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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 IC_{50} values ranging between 0.8 and 8 μM. Luteolin and quercetin were the strongest inhibitors with IC_{50} 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 IC_{50} values of the present study point at possible flavonoid–drug and/or flavonoid–xenobiotic interactions especially regarding transport processes involved in toxicokinetics.

Keywords: Flavonoids; Glutathione *S*-transferase; GS-X pump; MRP1; MRP2; Inhibition

1. 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

Abbreviations: GST, glutathione *S*-transferase; GSH, glutathione; GS-X, glutathione conjugate; MRP, multidrug resistance associated protein; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-SG, *S*-(2,4-dinitrophenyl)glutathione; IC_{50}, 50% inhibition concentration; P-gp, P-glycoprotein

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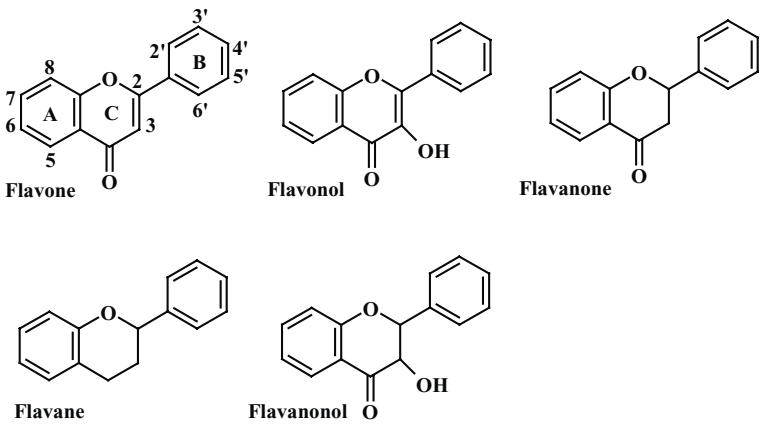
chemotherapeutic agents. Multidrug resistance is often associated with overexpression of glutathione *S*-transferases (GSTs) and efflux transporter proteins, such as P-glycoprotein (P-gp) and/or multidrug resistance associated protein (MRP) [1,2]. Therefore, when a particular drug is a substrate for 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, glutathione *S*-transferase P1-1 (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 tumors [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 [8–13]).

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



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'	-

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

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 (Fig. 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.

2. Materials and methods

2.1. 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).

2.2. 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 °C in a humidified 5% CO_2 atmosphere in MEM supplemented with 5% 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 plate and cultured for 24 h before exposure. Cells were exposed for 20 min in 2.0 ml HBSS containing different concentrations of the flavonoids and 0.5 mM acivicin (to prevent degradation of DNP-SG by γ-glutamyltranspeptidase). 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 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 min incubation at 37 °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 °C until further analysis for the CDNB–GSH (DNP-SG) content. Cells were trypsinized 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 °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 and 250 Ω 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, 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 MK-571 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 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 min 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.

2.3. 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 150 mm × 4.6 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 min. 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: $[\text{DNP-SG}]_{\text{excreted}}/[\text{DNP-SG}]_{\text{total}}$, in order to obtain data for IC_{50} 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.

2.4. 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. [30].

2.5. 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 was injected onto a 150 mm × 4.6 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

$$K' = \frac{t_r - t_o}{t_o}$$

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

2.6. 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.

3. Results

3.1. 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 (Fig. 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.

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

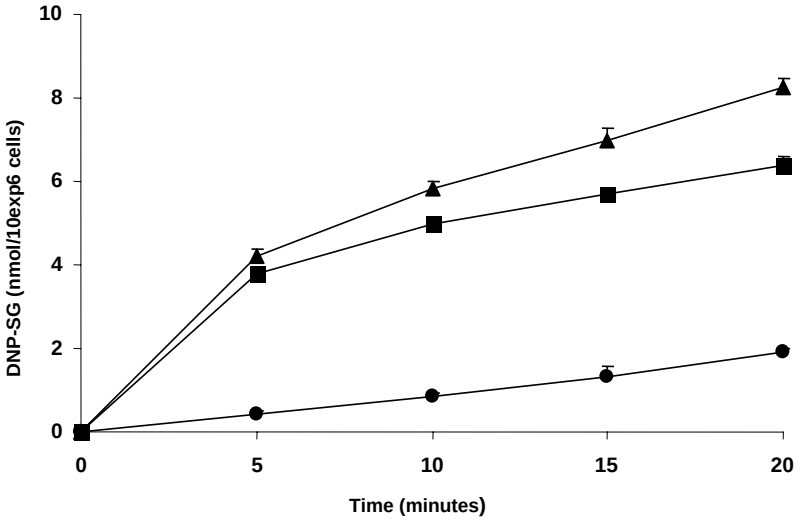


Fig. 2. The MCF7 pMTG5 cells as a model system. The formation of DNP-SG after addition of 10 μM CDNB to 10^6 cells. Samples were taken from the medium and cytosolic fraction at each timepoint, over a period of 20 min. The results are the means ± S.D. from triplicate measurements (excreted DNP-SG (●),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

