lab-Scan Ltd. (Dublin, Ireland). Fetal calf serum, Dulbecco's MEM, Dulbecco's MEM/F12 NutMix (HAM), fungizone, gentamycin and Hank's Balanced Salt Solution (HBSS) were purchased from Gibco Ltd Life Technologies (Paisley, UK). Calcein, adenosine-5'-triphosphate-disodium salt (ATP), adenosine 5'-monophosphate-sodium salt (AMP), creatine phosphate and DL-dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO, USA). Creatine kinase was purchased from Roche (Almere, The Netherlands) and $MgCl_2$.hexahydrate from Merck.

Cell lines.

The human cell line HT29 (colon carcinoma) and the rat cell line H4IIE (hepatocellular carcinoma) were purchased from the European Collection of Cell Cultures (ECACC). Both cell lines were grown in 75 cm^2 plastic cell culture flasks in MEM-alpha medium supplemented with 10% fetal calf serum, 1% fungizone and 0.1% gentamicin.

HPLC Analysis. HPLC was performed on a Waters M600 liquid chromatography system, using an Alltima C18 5U column (4.6 mm × 150 mm; Alltech, Breda, The Netherlands) as described before [24]. Before injection, the incubation mixtures were centrifuged for 4 min at 14000 rpm. In a typical run, aliquots of 10 µL of the supernatant were injected. Samples were eluted at a flow of 1 mL/min with the following gradient: from 20% acetonitrile in nanopure water containing 0.1% trifluoroacetic acid, to 25% acetonitrile in 15 min, to 35% acetonitrile in 5 min, isocratic elution for 15 min at 35% acetonitrile, followed by an increase to 80% acetonitrile in 2 min, keeping this percentage for 1 min, after which it was

decreased to 0% acetonitrile in 1 min. This was kept for 1 min, after which the column was equilibrated at the initial conditions. Detection was performed between 220 and 445 nm using a Waters 996 photodiode array detector. Chromatograms used are based on detection at 370 nm. The limit of detection of this HPLC method for quantification of quercetin and its metabolites was 0.1 μM (injection volume 10 μL).

Preparation of phase II metabolites of quercetin.

For metabolism studies, cells were grown to confluency in cell culture flasks (75 cm^2). Before exposure, medium was removed and cells were washed with 10 mL HBSS. Then, 10 mL exposure medium was added to the cells consisting of 1 mM ascorbic acid in Dulbecco's MEM/F12 NutMix (HAM) without phenol red, containing 15 mM HEPES, L-glutamine and pyridoxine was supplemented with 100 μM quercetin from a 200 times concentrated stock solution in DMSO. Control incubations were exposed to 0.5% DMSO in medium containing ascorbic acid. Cells were exposed in duplicate and samples were taken 24 hours after starting the incubation. Samples were freeze dried and stored at -80°C until analysis. After storage at -80°C, the freeze-dried samples were resolved in Tris-sucrose (TS buffer: 10 mM Tris, 250 mM sucrose, pH 7.4) suited for the vesicle experiments. Prior to use in the vesicle transport experiments, HPLC analysis of the thawed mixtures was performed essentially as described by Van der Woude et al. [24], to assure the quality and quantity of the metabolites in the samples.

Identification and quantification of quercetin metabolites.

To compare the quercetin phase II metabolite patterns made by the different cell lines, identification and quantification of the different metabolites was performed as previously described [24]. HPLC analysis was identical to the method described above for detection of the metabolites. Chromatograms were based on detection at 370 nm. For commercially available standards calibration curves were made by plotting the peak area against the concentration. To quantify the amount of quercetin phase II metabolites produced by the cell lines, the peak area for the identified metabolites, was compared with the peak area-concentration curve obtained for the commercially available quercetin-glucoside rutin (quercetin-3-O-rhamnosylglucoside). The quercetin glucuronides were quantified using the rutin calibration curve, based on the assumption that the glucuronides have a comparable extinction coefficient as rutin. The limit of detection of this HPLC method for quantification of quercetin and its metabolites was 0.1 μM (injection volume 10 μL).

Expression of MRP1 and MRP2 in insect cells. S*f*9 insect cells were infected with recombinant baculoviruses containing either *MRP1* cDNA or *MRP2* cDNA as described previously [25]. Briefly, cells were cultured in spinner flasks in Grace's insect medium with 10% fetal calf serum and 10 μg/mL gentamycin at 27°C. For infection, cells were cultured on 145 cm^2 culture disks and infected with a baculovirus (multiplicity of infection of 5) for three days essentially as described by Zaman *et al [26]*. Virus-infected *Sf*9 cells were harvested and frozen at -80°C until membrane preparation.

Vesicle preparation and immunoblotting.
Membranes from infected *Sf*9 cells were isolated as described by van Aubel *et al.* [27]. Membrane protein concentrations were determined according to Bradford [28] adapted for 96-wells measurements on a BioRad 3550 microplate reader. Vesicles were prepared by passing the suspension 30 times through a 26-gauge needle with a syringe. Aliquots of 25 μL membrane vesicles containing 1 mg protein/mL were quickly frozen in liquid nitrogen and stored at -80°C until use. The expression of MRP1 and MRP2 in *Sf*9 membranes was assessed using immunoblotting with monoclonal antibodies MRPr1 and M_2III-6, raised against human MRP1 and human MRP2, respectively. The results obtained indicate an apparent M_r of 150 kDa in the *Sf*9 transfected cells. This is in line with literature data describing that human MRPs are produced in an underglycosylated form in *Sf*9 cells, which has been demonstrated not to affect their transport functions [29, 30].

