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Review

Modulation by flavonoids of cell multidrug resistance mediated by P-glycoprotein and related ABC transporters

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Abstract. Cancer cell resistance to chemotherapy is often mediated by overexpression of P-glycoprotein, a plasma membrane ABC (ATP-binding cassette) transporter which extrudes cytotoxic drugs at the expense of ATP hydrolysis. P-glycoprotein (ABCB1, according to the human gene nomenclature committee) consists of two homologous halves each containing a transmembrane domain (TMD) involved in drug binding and efflux, and a cytosolic nucleotide-binding domain (NBD) involved in ATP binding and hydrolysis, with an overall $(TMD\text{-}NBD)_2$ domain topology. Homologous ABC multidrug transporters, from the same ABCB family, are found in many species such as *Plasmodium falciparum* and *Leishmania* spp. protozoa, where they induce resistance to antiparasitic drugs. In yeasts, some ABC transporters involved in resistance to fungicides, such as *Saccharomyces cerevisiae* Pdr5p and Snq2p, display a different $(NBD\text{-}TMD)_2$ domain topology and are classified in another family, ABCG. Much effort has been spent to modulate multidrug resistance in the different species by using specific inhibitors, but generally with little success due to additional cellular targets and/or extrusion of the potential inhibitors. This review shows that due to similarities in function and maybe in three-dimensional organization of the different transporters, common potential modulators have been found. An in vitro 'rational screening' was performed among the large flavonoid family using a four-step procedure: (i) direct binding to purified recombinant cytosolic NBD and/or full-length transporter, (ii) inhibition of ATP hydrolysis and energy-dependent drug interaction with transporter-enriched membranes, (iii) inhibition of cell transporter activity monitored by flow cytometry and (iv) chemosensitization of cell growth. The results indicate that prenylated flavonoids bind with high affinity, and strongly inhibit drug interaction and nucleotide hydrolysis. As such, they constitute promising potential modulators of multidrug resistance.

Key words. Flavonoids; multidrug resistance (MDR); P-glycoprotein; cancer cells; yeast; *Leishmania*; modulators; ABC transporters.

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Cancer cell resistance to chemotherapy and overexpression of P-glycoprotein

Chemotherapy occupies an important place among strategies to fight cancer. Unfortunately, tumor cells frequently continue to grow by developing resistance mechanisms, which constitutes a major problem for cancer treatment [1]. Among the different resistance mechanisms, a pleiotropic resistance against a series of drugs, or cellular MDR (multidrug resistance) phenotype, is often found. It is either intrinsic and immediate, or progressively develops during chemotherapeutic treatment [2].

The emergence of MDR phenotype is often correlated to overexpression of a membrane ATPase, P-glycoprotein [3] [ABCB1 according to the new nomenclature of ABC (ATP-binding cassette) proteins as defined on the Web site http://www.gene.ucl.ac.uk/users/hester/abc.html]. Other ABC transporters more recently discovered, such as MRP1 (multidrug resistance-associated protein 1, ABCC1) [4], MRP2 (ABCC2) and MRP3 (ABCC3) [5] as well as BCRP/MXR1 (ABCG2) [6, 7], also contribute to MDR, but P-glycoprotein is overexpressed to the highest level and plays the major role. It uses energy from ATP hydrolysis to extrude cytotoxic drugs out of cancer cells, causing them to become resistant to chemotherapy [8]. P-glycoprotein belongs to a small family of two isoforms in humans: MDR3 [ABCB4] selectively translocates phosphatidylcholine [9], whereas MDR1, which is constitutively involved in cellular detoxification [10] and responsible for the MDR phenotype, is also able to transport various phospholipids [9] and to catalyze hormone efflux. In rodents, MDR1 is replaced by two isoforms, Mdr1a and Mdr1b, whereas Mdr2 is equivalent to human MDR3 [11].

P-glycoprotein is located inside plasma membranes. It displays the schematic structural organization illustrated in figure 1 A, with two homologous halves each containing a transmembrane domain (TMD) preceding a cytosolic nucleotide-binding domain (NBD). Each TMD is composed of six transmembrane α-helix segments involved in drug binding and efflux [12]. Each NBD contains a nucleotide binding site with four consensus sequences: in addition to the well-known Walker A and B motifs [13] involved in the binding of the phosphate chain and the chelated magnesium ion, respectively, are found the ABC-transporter signature [14, 15] the role of which is still discussed, and a Q-containing loop recently reported to interact with the phosphate chain in Rad50 [16]. Both N- and C-terminal halves are linked by a phosphorylatable linker peptide.