was corrected for non-specific DNP-SG formation, i.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 min. 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.

3.2. 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 min in the presence of 25 μM of

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

Flavonoid	GST activity (nmol DNP-SG per 10^6cells)[a,b]	GSTP1-1 inhibition IC_{50} (μM)[b]	GS-X pump activity (nmol DNP-SG per 10^6cells)[a]	GS-X pump inhibition IC_{50} (μM)[c]
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′-Dihydroxyflavone	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

All values are the means of triplicate measurements ± S.D.

[a] Final concentrations for all flavonoids: 25 μM, and for CDNB: 10 μM.

[b] Amounts are corrected for chemical conjugation.

[c] The IC_{50} 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.

* Statistically significant difference from value for control ($P < 0.05$) according to Student's *t*-test.

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 IC_{50} was determined (Table 1). The strongest GSTP1-1 inhibitor is galangin with an IC_{50} of 14.4 μM. IC_{50}s of about 23–26 μM were obtained for eriodictyol, kaempferol and quercetin.

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.

3.3. 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. Fig. 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 IC_{50} values. The IC_{50} values obtained—expressing the potency of the flavonoids to inhibit the DNP-SG excretion by 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 IC_{50} values of 0.8 and 1.3 μM. The second group of flavonoid-type GS-X pump inhibitors consists of moderate inhibitors with IC_{50} 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 IC_{50} 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.

3.4. 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,

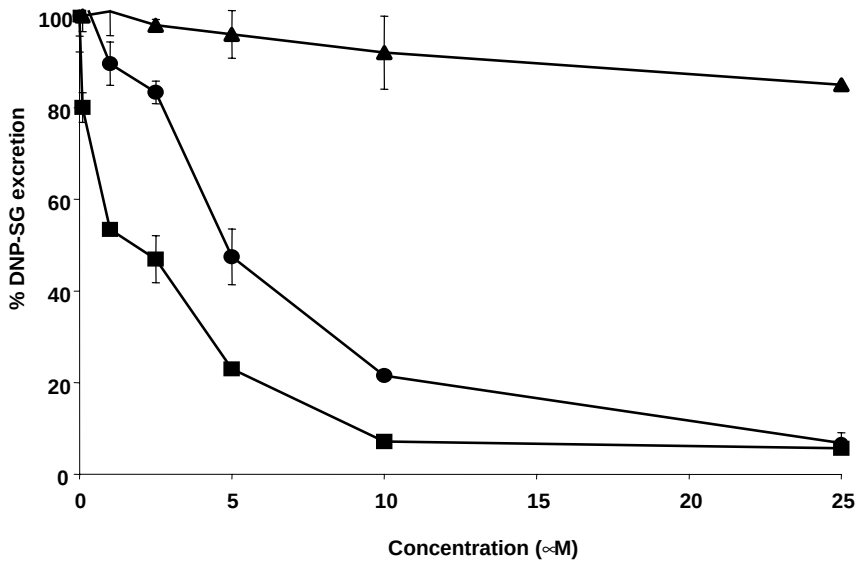


Fig. 3. The concentration-dependent effect of the flavonoids quercetin (■), kaempferol (●) and taxifolin (▲) on the excretion of DNP-SG in GSTP1-1 transfected pMTG5 cells following 20 min exposure to 10 μM CDNB. The results are the means ± S.D. from triplicate measurements.

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

Flavonoid	C3–C2–C1′–C2′ dihedral angle (°)[a]	Relative lipophilicity (K')[b]
Flavone	5.5	19.7
3′,4′-Dihydroxyflavone	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': $K' = (t_r - t_o)/t_o$.

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 IC_{50} 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°, reflecting loss of planarity between the B and C ring. Comparison of these data to the IC_{50} 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.

Finally, Fig. 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 IC_{50} 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.