Calcein uptake in Sf9 membrane vesicles.
The uptake of fluorescent MRP1 and MRP2 substrate calcein in isolated *Sf*9 cell membrane vesicles was performed as follows [25]. Vesicles were rapidly thawed and pre-incubated for 1 min at 37°C in Tris-sucrose buffer (TS buffer: 10 mM Tris, 250 mM sucrose, pH 7.4) containing 4 mM ATP or 4 mM AMP, 10 mM DTT, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg/mL creatine kinase and 20 μM of quercetin or its metabolites: 3'-O-methylquercetin (isorhamnetin), 4'-O-methylquercetin (tamarixetin), rutin (quercetin-3-rutinose), isoquercitrin (quercetin-3-O-ß-glucoside) and the metabolite mixtures produced by the HT29 and H4IIE cells. The reaction was started by addition of calcein to a final concentration of 40 μM. After 10 minutes incubation the reactions were stopped by adding 1 mL of ice-cold TS buffer. Then, the samples were rapidly filtered through pre-soaked nitrocellulose filters (0.45 μm pore size) (Schleicher and Schuell, Dassel, Germany) in a 1225 sampling Manifold filtration unit (Millipore, Ettenleur, The Netherlands). Filters were rinsed with 10 mL TS buffer and the filters containing the vesicles were put in a 6 wells plate (Corning) and 50 μL TS buffer was added on top of the filters. Fluorescence of the filters (excitation wavelength 485 nm, emission wavelength 530 nm) was measured using a Cytofluor 2300 (Millipore, Ettenleur, The Netherlands). In control experiments ATP was replaced by 4 mM AMP-PCP (α,β-methylene adenosine 5'-triphosphate) to measure background, ATP independent, uptake of calcein. ATP-dependent calcein uptake was calculated by subtracting the values obtained in the presence of AMP-PCP from those in the presence of ATP.

Data analysis.
A one-way analysis of variance test was used for all data analysis ($P<0.05$) using SPSS 10.1.0 software from SPSS Inc.

Results

MRP1 and MRP2 inhibition by quercetin

Figure 2 shows the effects of increasing concentrations of quercetin on MRP1 and MRP2 mediated calcein uptake. From the data presented it follows that 20 μM quercetin inhibits MRP1 mediated calcein uptake by 54 ± 6%. Using 20 μM quercetin, both stronger and weaker inhibitory potencies of quercetin metabolites can be detected. Therefore, 20 μM quercetin and/or its metabolites appeared the optimal concentration for studies on the consequences of quercetin phase II metabolism on its MRP1 inhibiting potential. Figure 2 also shows that MRP2 is only mildly inhibited by quercetin. Even at 50 μM, quercetin does not inhibit MRP2 mediated calcein uptake to 50%. Nevertheless, to allow comparison to the MRP1 inhibition experiments MRP2 inhibition experiments were also performed at 20 μM concentrations of quercetin or its metabolites. At this concentration of quercetin MRP2 was inhibited by 35 ± 8%.

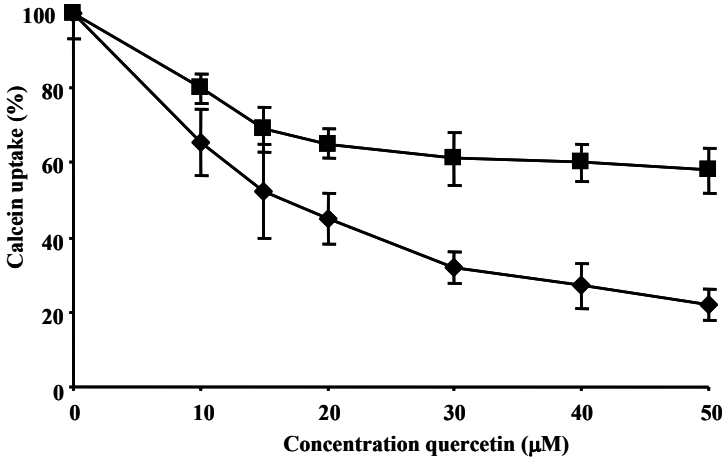

Figure 2. Concentration-dependent inhibition of MRP1 (◆) and MRP2 (■) mediated uptake of calcein by quercetin in inside out *Sf*9 vesicles. All values differ significantly from control (vehicle only) ($P<0.05$).

Formation of quercetin phase II metabolites by HT29 and H4IIE cells.

Table 1 gives the complete composition of the quercetin phase II metabolites synthesized by HT29 cells and H4IIE cells upon 24 h exposure. This table also summarizes the retention times of the peaks on HPLC as identified before [24] and used in the present study for their identification. The HT29 cells produced 3 major metabolites: 4'-O-glucuronosyl 3'-O-methylquercetin (15%), 3-O-glucuronosyl quercetin (26%) and 4'-O-glucuronosyl quercetin (27%). After 24 hours incubation, approximately 19% of the native quercetin is still present. The H4IIE cells produced one major metabolite: 7-O-glucuronosyl quercetin (84%) and a few other metabolites of which 3-O-glucuronosyl quercetin (10%) is the most abundant. After 24 hours incubation, almost all quercetin appears to be converted as illustrated by the fact that only 1 % of the native quercetin is still present.

Table 1: Phase II metabolites synthesized by HT29 cells and H4IIE cells. The cell lines were exposed to 100 μM quercetin in presence of 1 mM ascorbic acid for 24 hours. The samples were analysed by HPLC. The percentage of the metabolites is based on the average concentration ± SD of two samples.

Compound	Retention time (min)	Concentration (μM)
HT29 cells		
3-O-glucuronosyl quercetin	7.4	26 ± 0.6
3-O-glucuronosyl 3'-O-methylquercetin	12.3	3 ± 0.1
4'-O-glucuronosyl quercetin	13.6	27 ± 0.2
3'-O-glucuronosyl quercetin	15.3	5 ± 0.3
4'-O-glucuronosyl 3'-O-methylquercetin	16.4	15 ± 0.4
3'-O-glucuronosyl 4'-O-methylquercetin	17.8	3 ± 0.3
Quercetin	24.0	19 ± 0.3
3'-O-methylquercetin	30.7	2 ± 0.1
4'-O-methylquercetin	31.2	1 ± 0.1
H4IIE cells		
7-O-glucuronosyl quercetin	7.3	84 ± 6.5
3-O-glucuronosyl quercetin	13.9	10 ± 0.4
7-O-glucuronosyl 4'-O-methylquercetin	15.1	2 ± 0.3
3'-O-glucuronosyl quercetin	15.6	6 ± 0.9
Quercetin	24.0	1 ± 0.2

Inhibition of MRP1 and MRP2 activity by quercetin and its metabolites.