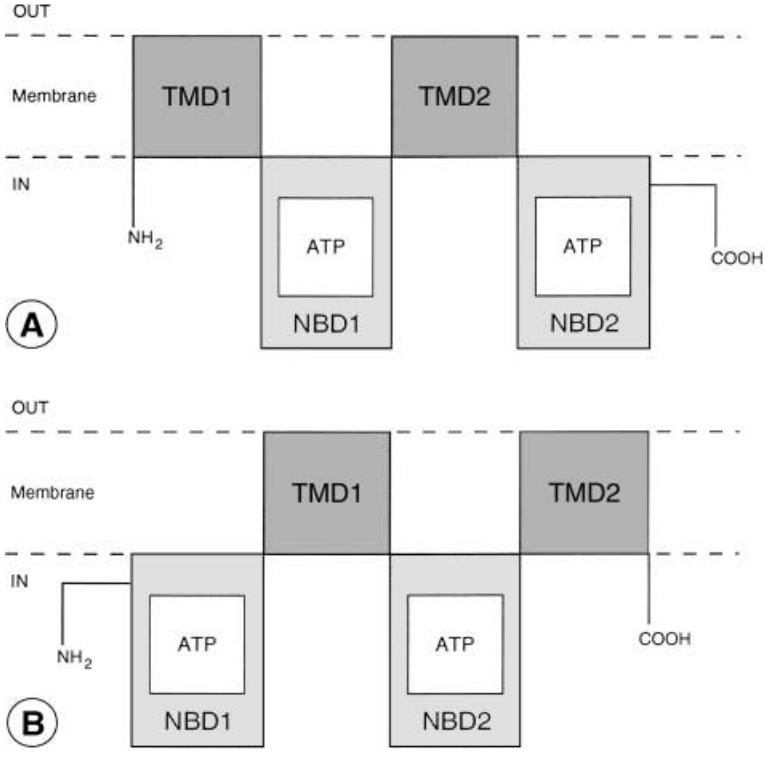


Figure 1. Schematic domain topology of cancer cell P-glycoprotein and related multidrug transporters from other species. Each transporter contains two cytosolic, N-terminal and C-terminal, nucleotide-binding domains (NBD1 and NBD2, respectively) and two hydrophobic transmembrane domains (TMD1 and TMD2, respectively). The domain topology within the sequences of cancer cell P-glycoprotein and *Leishmania tropica* Ltrmdr1, where NBDs follow TMDs (*A*, ABCB family), differs from that of yeast Pdr5p and Snq2p, where NBDs precede TMDs (*B*, ABCG family).

Up to now, only a rather low-resolution structure at 25 Å has been obtained for P-glycoprotein by electron microscopy and image analysis [17], mainly providing information about the overall domain spatial organization. In contrast, high-resolution structures have been obtained for several ATP-binding subunits of bacterial ABC proteins, such as HisP [18], MalK [19] and Rad50 [16]. Apart from the Walker A and B motifs, which always display the same interactions with bound nucleotide, the two other consensus sequences, ABC signature and Q-loop, exhibit protein-specific interactions, and the adenine-interacting residues vary in identity and position within the sequences. The 'dimeric' NBD/NBD interactions also diverge among the different bacterial proteins. Structurally unrelated substrates, histidine, maltose and double-stranded DNA, evidently have different protein structural requirements for function.

The two ATP binding sites in P-glycoprotein appear to be strictly cooperative and to alternate during pump turnover [20], with a maximal rate of a few μmoles of ATP hydrolyzed/min × mg of protein and a K_m(MgATP) in the millimolar range. *Ortho*-vanadate is a strong inhibitor of ATP hydrolysis, by mimicking phosphate and preventing the release of ADP, which is a rate-limiting step [21]. In this way, vanadate stabilizes a catalytic transition state of the enzyme, which has been used both to quantify 'nucleotide trapping' after photolabeling with radioactive 8-azido-ATP [22] and to identify a derivatized amino acid [23].

Cancer cells overexpressing P-glycoprotein exhibit cross-resistance to a number of structurally divergent drugs [2, 3, 8], including *vinca*-alkaloids, anthracyclins, taxans, epipodophyllotoxins, antibiotics, other cytotoxic compounds, peptides, hormones, human immunodeficiency virus (HIV) protease inhibitors. Most of these substrates share three common parameters: hydrophobicity, large size and positively charged nitrogen atom at neutral pH [24]. Two positively cooperative sites, with overlapping specificities, appear to be involved in drug binding and transport in P-glycoprotein [25], as well as in LmrA, a bacterial hemitransporter described below [26]. Drug extrusion from the inner leaflet of the membrane favors a 'hydrophobic vacuum cleaner' mechanism [8, 27]. MDR1 P-glycoprotein is able to translocate the NBD derivatives of various lipids [9, 28], but it remains to be seen whether normal phospholipids are indeed transported. A flippase mechanism has been proposed [29] and demonstrated for the human MDR3 [9] and mouse Mdr2 [30] isoforms, which are specific for phosphatidylcholine. A number of mutations have been introduced within the transmembrane α helices and the connecting loops to understand drug-substrate specificity, as summarized in a recent review [31]. Both mutations and affinity labelings have shown that the two last pairs of transmembrane segments, 5 and 6 within TMD1 together with 11 and 12 within TMD2, constitute major sites for drug interaction [32, 33]. A stoichiometry close to one-to-one for the coupling between hydrolyzed ATP and transported drugs has been found with a series of substrate drugs [22, 34].

P-glycoprotein homologous transporters are found in many species. In plants, such as *Arabidopsis thaliana*, AtPGP1 is involved in detoxification and in hypocotyl elongation in light-grown seedings [35]. In parasites, Pgh1 is responsible for *Plasmodium falciparum* resistance to multiple antimalarials [36], and mdr1-type transporters play similar roles in *Leishmania* [37, 38]. Ltrmdr1 from *Leishmania tropica* displays the same domain topology $(TMD\text{-}NBD)_2$ as cancer cell P-glycoprotein (fig. 1A). In bacteria, the hemitransporter LmrA from *Lactococcus lactis*, which functions as a homodimer [26] and confers the same phenotype as P-glycoprotein in human transfected cells [39], has been involved in bacterial resistance to antibiotics [40]. A number of bacteria also contain a highly homologous ABC hemitransporter [41]. In contrast, no obvious P-glycoprotein homologue is found in yeasts [42–44], whereas a number of ABC transporters such as Pdr5p [45, 46], Snq2p [47, 48], and probably also Pdr10p, Pdr11p and Pdr15p in *Saccharomyces cerevisiae*, belong to the PDR (pleiotropic drug resistance) subfamily, among the ABCG family, characterized by a mirror domain topology $(NBD\text{-}TMD)_2$ as illustrated in figure 1B. Despite such a variation in domain topology and significant differences in NBD1 Walker A and B motifs, and in NBD2 ABC-transporter signature [45], Pdr5p, which is overexpressed to a high level in strains with *pdr1-3* gain-of-function mutations and confers resistance to multiple antifungals, is able to bind and/or transport similar drugs and modulators as cancer cell P-glycoprotein [49]. Single-point mutations have been identified to either induce overexpression of Pdr5p [50] or to alter the drug-efflux activity of the transporter [51]. Very close homologues, Cdr1p and Cdr2p, are found in pathogenic *Candida albicans* [52, 53].