3.5. 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]. Fig. 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

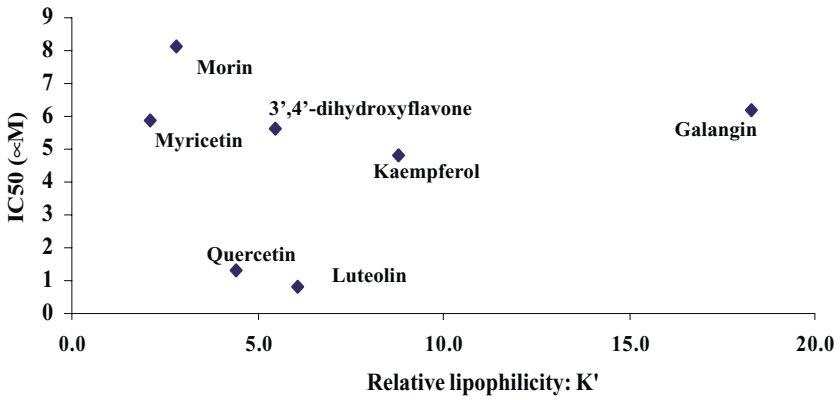


Fig. 4. Comparison between the relative lipophilicity (expressed as capacity factor K') of flavonoids which did show GS-X pump inhibition and their IC_{50} values for this GS-X pump inhibition.

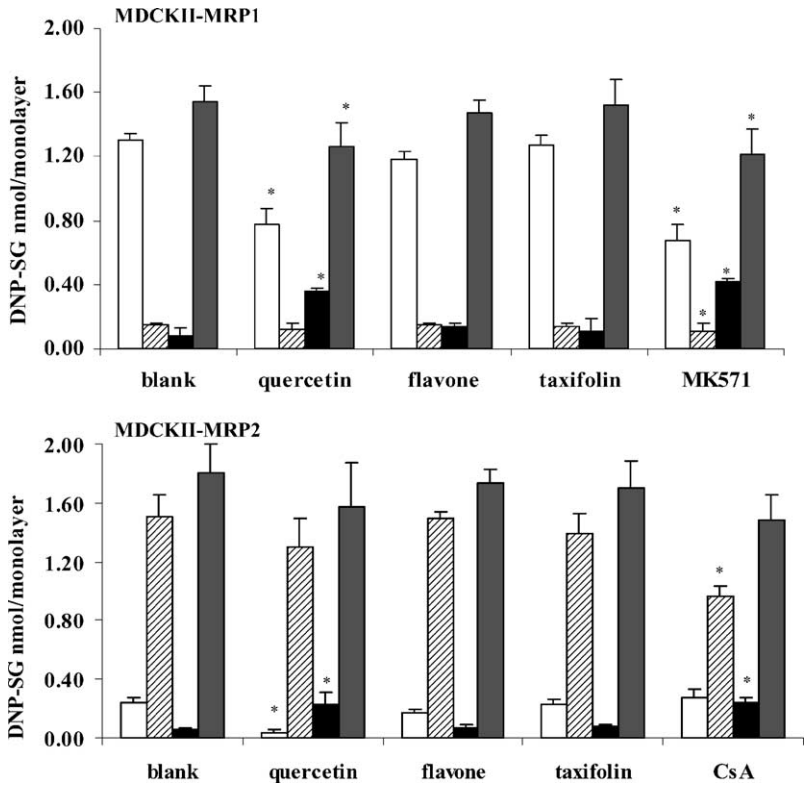


Fig. 5. Effect of the flavonoids quercetin, flavone and taxifolin (all 50 μM concentrations) on the formation and distribution of DNP-SG by MDCKII-MRP1 and MDCKII-MRP2 cells. Results present DNP-SG concentrations (nmol/monolayer) in the basolateral compartment (first bar), apical compartment (second 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 ± S.D. of incubations performed in duplicate. Those marked with asterisks differ significantly (ANOVA + Dunnetts' test) from the corresponding value in DMSO-treated cells ($P < 0.05$).

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.

4. 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 min) 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 IC_{50} 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, this 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 antioxidants 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 IC_{50} 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 IC_{50} 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°).

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, shows 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. [17] 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. [17] 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. [17]. Another and even more important difference between the study of Leslie et al. [17] 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 ATPase activity might affect the ATP-dependent GS-X pump activity. Structure activity studies for the inhibition of P-gp ATPase 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 sulfate-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 100–500 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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