Figure 3A shows the effects of quercetin and its metabolites on MRP1-mediated uptake of the fluorescent substrate calcein. From the results presented it can be seen that 20 μM quercetin inhibits MRP1 activity by approximately 54%. Of the metabolites tested only 20 μM of the H4IIE mixture showed a significantly stronger inhibition (68%) compared to 20 μM quercetin. One metabolite tested, 4'-O-methylquercetin inhibited calcein uptake by MRP1 by 36% which is significantly less than the MRP1 inhibition by 20 μM quercetin. Moreover, the quercetin-glycoside isoquercitrin did not inhibit MRP1-mediated calcein uptake at all whereas the other quercetin-glycoside tested, rutin, was a significant stronger inhibitor of MRP1 than 20 μM quercetin. The other metabolites tested showed MRP1 inhibitory potencies similar to 20 μM quercetin.

Figure 3B shows the effects of quercetin and its metabolites on MRP2-mediated uptake of the fluorescent substrate calcein. The data presented reveal that 20 μM quercetin inhibits MRP2-mediated calcein uptake by approximately 35%. Of the metabolites tested both the HT29 and H4IIE mixtures containing 20 μM quercetin and its metabolites in total inhibited MRP2 mediated calcein uptake by 61% and 95% respectively which is significantly stronger than the MRP2 inhibition by 20 μM quercetin ($P<0.05$). Interestingly, the quercetin metabolite mixture formed by the H4IIE cells, containing especially (84 %) 7-O-glucuronosyl quercetin showed almost complete inhibition of MRP2-mediated uptake of calcein. Two compounds tested, the quercetin-glycosides rutin and isoquercitrin inhibited MRP2 mediated calcein uptake only by respectively 7 and 17% which is significantly less than the MRP2 inhibition by 20 μM quercetin. Interestingly, one metabolite: 4'-O-methylquercetin, did even stimulate calcein uptake compared to vehicle only incubations (control) by 20% ($P<0.05$). The other metabolites tested showed MRP2 inhibitory potencies similar to 20 μM quercetin

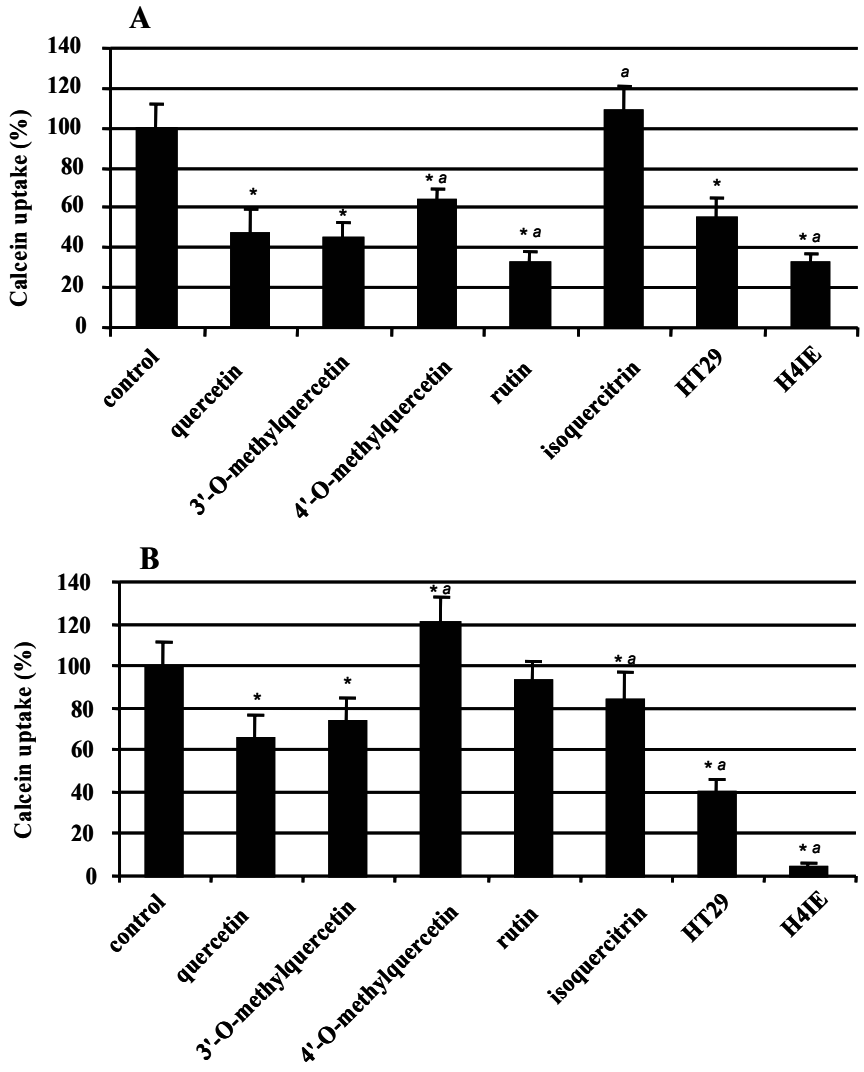

Figure 3. Calcein uptake by MRP1 (A) and MRP2 (B) upon exposure to vehicle only (control), quercetin and the metabolites or metabolite mixtures tested (all at 20 µM total final concentration). The metabolites produced by the HT29 and H4IIE mixtures are described in Table 1. The bars represent the mean ± SD (n=6). Asterisks (*) represent values that differ significantly from control (vehicle only) ($P<0.05$). [a] values that differ significantly from 20 µM quercetin ($P<0.05$).

Discussion

Phase II metabolism is a process which determines the fate and biological activity of quercetin in the human body [14]. Not only are phase II metabolites of quercetin the most important forms present in human plasma, phase II metabolism is also known to influence the biological activity of the flavonoid [15].