Problems in MDR modulation

Much effort has been spent to find out efficient modulators, or chemosensitizers, able to inhibit P-glycoprotein-mediated drug efflux and to restore drug cytotoxic effects in cancer cells. A number of modulators are able to reverse MDR in vitro, such as calcium channel blockers, calmodulin antagonists, hydrophobic peptides, protein kinase inhibitors, antibiotics, hormone derivatives and flavonoids [2, 8, 54]. Some of these modulators have in common a nitrogen atom and two planar aromatic rings [55]. More precise structure-activity relationships have been defined within the different classes of compounds by studying derivatives of quinacrine [55], phenothiazine [56], reserpine [57], phenoxazine [58], colchicine [24],

verapamil [59], prenylcysteine [60], propafenone [61], staurosporine [62, 63] or peptides [64, 65]. Some reviews have dealt with the classification of all the compounds reported to behave as modulators [66, 67].

A problem with most modulators is that they also become transported by P-glycoproteins. Therefore, their inhibitory effect is through competition, and the concentration has to be extremely high to be effective in vivo generating unendurable side effects, such as cardiotoxicity for verapamil or immunosuppression for cyclosporin A. It is also remarkable that these classical P-glycoprotein modulators are not able to efficiently overcome in vivo MDR in *Leishmania* spp. at noncytotoxic concentrations for the parasites [38]. A few modulatory compounds do not appear to be transported. This is the case for hydrophobic steroids like progesterone, megestrol acetate or medroxyprogesterone, which strongly modulate vinblastine efflux, as opposed to more hydrophilic derivatives, which are transported [68]. The antiprogestin RU486 is a potent modulator in vitro [69, 70], but due to its hormonal properties, clinical use might be at risk. Similarly, hydrophobic antiestrogens such as tamoxifen and derivatives bind with high affinity to P-glycoprotein and are not transported [71]. They are being tested in clinical trials against breast cancer, but have been reported to behave as agonists in some ovarian cells and are therefore suspected to favor endometrial cancers [72].

Another interesting, and promising, family of compounds as efficient modulators are the flavonoids.

Flavonoids: a large family of natural compounds with a number of healthy properties

A total of more than 6500 different flavonoids have been identified from plant sources [73] of which at least 400 appear to be prenylated [74]. Flavonoid compounds are particularly abundant in fruits (especially in *Citrus* [75]), vegetables, nuts, stems, flowers, wine and tea, and constitute important components of normal human food, with an average of 200 mg consumed in the daily Western diet [76]. The structures of a number of flavonoid classes and substituents are indicated in figure 2. Flavones are constituted of three conjugated rings, A and C being juxtaposed and B branched at position 2. Flavonols additionally contain a hydroxyl substituent at position 3 of C-ring, and dehydrosilybin is further substituted by a high-size monolignol unit consisting of two additional rings branched at positions 3′ and 4′ of B-ring. In isoflavones, the B-ring is branched at position 3, whereas the 2,3-bond of flavanones is reduced, thereby losing electron conjugation and ring planarity. In chalcones, the C-ring is open and the numbering is different. Apart from halogens and *O*-alkyl groups, all the other indicated substituents are natural, including glycosylation and prenylation.

Flavonoids are known to exhibit a number of beneficial properties for human health due to their interactions with a number of cellular targets [77], such as antioxidant and free-radical scavenger activities, as well as antiinflammatory, antiviral and especially anticancer properties.

The anticarcinogenic properties are of different natures: antimutagenic effects related to the ability of polyphenols to absorb ultraviolet radiation, inhibitory effects of carcinogenic cell invasion due to lowered cell motility and especially antiproliferative effects [75, 77]. The latter effects are mediated through two types of action: antiestrogen activity [78] due to mimicking of hormones (flavonoids are therefore often considered as 'phytoestrogens'), and inhibition of a series of protein kinases and ATP(adenosine 5′-triphosphate)ases due to mimicking of the ATP-adenine base. Among the latter are serine/threonine kinases [79, 80], tyrosine kinases [81, 82], topoisomerase II [83], myosin [84] as well as various membrane ATPases: mitochondrial H^+-ATPase [85], Na^+/K^+-ATPase [86], Ca^{2+}-ATPase [87] or H^+/K^+-ATPase [88].

Different flavonoid structure-activity relationships for distinct cellular targets

The multiple cell targets for flavonoids are not recognized by the same types of flavonoid and therefore display specific structure-function relationships. For example, flavonoid binding to estrogen receptors responsible for cell proliferation requires hydroxyl groups at B-ring positions 2′ and 3′, a double bond at C-ring positions 2–3, and the absence of any hydrophobic prenylated substituent [78]. In contrast, the free-radical scavenger activity is dependent on a hydroxyl at A-ring position 7, but not at C-ring position 3, which excludes flavonols [89].

Recognition of the ATP-binding site in various ATPases requires the presence of three hydroxyl groups at A-ring positions 5 and 7, and C-ring position 3, which favors some flavonols [88]. On the contrary, protein kinases exhibit different requirements: an isoflavone structure for tyrosin kinases [81], or flavones substituted at A-ring position 8 for CDK2 [80].