The phase II metabolism by the H4IIE and HT29 cell lines was described before [24]. Comparison of the data set in Table 1 with the data obtained from this previous study reveals that the quercetin metabolite pattern formed by the two cell lines was similar to that observed previously. The present study shows that phase II metabolism of quercetin results in metabolites that are at least equally good inhibitors of MRP1 and MRP2 activity as quercetin itself. The results obtained in the present study reveal that methylation of the catechol moiety does not affect the MRP inhibitory potential of quercetin, with the exception of 4'-O-methylation that appeared to reduce the potential to inhibit MRP2. The results obtained for the MRP1 inhibitory potential of the different glucuronosyl mixtures produced by the two cell lines indicate that not only the type of conjugation is of importance for quercetin mediated MRP1 inhibition but also the regioselectivity of the phase II metabolism. Apparently, 7-O-glucuronosyl quercetin is a stronger inhibitor of MRP1 than the glucuronosyl metabolites present in the HT29 mixture containing 3-O-glucuronosyl quercetin, 4'-O-glucuronosyl 3'-O-methylquercetin and 4'-O-glucuronosyl quercetin. Glucuronidation of quercetin also improves the MRP2 inhibitory potential of quercetin as demonstrated by the HT29 and H4IIE mixtures. Again, regioselectivity of glucuronidation plays a role since the H4IIE mixture, containing 7-O-glucuronosyl quercetin, is a stronger inhibitor of MRP2 activity than the HT29 mixture. One metabolite: 4'-O-methylquercetin, did stimulate calcein uptake by MRP2 compared to control incubations. The mechanism responsible for this phenomenon is unknown. However it should be noted that 4'-O-methylquercetin, as well as 3'-O-methylquercetin, is hardly present in human plasma because it is readily excreted via the bile [21, 24]. In a previous study using MRP1 transfected MDCKII cells, it was found that methylation of quercetin resulted in stronger MRP1 inhibitors compared to quercetin itself [12]. This effect seems therefore to be the result of higher uptake of the methylated form by the cells, rather than higher inhibitory capacity as a result of this methylation.

For MRP2, rutin showed significantly reduced (weaker) inhibition than 20 μM quercetin. This is comparable to MRP1 where the quercetin-glycoside isoquercitrin showed reduced (weaker) inhibition than 20 μM quercetin. Isoquercitrin and rutin are examples of important types of glycosylated quercetin available in food. These glycosides may become deconjugated during passage across the small intestine [19] or by bacterial activity in the colon [20]. It is not likely that glycosylated forms of quercetin are present in human plasma [31].

Altogether the results of the present study demonstrate for both transporters that quercetin phase II metabolism is an important factor influencing its MRP1 and MRP2 inhibiting potential. Glucuronidated quercetin metabolites seem to be better inhibitors of both MRP1

and MRP2 than the parent compound or its methylated metabolites. The effect of quercetin sulfation remains a topic for future study although it is likely that also these conjugates will be able to inhibit MRP1 and MRP2 activity since sulfate conjugates are known to be common substrates of these transporters [4]. An important observation in this study was that all metabolites were at least equally good inhibitors of MRP1 and MRP2 as quercetin. This implies that quercetin will not loose its inhibitory capacity for MRP1 and MRP2 *in vivo* upon its conversion to phase II type metabolites. Also for other flavonoids, it is likely that phase II metabolism will not reduce their inhibitory capacity for MRP1 and MRP2 since their pattern of phase II metabolism is comparable to quercetin [14].

A possible mechanism by which flavonoids interact with MRPs has been described for human colonic carcinoma Caco-2 cells [32, 33]. These reports show that flavonoids as well as their glucuronide- and sulfate-conjugates can act as MRP2 substrates and are efficiently transported by this transporter. This observation indicates an interaction of flavonoid and its metabolites with the substrate binding site of MRP2. In a previous study, the flavonoid robinetin was shown to be a competitive inhibitor of MRP1- and MRP2-mediated efflux of calcein from MRP1 and MRP2 transfected MDCKII cells, thereby corroborating the possible interaction of the flavonoid with the substrate binding site [12].

Overall, the results of this study reveal that the major phase II metabolites of quercetin are equally potent or even better inhibitors of MRP1 and MRP2**,** indicating that phase II metabolism is unlikely to reduce the potential for use of quercetin as an inhibitor to overcome MRP mediated multidrug resistance.

Acknowledgments

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8

Summary, conclusions and perspectives.

In this thesis the use of flavonoids for inhibition of two important players in the glutathione related biotransformation system involved in multidrug resistance was investigated. This included the phase II glutathione S-transferase enzyme GSTP1-1, able to detoxify anticancer agents through conjugation with glutathione, and the two multidrug resistance proteins MRP1 and MRP2 involved in glutathione mediated cellular efflux of, amongst others, anticancer drugs. To this end, the flavonoid mediated inhibition of GSTP1-1 and the efflux proteins MRP1 and MRP2 was explored using several model systems. The model systems used in this thesis are purified enzymes (GSTP1-1) (chapter 2), transfected cell lines including MCF7 cells transfected with GSTP1-1 (chapter 3) and MDCKII cells transfected with MRP1 or MRP2 (chapter 4, 5 and 6) as well as MRP1 and MRP2 containing inside-out vesicles (chapter 7). The transfected cell lines were considered an important *in vitr*o model system, because cells are one step closer to the *in vivo* situation than other *in vitro* systems like purified enzymes and inside-out vesicles. In a cellular system the interaction of flavonoids with the protein of interest is studied under normal cellular conditions including characteristics for uptake, metabolism and elimination of the flavonoid by the cells. Results of the present thesis corroborated that the model system used can be of crucial importance on the results obtained. Good inhibitors of purified GSTP1-1 or of MRP1 and MRP2 in inside-out vesicles appeared not necessarily good inhibitors of these proteins in intact cell models. Using purified GSTP1-1 it was demonstrated for example that quercetin was a good inhibitor (chapter 2). Detailed studies on the mechanism underlying this inhibition revealed the involvement of quinone-type oxidation products of quercetin likely acting as specific active site inhibitors of GSTP1-1, thereby completely inhibiting the GSTP1-1 activity. The cellular and *in vivo* relevance of these results obtained with purified GSTP1-1 inhibited by quercetin quinone-type oxidation products can be expected to be dependent on two factors that are likely to influence the cellular oxidation of quercetin to its quinone-type products, including (1) the antioxidant status of the cells and (2) the cellular presence of tyrosinase and/or peroxidases (like in melanoma cells). The presence of cellular antioxidants like glutathione and vitamin C may prevent the formation of significant levels of cellular quercetin quinone-type products [1, 2], whereas the cellular presence of tyrosinase and/or peroxidases may stimulate quercetin-quinone formation (chapter 2). In line with these considerations it appeared that in the MCF7 cells which contain normal antioxidant levels and not especially high levels of tyrosinase or peroxidase enzymes. GSTP1-1 is only marginally susceptible towards inhibition by quercetin and also other flavonoids (chapter 3). It is likely that the presence of natural antioxidants in the cells prevent the formation of significant levels of quercetin oxidation products that are high enough to result in covalent GSTP1-1 inhibition by these oxidation products. The moderate GSTP1-1 inhibition by quercetin found in the studies with MCF7 cells (chapter 3) is more likely to be caused by the reduced form of the flavonoid. However, based on this, only moderate inhibition of GSTP1-1 by flavonoids in a cellular model (chapter 3), it is concluded that flavonoids are unlikely to provide efficient cellular or *in vivo* GSTP1-1 inhibiting agents useful to reverse this aspect of multidrug resistance.

The second possible glutathione-related mechanism involved in multidrug resistance for which inhibition by flavonoids was investigated in this thesis was inhibition of MRP1 and MRP2-mediated efflux. An important conclusion from this thesis is that the major site for flavonoids mediated interaction with GSH-dependent multidrug resistance processes are the GS-X pumps MRP1 and MRP2 rather than the conjugating GSTP1-1 activity (chapter 3 and 4). Importantly, MRP2 displays higher selectivity for flavonoid-type inhibition than MRP1. For inhibition of MRP1, a quantitative structure activity relationship (QSAR) was obtained that indicates three structural characteristics to be of major importance for MRP1 inhibition by flavonoids: the total number of methoxylated moieties, the total number of hydroxyl groups and the dihedral angle between the B- and C-ring. The employability of flavonoids to reverse MRP mediated multidrug resistance was studied *in vitro* using vincristine as model anticancer drug in transfected MDCKII cells (chapter 5). For this study, the flavonoid myricetin was used as a model compound since it appeared able to inhibit both MRP1 and MRP2 (Chapter 4). In Chapter 5 it was shown that myricetin can inhibit vincristine efflux by MRP1 and MRP2 thereby sensitizing the cells towards vincristine, indicating that flavonoids like myricetin might be a valuable adjunct to chemotherapy to reverse MRP mediated resistance. After demonstrating the increase in vincristine sensitivity of MRP1 and MRP2 cells by myricetin it was investigated whether myricetin, at the concentrations required for reversal of vincristine sensitivity, would also affect the efflux of endogenous metabolites. To this end, the effects of myricetin on the efflux of PGA_2-SG from the MRP1 and MRP2 cells were characterised (Chapter 6). Myricetin, shown to be a strong inhibitor of vincristine efflux (Chapter 5), was only a weak inhibitor of PGA_2-SG efflux (Chapter 6). This discrepancy in myricetin-mediated inhibition of vincristine and PGA_2-SG efflux could best be explained by the hypothesis that myricetin is unlikely to affect MRP-mediated transport of glutathione conjugates to a significant extent, because glutathione conjugates, including PGA_2-SG are known to be high affinity substrates of MRP1 and MRP2 [3, 4]. These results also indicate that myricetin supplementation, and perhaps even flavonoid supplementation in general, may not significantly affect MRP-mediated transport of endogenous or other GSH conjugates in such a way that adverse health effects, as a result of this inhibition, are to be expected. Interestingly, these results also provide an argument for the possible absence of specific negative side effects on the kinetics and physiology of natural MRP substrates, to be expected upon use of these natural MRP inhibitors in the reversal of multidrug resistance.

If flavonoids are to be used in a clinical setting to revert MRP-mediated multidrug resistance, an important factor determining the therapeutic outcome will be the effects of flavonoid phase II metabolism on the capacity of the flavonoid to inhibit MRP1 and MRP2 activity. This aspect was investigated in the present thesis using quercetin as the model compound, because for this flavonoid phase II metabolites to be expected were recently identified and quantified [5]. In Chapter 7 it was shown that the major phase II metabolites of quercetin are equally potent or even better inhibitors of MRP1 and MRP2, indicating that phase II metabolism is unlikely to reduce the potential for use of this flavonoid as an inhibitor to overcome MRP mediated multidrug resistance.