Flavonoid modulation of MDR in cancer cells, and interaction with multidrug transporters

Various and contradictory results have been reported for flavonoid effects on MDR and its reversal and on the proteins themselves, depending on the type of cancer cells and the chemotherapeutic drug used. Flavonols such as quercetin, kaempferol and galangin were reported to increase adriamycin efflux from HCT-15 colon cancer cells [90], whereas a hydrophobic quercetin derivative was able to both inhibit rhodamine 123 efflux from MCF-7

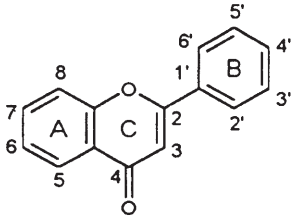
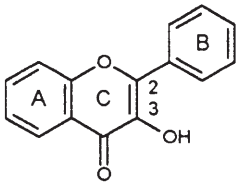
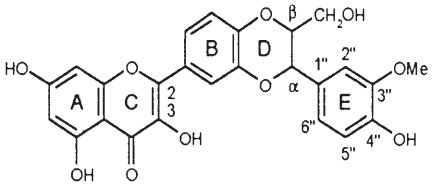
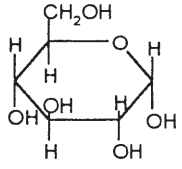
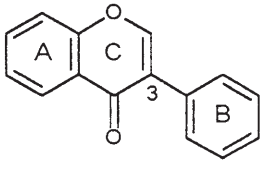
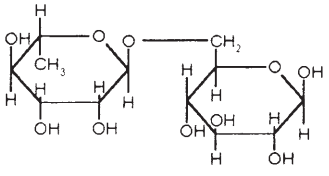
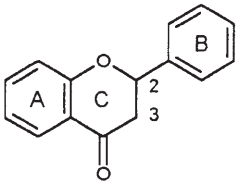
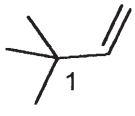
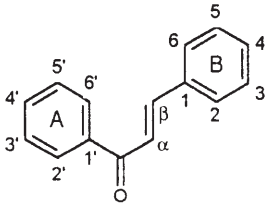
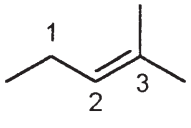
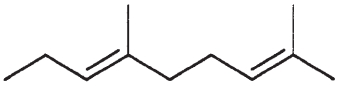
Flavonoid class		Substituents	
Flavones		Hydroxyle	OH
		Methyl	CH_3
		Methoxy	OCH_3
		Halogen	F Cl Br I
Flavonols		O-alkyl	O-*n*-C_2H_5 O-*n*-C_4H_9 O-*n*-C_6H_{13} O-cyclohexyl O-*n*-C_8H_{17} O-*n*-$C_{10}H_{21}$ O-*n*-$C_{14}H_{29}$
Dehydrosilybin		Glucoside	
Isoflavones		Rutinose (= gluco-rhamnose)	
Flavanones		Dimethylallyl (= DMA)	
Chalcones		Prenyl (= 3,3-DMA)	
		Geranyl	

Figure 2. Different classes and substituents of the studied flavonoids. The main classes of flavonoids studied here, with the corresponding numberings, are shown in the left panel. Their main substituents are described in the right panel.

breast cancer cells and abolish their MDR phenotype [91], and various favonols inhibited drug efflux from hepatocytes overexpressing P-glycoprotein [92]. The isoflavone genistein at higher concentration was also reported to inhibit drug efflux [93].

This discrepancy was probably, at least partly, related to multiple cellular targets, as detailed above. An alternative possibility might be the existence of different flavonoid binding sites within the same multidrug transporter. Indeed, quercetin was found not only to inhibit P-glycoprotein ATPase activity [94] but also to prevent the binding of transported drugs such as colchicin or Hoechst 33342, or on the contrary, to activate the binding of rhodamine 123 [25]. This further demonstrates the involvement of several interacting binding sites for drugs and modulators.

Flavonoid molecular interactions with P-glycoprotein and related multidrug transporters

We have investigated a large number of flavonoids, as listed in table 1, for their direct binding either to the NBD2 cytosolic domain of mouse P-glycoprotein or *Leishmania* Ltrmdr1, or to the whole Pdr5p yeast transporter by quenching of protein intrinsic fluorescence. This fluorescence was due to tryptophan residues: 1 in P-glycoprotein NBD2 [95, 96], 3 in Ltrmdr1 NBD2 [97] and 22 in Pdr5p [45].

An extensive study of flavonoids was performed with mouse P-glycoprotein NBD2. First, different classes of flavonoids were investigated for their binding ability, as estimated by determining K_D with the Grafit program (Erithacus software), the maximal quenching of fluorescence being generally high (80–100%). The following sequence in affinity was obtained: dehydrosilybin > chalcone > flavonol > flavone > isoflavone > flavanone, when comparing, for example, dehydrosilybin to 2′,4′,6′-tri*OH*-chalcone, galangine or kaempferol, chrysin or apigenin, genistein, and naringenin or silybin, respectively [96, 98, 99]. Second, inside the same class, the following efficiency of substituents was observed: alkoxyl, geranyl > dimethylallyl > halogen > monolignol > methoxy > hydroxyl > glycosyl. Hydrophobicity of the substituents was an important parameter since (i) alkoxylation up to 8–10 carbon atoms gradually increased the chalcone binding affinity [100], (ii) geranylation was better than prenylation in both chrysin and dehydrosilybin [98, 101] and (iii) halogens were better than H or OH in chalcones and the sequence I > Br > Cl > F correlated to increase in lipophilicity [99]. The positive effects of *n*-octyl and iodine substituents were also observed in galangin derivatives [102]. The positive effects of prenylation were observed in all classes, including flavones [101] and chalcones [103], as well as in other polyphenol compounds such as xanthones [104]. Other hydrophobic substituents were studied, such as isopropyl and benzyl, but gave more variable effects [101]. A positive effect was produced by the high-size monolignol unit when comparing silybin to taxifolin and dehydrosilybin to galangin [98]. Slightly positive effects were produced by methyl/methoxy groups, for example at either position 6, 7 or 8 of chrysin, position 4′ of galangin or position 4 of chalcone, but not at position 3 of galangin. The hydroxyl groups appeared to be important at position 3, when comparing flavonols to flavones, and at position 5 [96]. In contrast, all forms of glycosylation at different positions dramatically altered the binding affinity, as observed for rutin [86], and as well for apigenin-7-*O*-glucoside and vitexin.

Table 1. Direct binding of flavonoids to purified multidrug transporters or cytosolic domains, as monitored by quenching of intrinsic fluorescence.

Flavonoids	K_D (μM)		
	P-glycoprotein NBD2	Ltrmdr1 NBD2	Full-length Pdr5p
Flavones			
7-*OH*-flavone	34.9 ± 4.5	83.7 ± 15.5	
Chrysin (5,7-di-*OH*-flavone)	8.9 ± 0.3	17.6 ± 5.9	9.3 ± 1.8
6-Methyl-chrysin	3.1 ± 0.97		
Tectochrysin (5-*OH*,7-methoxy-flavone)	6.3 ± 4.3		
6,7-Dimethyl-chrysin	1.3 ± 0.35		
Apigenin (4′-*OH*-chrysin)	10.1 ± 1.9	15.7 ± 1.0	7.7 ± 1.4
Apigenin-7-*O*-glucoside	low quenching*		
Vitexin (apigenin-8-*C*-glucoside)	low quenching		
Acacetin (4′-methoxy-chrysin)		20.9 ± 2.0	
3′,4′-Difluoro-chrysin	6.3 ± 0.5		
4′-Iodo-chrysin	2.2 ± 0.4		
6-Isopropyl-chrysin	low quenching		
7-*O*-Isopropyl-chrysin	1.3 ± 0.34		
6,7-Diisopropyl-chrysin	low quenching		

Table 1. continued

Flavonoids	K_D (μM)		
	P-glycoprotein NBD2	Ltrmdr1 NBD2	Full-length Pdr5p
6,7,8-Triisopropyl-chrysin	low quenching		
6-Benzyl-chrysin	0.34 ± 0.04		
8-Benzyl-chrysin	0.99 ± 0.14		
6,8-Dibenzyl-chrysin	low quenching		
7-*O*-Benzyl-chrysin	low quenching		
6-Prenyl-chrysin	0.30 ± 0.03		1.1 ± 0.1
7-*O*-Prenyl-chrysin	low quenching		
8-DMA-chrysin	0.20 ± 0.05	1.4 ± 0.2	
8-Prenyl-chrysin	0.28 ± 0.04	2.9 ± 0.3	3.1 ± 0.2
6,8-Diprenyl-chrysin	low quenching		low quenching
6-Geranyl-chrysin	0.045 ± 0.005		3.4 ± 0.5
8-Geranyl-chrysin	0.025 ± 0.005		4.6 ± 0.4
8-DMA-apigenin	0.7 ± 0.1	0.7 ± 0.1	
Flavonols and 3-methyl derivatives			
3-*OH*-flavone	10.1 ± 2.5	18.7 ± 3.2	
Galangin (3,5,7-tri*OH*-flavone)	5.3 ± 0.1	9.2 ± 1.0	6.3 ± 0.5
Kaempferol (3,5,7,4′-tetra*OH*-flavone)	6.7 ± 0.3		
Kaempferide (3,5,7-tetra*OH*,4′-methoxy-flavone)	4.5 ± 0.2	13.6 ± 2.3	5.1 ± 0.6
Quercetin (3,5,7,3′,4′-penta*OH*-flavone)	7.0 ± 0.5		13.0 ± 2.9
8-DMA-kaempferide	0.20 ± 0.08	0.7 ± 0.1	1.6 ± 0.2
8-DMA-galangin	0.45 ± 0.07	0.34 ± 0.09	
6-Prenyl-galangin	0.21 ± 0.06		1.6 ± 0.1
8-Prenyl-galangin	0.22 ± 0.05	0.62 ± 0.24	2.2 ± 0.2
4′-Fluoro-galangin	6.8 ± 0.28		
2′,4′-Dichloro-galangin	4.0 ± 0.23		
4′-Iodo-galangin	1.1 ± 0.08		
4′-*n*-C_8H_{17}-galangin	0.06 ± 0.02		
Rutin (quercetin-3-*O*-rutinose)	low quenching	low quenching	low quenching
3-methyl-galangin	8.9 ± 0.6		6.4 ± 1.1
8-Prenyl-3-methyl-galangin			2.6 ± 0.3
8-DMA,3,7-dimethyl-galangin	0.15 ± 0.02		
Dehydrosilybin derivatives			
Dehydrosilybin	2.2 ± 0.1	2.3 ± 0.2	
6-Prenyl-dehydrosilybin	0.37 ± 0.02	0.27 ± 0.05	
8-Prenyl-dehydrosilybin	0.25 ± 0.01	0.11 ± 0.02	
6-Geranyl-dehydrosilybin	0.18 ± 0.01	0.67 ± 0.06	
8-Geranyl-dehydrosilybin	0.12 ± 0.01	0.31 ± 0.05	
Isoflavones			
Genistein	26.5 ± 5.5	64.2 ± 15	30.0 ± 7.8
Flavanones			
Naringenin (5,7,4′-tri*OH*-flavanone)	36.5 ± 3.5	59.7 ± 18.6	26.5 ± 5.1
Taxifolin (3,5,7,3′,4′-penta*OH*-flavanone)	37.4 ± 3.3		
Silybin (3,5,7-tri*OH*,3′,4′-monolignol-flavanone)	6.8 ± 0.3	9.2 ± 1.0	
Chalcones			
Chalcone (2′,4′,6′-tri*OH*)	4.6 ± 0.3		
4-*OH*-chalcone	4.8 ± 0.5		
4-Methoxy-chalcone	2.3 ± 0.2		
4-Fluoro-chalcone	3.6 ± 0.4		
4-Chloro-chalcone	1.3 ± 0.1		
4-Bromo-chalcone	0.57 ± 0.08		
4-Iodo-chalcone	0.25 ± 0.06		
4-*n*-C_2H_5-chalcone	2.1 ± 0.2		
4-*n*-C_4H_9-chalcone	1.0 ± 0.08		
4-*n*-C_6H_{13}-chalcone	0.27 ± 0.05		
4-*n*-Cyclohexyl-chalcone	0.53 ± 0.07		
4-*n*-C_8H_{17}-chalcone	0.02 ± 0.04		
4-*n*-$C_{10}H_{21}$-chalcone	0.06 ± 0.04		
4-*n*-$C_{14}H_{29}$-chalcone	14.2 ± 2.5		
4-*OH*-3-prenyl-chalcone	0.53 ± 0.09		
Broussochalcone A (3,4-di*OH*,5′-prenyl-chalcone)	0.44 ± 0.07		

* When the quenching was low (<40%), the K_D was not determined.