In addition to the influence of *in vivo* metabolism of the flavonoids, the extrapolation of the results presented in this thesis to the *in vivo* situation raises several other questions that should be answered before clinical use of flavonoids as MRP inhibitors should be undertaken. One of these questions concerns the possible side effects of flavonoids. The use of relatively non-specific MRP inhibitors in clinical therapy might give disappointing results due to drug-drug interactions and negative side effects [6]. For another multidrug resistance-related efflux protein like P-glycoprotein, several clinical trials with inhibitors like, among others, verapamil demonstrated this behaviour [7] including high overall toxicity. Side effects upon the use of flavonoids in clinical settings may come from the fact that flavonoids are compounds that can interact with many different enzymes, among which: phosphoinositide 3-kinase (PI 3-kinase), Akt/protein kinase B (Akt/PKB), tyrosine kinases, protein kinase C (PKC), and mitogen activated protein kinase (MAP kinase) reviewed by Williams et al. (2004) [8], cytochrome P450, DT-diaphorase, NADPH cytochrome c reductase and glutathione reductase [9] and also other transport proteins like ABCG2 [10] and P-glycoprotein [11]. These possible effects need to be examined and evaluated *in vivo* in further detail. Nevertheless, increased plasma levels of flavonoids as a result of supplementation of the diet or from phase I clinical trials have previously been considered safe and relatively non-toxic [12, 13]. Furthermore, the results described in Chapter 6 of this thesis provided an argument for the absence of possible adverse effects due to interference with the physiological MRP1 and MRP2 substrate PGA_2-SG, know to be transported by these GS-X (of glutathione conjugate) efflux pumps. When this absence of inhibition of PGA_2-SG transport by MRP1 and MRP2 transport is indeed related to the fact that flavonoids will not interfere with MRP1 and MRP2 mediated transport of glutathione conjugates due to their higher affinity for the transport proteins than the flavonoids, this implies that interference of flavonoids with normal GS-X transport is not foreseen. This also implies that the flavonoids can only be used for the reversal of multidrug resistance towards drugs that are no glutathione conjugates.

Another matter that should be considered in more detail before clinical trials on reversal of multidrug resistance by flavonoids may be undertaken, concerns the type of flavonoid to be used as therapeutic adjuvant. Although myricetin was used in several chapters of this thesis as model compound, since it appeared to be able to inhibit both MRP1 and MRP2 activity, it is not the strongest MRP1 inhibiting flavonoid as can be concluded based on the results presented in Chapter 4. In this chapter, the methoxylated flavonoids chrysoeriol and diosmetin were demonstrated to be the best MRP1 inhibitors. Interestingly, also some phase II metabolites of the flavonoid quercetin, especially some glucuronides, were demonstrated to be superior inhibitors of MRP1 and MRP2 activity as compared to the native flavonoid (Chapter 7). Also other flavonoid structural characteristics might increase its inhibitory potency. As an example, isoprenylation of flavonoids might result in stronger inhibitors as demonstrated for P-glycoprotein and MRP1 [14, 15]. The selection of the best suited flavonoid should also take into account possible side effects, cellular uptake characteristics and phase II metabolism of the flavonoid to be selected. Nevertheless, the model flavonoids in several chapters of this thesis, myricetin and quercetin, might be a good starting point for

in vivo experiments because they are well described in literature (even in phase I clinical trials), relatively safe and can become more potent inhibitors of MRP1 and MRP2 upon their biotransformation expected to occur in the intestine upon absorption, as seen in Chapter 7.

A final matter to be considered for extrapolation of the results presented in this thesis to the *in vivo* situation, concerns the type of efflux proteins actually involved in the multidrug resistance of the tumour to be treated. Inhibitors of MRP1 or MRP2 are only applicable if the tumour drug resistance is directly caused by MRP overexpression. This relation is known for several tumour types, among which are non-small-cell lung carcinomas (NSCLC) [16] and myeloid leukemia [17]. For MRP2 such a relation has been described for hepatocellular carcinoma (HCC) [18] and acute myeloid leukemia [19]. This implies that the involvement of these MRPs in specific cases of clinical drug resistance has to be confirmed prior to therapeutic use of MRP inhibitors. Although trivial, such clinical screening is not yet common practice [7, 20]. For P-glycoprotein for example, early clinical resistance reversal trials, revealed disappointing results. In part this was caused by the fact that the patients were not specifically selected based upon P-glycoprotein resistance. Despite this, three randomized trials have shown statistically significant benefits with the use of a P-glycoprotein inhibitor in combination with chemotherapy [7]. Improved diagnostic techniques aimed at the selection of patients with tumours that express P-glycoprotein or MRPs should result in more successful outcomes.

Altogether, the results of the present thesis reveal the potential of flavonoids to act as inhibitors of two important players in the glutathione-related biotransformation system involved in multidrug resistance namely the phase II enzyme GSTP1-1 and the efflux proteins MRP1 and MRP2. The major site for flavonoid mediated interaction with GSH-dependent multidrug resistance appeared to be the GS-X pumps MRP1 and MRP2 rather than the conjugating GSTP1-1 activity. Testing of the *in vitro* outcomes of the present study in clinical settings may start with flavonoids that have already a safe history of use in for example food supplements and requires the confirmation of involvement of the MRPs in specific cases of clinical drug resistance prior to therapeutic use of the flavonoids as MRP inhibitors