Slightly lower affinities were obtained with the N-terminal cytosolic domain, NBD1, of mouse P-glycoprotein for quercetin and dimethylallyl derivatives of apigenin, kaempferide and ermanin [105]. However, the experiments were performed at pH 9.0 instead of 6.8 for NBD2, due to the much higher solubility of NBD1 at alkaline pH [106, 107]. Such an increase in pH lowered the kaempferide binding affinity for NBD2 [108]; therefore, both NBD1 and NBD2 exhibited similar binding affinities for flavonoids under comparable conditions. The same preference for prenylated flavonoids as compared with unsubstituted compounds was observed for the human P-glycoprotein NBD2 [109].

Similar structure-activity relationships were obtained with parasite Ltrmdr1 NBD2, despite a lower solubility of the recombinant protein which required (i) renaturation from inclusion bodies, (ii) a need for a residual 10-mM imidazole concentration and (iii) a requirement to perform excitation at 288 nm instead of 295 nm [97]. In addition, the 1,1-dimethylallyl (DMA) isomer appeared to be better than the 3,3- one (prenyl) in both chrysin and galangin, prenylation at position 8 was found slightly more efficient than at position 6 and prenylation appeared better than geranylation [110]. The recombinant NBD from the *Bacillus subtilis* YvcC ABC transporter, highly homologous to *Lactococcus lactis* LmrA [26], also bound the 8-dimethylallyl derivative of kaempferide with much higher affinity than unsubstituted kaempferide [111].

The prenylation effects produced in full-length Pdr5p from yeast were qualitatively comparable, but quantitatively much lower [112]. This might be attributable, at least partly, to the presence of residual detergent, 0.02% *n*-dodecyl β-D-maltoside, required to keep the transporter soluble, which is expected to lower protein interactions with hydrophobic ligands.

Both NBD1 [107] and NBD2 [96] from P-glycoprotein, as well as NBD2 from Ltrmdr1 [97], contain a region interacting with hydrophobic steroid derivatives such as RU486. This region is probably located in close proximity to the ATP binding site since RU486 completely prevents or displaces the hydrophobic nucleotide derivative 2′(3′)-methylanthraniloyl-ATP (MANT-ATP) [107]. The binding of kaempferide to P-glycoprotein NBD2 was partly prevented by preincubation with either ATP or RU486, or additively by both ATP and RU486, which suggests that kaempferide displays bifunctional interactions with the ATP binding site and the hydrophobic steroid-interacting region [96]. The binding of flavonoids to the ATP binding site was also monitored by studying their ability to prevent photoaffinity labeling by [γ-^{32}P]TNP-8azido-ATP, which was shown to label the ATP site of Ca^{2+}-ATPase [113]. Table 2 shows that kaempferide indeed bound to the ATP binding site since it prevented NBD2 photolabeling with a IC_{50} value (concentration producing 50% inhibition of labeling) similar to the K_D for direct binding [114]. However, among a total of 29 flavonoids tested, only 3 were found to bind to the ATP site. Galangin and dehydrosilybin are also flavonols, indicating that the hydroxyl at position 3 is critical; the higher IC_{50} value for dehydrosilybin as compared with the K_D for direct binding suggests that extension of B-ring is possible but that the high size of the monolignol unit might produce some steric hindrance. In addition to the importance of hydroxyl group at position 3, since neither flavones nor chalcones had any preventive effect, the oxidation of the 2, 3-bond was critical since silybin was inefficient. These requirements are similar to those observed for quercetin binding to the Hck tyrosine kinase as demonstrated by cocrystallization [82], and for other ATPases by inhibition kinetics [88]. In contrast, they differ from those concerning the cyclin-dependent CDK2, the crystal structure of which was determined with bound chrysin derivatives [80]. Interestingly, hydrophobic substitution by prenylation at either position 6 or 8, which considerably increased the binding affinity for P-glycoprotein NBD2, shifted flavonol binding outside the ATP binding site as it did not compete anymore with nucleotides. It is likely that the binding of prenyl-flavonols might better overlap the hydrophobic steroid-interacting region than the binding of unsubstituted ones.

The effects of flavonoids on nucleoside triphosphate hydrolysis was studied in vitro, on the yeast Pdr5p transporter [112] within enriched plasma membranes (table 3). Chrysin and quercetin behaved as poor inhibitors, but flavonoid prenylation markedly increased the efficiency of inhibition, up to a IC_{50} value of 4.9 μM for 6-prenyl-galangin towards UTP(uridine 5′-triphosphate)ase activity; the inhibition appeared to be noncompetitive. In the case of cyclic AMP-dependent kinase, a prenylated derivative, waranglone, also behaved as a much more potent inhibitor than the corresponding unsubstituted flavonoid, and produced a noncompetitive inhibition with respect to ATP [115].

The same flavonoids and prenylated derivatives were very efficient for inhibiting the energy-dependent interaction of rhodamine 6G with the Pdr5p-enriched plasma membranes [112]. Here, also, the prenylated derivatives were much more potent inhibitors than the unsubstituted flavonoids for both chrysin, kaempferide, 3-methyl-galangin and galangin. The best affinity was also obtained for 6-prenyl-galangin, with a IC_{50} value of 0.24 μM, indicating a 200-fold higher inhibition efficiency than for ATPase activity. In addition, the inhibition was always found to be competitive with respect to rhodamine 6G. Therefore, prenyl-flavonoids exhibit a marked preference for binding to the drug binding site(s) of Pdr5p over the ATP binding site(s).

Table 2. Effects of flavonoid substituents on binding to the ATP site of P-glycoprotein NBD2, as monitored by protection against photolabeling of the recombinant domain by radioactive TNP-8N_3-ATP.
The purified domain was preincubated with increasing concentrations of each flavonoid and then photolabeled by the ATP derivative.

Flavonoids	Substituents							IC_{50} of TNP-8N_3-ATP photolabeling*
	3	5	6	7	8	3′	4′	
Flavones								
Chrysin		OH		OH				n.i.†
6-Prenyl-chrysin		OH	prenyl	OH				n.i.
7-Prenyl-chrysin		OH		O-prenyl				n.i.
Tectochrysin		OH		OCH_3				n.i.
8-DMA-chrysin		OH		OH	DMA			n.i.
8-Prenyl-chrysin		OH		OH	prenyl			n.i.
6,8-Diprenyl-chrysin		OH	prenyl	OH	prenyl			n.i.
6-Geranyl-chrysin		OH	geranyl	OH				n.i.
8- Geranyl -chrysin		OH		OH	geranyl			n.i.
4′-Fluoro-chrysin		OH		OH			F	n.i.
4′-Iodo-chrysin		OH		OH			I	n.i.
Flavonols								
Galangin	OH	OH		OH				20 μM
6-Prenyl-galangin	OH	OH	prenyl	OH				n.i.
8-Prenyl-galangin	OH	OH		OH	prenyl			n.i.
Kaempferide	OH	OH		OH			OCH_3	1.9 μM
8-DMA-kaempferide	OH	OH		OH	DMA		OCH_3	n.i.
8-DMA-3-Me-kaempferide	OCH_3	OH		OH	DMA		OCH_3	n.i.
Dehydrosilybin derivatives								
Dehydrosilybin	OH	OH		OH		monolignol unit		32 μM
6-Prenyl-dehydrosilybin	OH	OH	prenyl	OH		monolignol unit		n.i.
8-Prenyl-dehydrosilybin	OH	OH		OH	prenyl	monolignol unit		n.i.
Silybin (reduced 2,3-bond)	OH	OH		OH		monolignol unit		n.i.
		2′	3′	4′	5′	6′	3	4	
Chalcones									
4-OH-chalcone		OH		OH		OH		OH	n.i.
4-C_2H_5-chalcone		OH		OH		OH		C_2H_5	n.i.
4-C_4H_9-chalcone		OH		OH		OH		C_4H_9	n.i.
4-C_6H_{13}-chalcone		OH		OH		OH		C_6H_{13}	n.i.
4-C_8H_{17}-chalcone		OH		OH		OH		C_8H_{17}	n.i.
4-Fluoro-chalcone		OH		OH		OH		F	n.i.
4-Iodo-chalcone		OH		OH		OH		I	n.i.
4-OH-3-prenyl-chalcone		OH		OH		OH	prenyl	OH	n.i.

* The IC_{50} corresponds to the flavonoid concentration producing a 50 % inhibition of labeling.
† n.i. meams that no inhibition was observed up to a 50 μM concentration.

Cellular effects of flavonoids

Prenyl-flavonoids produced an efficient inhibition of P-glycoprotein-mediated drug efflux within leukemic K562/R7 cells [101], as monitored by flow cytometry (fig. 3). The greatest effect was produced by 8-prenyl-chrysin, but significant effects were also produced by 6-prenyl, 8-geranyl, 6-geranyl and 6,8-diprenyl derivatives. Clearly, although hydrophobicity is a critical parameter for both binding affinity towards NBD2 and inhibition of cellular P-glycoprotein activity, other determinants are also important for the inhibition. Indeed, the most efficient inhibitor, 8-prenyl-chrysin, is a moderately hydrophobic compound, characterized by a high-affinity binding for, and a high maximal fluorescence quenching (>80%) of, P-glycoprotein NBD2. In contrast, more hydrophobic compounds with lower inhibitory effects, such as isopropyl-, diprenyl- and geranyl- derivatives, probably bound differently to NBD2 since the maximal quenching was significantly lower [101]. Hydrophobic substitution at position 7 with a *N*-benzylpiperazine chain was also found to increase the potency of inhibiting P-glycoprotein, and overall lipophilicity was also concluded to be an important determinant, although not the only one [116].

Prenylation was also critical in vivo to improve flavonoid inhibiton in the case of the *Leishmania tropica* multidrug transporter Ltrmdr1 since 8-DMA-kaempferide was a better modulator than either cyclosporin A or verapamil [97], whereas apigenin produced a significant but much

Table 3. Inhibition by flavonoids of nucleotide hydrolysis by, and energy-dependent rhodamine 6G interaction with, yeast plasma membranes enriched with Pdr5p.

Flavonoids	IC_{50} nucleotide hydrolysis (μM)		
	ATPase activity	UTPase activity	I_{50} rhodamine 6G interaction (μM)
Chrysin	high*	high*	8.5 ± 0.6
8-Prenyl-chrysin			0.94 ± 0.05
6,8-Diprenyl-chrysin			0.39 ± 0.01
8-Geranyl-chrysin	high*	180	1.5 ± 0.01
6-Geranyl-chrysin			0.43 ± 0.05
Quercetin	high*	450	1.5 ± 0.01
Kaempferide			1.3 ± 0.1
8-DMA-kaempferide	high*	38.6 ± 5.3	0.33 ± 0.04
3-Methyl-galangin			5.9 ± 0.2
8-Prenyl-3-methyl- galangin	80.4 ± 5.6	14.4 ± 5.1	0.67 ± 0.05
8-DMA-3-methyl-galangin			0.49 ± 0.02
Galangin			2.2 ± 0.1
8-Prenyl-galangin			0.57 ± 0.03
8-DMA-galangin			0.39 ± 0.01
6-Prenyl- galangin	50 ± 10	4.9 ± 0.8	0.24 ± 0.03

* High IC_{50} was due to insufficient inhibition, preventing accurate determination of a precise value.