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Samenvatting

In dit proefschrift is onderzocht in welke mate flavonoïden, stoffen die veel voorkomen in bijvoorbeeld groenten en fruit, de werking van het cellulaire detoxificatie-systeem kunnen beïnvloeden dat betrokken is bij mulidrug resistentie. Het onderzoek richtte zich vooral op het fase II enzym glutathion S-transferase P1-1 (GSTP1-1), dat betrokken is bij de detoxificatie van antikanker-medicijnen door deze te conjugeren aan glutathion, en twee van de transporteiwitten die betrokken zijn bij de cellulaire efflux van deze glutathion conjugaten, de multidrug resistance proteins 1 en 2 (MRP1 en MRP2). Voor dit onderzoek is een aantal verschillende *in vitro* test systemen gebruikt, te weten: zuivere enzymen (GSTP1-1 in hoofdstuk 2), een GSTP1-1 getransfecteerde cellijn (hoofdstuk 3), MRP1 en MRP2 getransfecteerde cellijnen (hoofdstuk 4, 5 en 6) en MRP1 of MRP2 bevattende inside-out vesicles (hoofdstuk 7). Vooral de getransfecteerde cellijnen vormen een belangrijk test systeem omdat in dit systeem ook enkele factoren een rol spelen, die het uiteindelijke effect van de flavonoïden op GSTP1-1 en de efflux pompen MRP1 en MRP2 bepalen. Deze factoren zijn bijvoorbeeld de opname van de flavonoïden door de cellen, en de omzetting van de flavonoïden in metabolieten. Uit het hier beschreven onderzoek is gebleken dat het gebruikte test systeem van invloed kan zijn op het uiteindelijke gemeten effect. Potente remmers van zuiver GSTP1-1 of MRP1 en MRP2 in de inside-out vesicles zijn niet per definitie ook goede remmers van GSTP1-1, MRP1 en MRP2 wanneer deze zich in een cellulair systeem bevinden. Een mooi voorbeeld van dit verschijnsel staat in de hoofdstukken 2 en 3. Uit experimenten met zuiver GSTP1-1 blijkt dat quercetine een zeer goede remmer is van de enzym-activiteit. Nader onderzoek toonde aan dat vooral de quercetine-chinonen, producten van quercetine oxidatie, specifieke remmers zijn van GSTP1-1 door covalent te binden aan een belangrijk cysteïne residue. Echter, het effect van quercetine *in vivo* zal onder meer afhankelijk zijn van de mate van oxidatie van quercetine en dus van de antioxidant status van de cellen en de eventuele aanwezigheid van oxiderende enzymen zoals tyrosinases of peroxidases (aanwezig in bijvoorbeeld melanoma cellen). De aanwezigheid van antioxidanten zoals vitamine C en glutathion kunnen mogelijk de vorming en/of beschikbaarheid van de quercetine-chinonen verminderen, terwijl tyrosinases en peroxidases juist de vorming van deze chinonen kunnen stimuleren [1,2]. Deze hypothese wordt bevestigd door de experimenten met de GSTP1-1 getransfecteerde MCF7 cellen in hoofdstuk 3. In deze cellen, die normale hoeveelheden antioxidanten bevatten en niet veel tyrosinase en/of peroxidase activiteit hebben, is quercetine slechts een matige remmer van GSTP1-1 activiteit. Gebaseerd op de wat beperkte GSTP1-1 remmende potentie van de geteste flavonoïden in de MCF7 cellen kan worden gesteld dat het niet waarschijnlijk is dat flavonoïden geschikt zijn om GSTP1-1 activiteit, een mogelijke factor van multidrug resistentie, in intacte cellen of *in vivo* te remmen.

Een ander deel van dit proefschrift beschrijft de invloed van flavonoïden op transport eiwitten MRP1 en MRP2. Een belangrijke conclusie van hoofstuk 3 en hoofdstuk 4 is dat flavonoïden betere remmers zijn van de transporteiwitten MRP1 en MRP2 dan van GSTP1-1. Voor de remming van MRP1 door flavonoïden is in hoofdstuk 4 een kwantitatieve structuur-activiteits relatie opgesteld. Deze structuur activiteits relatie toont aan dat drie

flavonoïd-moleculaire karakteristieken van belang zijn voor goede MRP1 remming: te weten het aantal gemethoxyleerde groepen, het aantal gehydroxyleerde groepen en de dihedrale hoek tussen de B- en C-ring van de flavonoïden. De mogelijke toepasbaarheid van flavonoïden als remmers van MRP-gemedieerde multidrug resistentie is bestudeerd in MDCKII cellen met vincristine als antikanker medicijn. Voor deze experimenten is myricetine gebruikt als modelstof omdat dit flavonoïd zowel MRP1 als MRP2 activiteit kan remmen (hoofdstuk 4). In hoofdstuk 5 staat beschreven dat myricetine inderdaad de cellulaire efflux van vincristine door MRP1 en MRP2 kan remmen en dat, als gevolg van deze verminderde efflux, de cellen ook gevoeliger worden voor de antikanker werking van vincristine. Met andere woorden, *in vitro* kan MRP-gemedieerde multidrug resistente worden verminderd door myricetine. Vervolgens is ook onderzocht in hoeverre deze concentraties myricetine de efflux van endogene substraten kan verstoren. Als model substraat voor deze experimenten is prostaglandine PGA_2 gebruikt. Deze verbinding wordt intracellulair geconjugeerd met glutathion tot PGA_2-SG en vervolgens door MRP1 en MRP2 de cel uit getransporteerd (hoofdstuk 6). Uit deze experimenten blijkt dat myricetine -een sterke remmer van vincristine efflux- slechts marginaal de efflux van PGA_2-SG verstoord. Deze discrepantie kan het best worden verklaard door te veronderstellen dat, in het algemeen, glutathion conjugaten zoals PGA_2-SG, met hoge affiniteit door MRP1 en MRP2 worden gebonden, waardoor deze interactie ongevoelig(er) is voor remming door flavonoïden [3,4]. Uit deze experimenten kan worden geconcludeerd dat verrijking van het dieet met myricetine, of met flavonoïden in het algemeen, waarschijnlijk niet het transport van endogene substraten zoals PGA_2-SG door MRP1 en MRP2 in dusdanige mate zal verstoren dat negatieve gezondheidseffecten kunnen worden verwacht. Tevens leveren deze experimenten bewijs voor de stelling dat mogelijk het gebruik van myricetine om MRP-gemedieerde multidrug resistentie tegen te gaan niet zal leiden tot een ernstige verstoring van de kinetiek en fysiologie van endogene glutathion geconjugeerde MRP substraten.

Of flavonoïden ook *in vivo* geschikt zijn om MRP1 en MRP2 efflux te remmen is onder andere afhankelijk van het fase II metabolisme van deze flavonoïden in het lichaam en de effecten van deze metabolieten op MRP1 en MRP2 activiteit. In hoofdstuk 7 staat beschreven dat de belangrijkste fase II metabolieten van quercetine [5] in de regel even goede, zo niet betere remmers zijn van MRP1 en MRP2. Dit impliceert dat het metabolisme van flavonoïden in het lichaam waarschijnlijk geen beperkende factor is wat betreft het effect van de flavonoïden op MRP1 en MRP2.

Uiteraard is het een belangrijke vraag of het onderzoek uit dit proefschrift extrapoleerbaar is naar de *in vivo* situatie. Voordat flavonoïden klinisch kunnen worden toegepast zal eerst een aantal additionele belangrijke vragen moeten worden onderzocht. Een voorbeeld van zo'n vraag betreft de mogelijke negatieve neven-effecten van flavonoïden *in vivo*. Flavonoïden zijn relatief aspecifieke remmers van MRP1 en MRP2. Toepassing van deze flavonoïden in de kliniek kan mogelijk teleurstellende resultaten opleveren als gevolg van drug-drug interacties en andere neven-effecten [6]. Ter illustratie, klinische studies met verapamil, een niet-specifieke remmer van een ander transporteiwit, P-glycoproteine, resulteerden in dergelijke ongewenste effecten [7]. De effecten van flavonoïden op andere

enzymen, zoals phosphoinositide 3-kinase (PI 3-kinase), Akt/protein kinase B (Akt/PKB), tyrosine kinases, protein kinase C (PKC), en mitogen activated protein kinase (MAP kinase) [8], cytochrome P450, DT-diaphorase, NADPH cytochrome c reductase en glutathion reductase [9] en ook andere transporters zoals ABCG2 [10] en P-glycoproteine [11] zullen moeten worden bestudeerd voordat therapeutisch gebruik van flavonoïden als MRP1 en MRP2 remmers mogelijk is. Opgemerkt dient te worden dat er al enkele studies zijn verricht naar hoge flavonoïd bloed-plasma gehaltes door middel van verrijking en klinische studies waarbij geen negatieve gezondheids effecten zijn geconstateerd [12, 13].
Een andere vraag die moet worden besantwoord voordat flavonoïden kunnen worden gebruikt als klinisch adjuvant is de keuze van het te gebruiken flavonoïd [14,15]. Hoewel in dit proefschrift myricetine (remmer van zowel MRP1 als MRP2) in een aantal hoofdstukken is gebruikt als model-flavonoïd, is myricetine voor MRP1 niet de meest potente remmer (hoofdstuk 4). Hier staat immers beschreven dat een aantal gemethoxyleerde flavonoïden, zoals chrysoeriol en diosmetine sterkere MRP1 remmers zijn dan myricetine. Opvallend genoeg waren ook enkele van de fase II metabolieten van quercetine betere MRP1 en MRP2 remmers dan quercetine zelf. Desalniettemin kunnen de hier bestudeerde flavonoïden myricetine en quercetine prima worden gebruikt als beginpunt bij het *in vivo* onderzoek omdat deze flavonoïden relatief goed zijn onderzocht (zelfs in fase I klinische tests) waarin ze relatief veilig zijn bevonden. Tevens zijn de fase II metabolieten van deze flavonoïden mogelijk nog sterkere remmers van MRP1 en MRP2 (hoofdstuk 7).
Als laatste dient een goede selectie te worden gemaakt van patiënten die in aanmerking komen voor mogelijk klinisch gebruik van flavonoïden om multidrug resistentie te moduleren door remming van MRP1 en MRP2. Er zijn meerdere transporteiwitten bekend die multidrug resistentie kunnen veroorzaken waardoor er eerst bewijs dient te zijn dat MRP1 of MRP2 een rol speelt in de resistentie van de tumor onder behandeling. Een dergelijk bewijs is er bijvoorbeeld voor non-small-cell long carcinomas (NSCLC) [16] en myeloide leukemie [17] voor MRP1; en voor hepatocellulaire carcinoma (HCC) [18] en acute myeloide leukemie [19] voor MRP2. Screening naar de rol van MRP1 en MRP2 in tumor resistentie is op dit moment nog niet gebruikelijk in de kliniek [7,20].

De resultaten beschreven in dit proefschrift tonen aan dat flavonoïden mogelijk gebruikt kunnen worden om enkele enzymen die verantwoordelijk zijn voor het ontstaan van multidrug resistentie, te weten GSTP1-1 en de cellulaire transporters MRP1 en MRP2, te remmen in hun activiteit. Het belangrijkste effect van flavonoïden lijkt vooral remming van de transporters MRP1 en MRP2 en niet van de GSTP1-1 activiteit. Toepassing van flavonoïden in de kliniek op basis van deze *in vitro* studies kan mogelijk het beste geschieden met flavonoïden die goed zijn onderzocht en relatief veilig zijn bevonden in humane studies zoals myricetine en quercetine, die gebruikt zijn in dit proefschrift. Voorwaarde voor klinische toepassing van flavonoïden als remmers van MRP1 en MRP2 voor het moduleren van multidrug resistentie is dat de betrokkenheid van deze transporters bij de multidrug resistentie vast staat.

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Curriculum Vitae

Jelmer Jelle van Zanden werd geboren op 17 augustus 1977 te Utrecht. In 1995 behaalde hij zijn VWO diploma aan katholieke scholengemeenschap “de Breul” te Zeist. In dat zelfde jaar begon hij aan de studie Milieuhygiëne aan de Wageningen Universiteit. Tijdens zijn studie koos hij voor een specialisatie in de toxicologie en biochemie waar hij onder andere afstudeervakken deed bij Dr. I.A. Meerts (toxicologie) en Prof. I.M.C.M. Rietjens (biochemie). Als afsluiting heeft hij nog 6 maanden stage gelopen aan het Moleculair Biologisch Instituut aan de universiteit in Bergen (Noorwegen). In oktober 2000 behaalde hij zijn doctoraal diploma.
Vanaf oktober 2000 werd hij aangesteld op een promotieonderzoek, dat beschreven is in dit proefschrift, aan de Wageningen Universiteit, sectie toxicologie op een KWF gesubsidieerd samenwerkingsproject met TNO, Kwaliteit van Leven in Zeist. Sinds 1 februari 2005 is hij werkzaam als postdoc aan het Universitair Medisch Centrum in Groningen bij Prof. A. van der Zee en Dr. E. Schuuring.

List of Publications

published articles

Zanden van JJ, Mul A, Wortelboer H, Usta M, Bladeren PJ, Rietjens IMCM and Cnubben NHP, Reversal of *in vitro* cellular MRP1 and MRP2 mediated vincristine resistance by the flavonoid myricetin. Biochemical Pharmacology, 2005 in press.

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Training and Supervision plan

Courses -2004

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