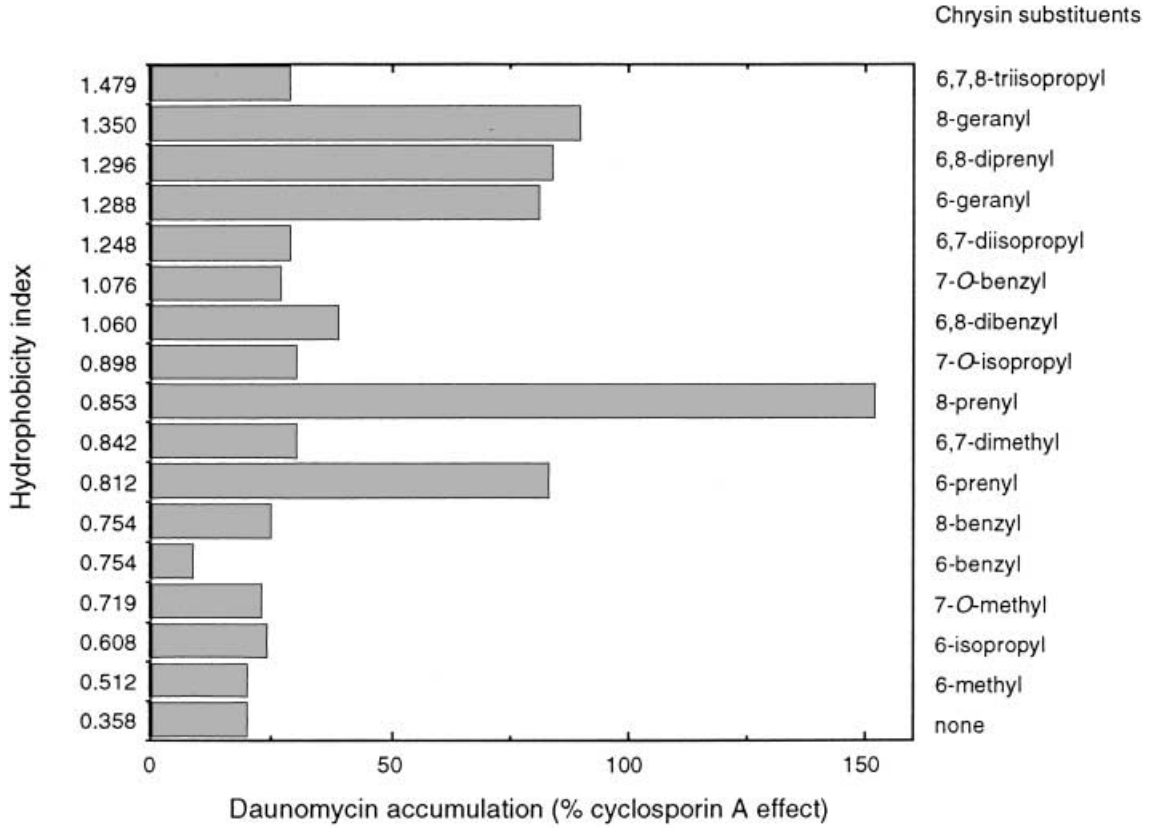


Figure 3. Intracellular daunomycin accumulation in leukemic K562/R7 human cells upon addition of chrysin derivatives. P-glycoprotein-overexpressing K562/R7 cells were preincubated with 10 μM of each chrysin derivative, mixed with daunomycin and assayed by flow cytometry for the intracellular remaining fluorescence. The modulatory effect was expressed with respect to that produced by 2 μM cyclosporin A. The hydrophobicity index of each chrysin derivative was determined by thin-layer chromatography on C18 reverse-phase silica gel [91].

less effect, and no inhibition at all was observed with rutin (table 4). Therefore, prenylation appears to be important for both increasing the binding affinity towards the cytosolic domain of this parasite transporter and inhibiting drug efflux to the extracellular medium.

Finally, prenylation was quite determinant for chemosensitizing the parasite growth [110] to the presence of cytotoxic drugs such as daunomycin at high concentration (table 5). This was true for any flavonoid tested such as chrysin, galangin or dehydrosilybin, as well for DMA, prenyl and geranyl, at either position 6 or 8. The best effect was produced by 8-prenyl-dehydrosilybin at 10 μM, since almost no growth inhibition was observed for the wild-type strain, whereas a marked effect was seen with the drug-resistant strain. However, the DMA isomer substituent was better in chrysin and galangin, suggesting that DMA-dehydrosilybin would be expected to behave as an even stronger chemosensitizer. Unsubstituted dehydrosilybin appeared to be highly cytotoxic, as compared with silybin; this might be attributable to its ability to interact with ATP binding sites which are present on a number of other cellular targets.

Therefore, we observed a strong correlation between the affinity of in vitro binding to the *Leishmania* Ltrmdr1 NBD2, and the efficiency of both in vivo modulation of drug accumulation and reversion of the resistant phenotype in the MDR *L. tropica* line [97, 110].

A tentative mechanism for the interaction of flavonoids with P-glycoprotein and related multidrug transporters is proposed on figure 4. Unsubstituted flavonols, such as galangin, kaempferol, kaempferide or dehydrosilybin, appear to interact bifunctionnally with cytosolic NBDs: the hydroxyl groups at positions 3 and 5, in addition to the ketone at position 4, would bind to the ATP binding site, whereas other parts of the molecule would bind to a vicinal region able to interact with hydrophobic steroid derivatives. Prenylation at either position 6 or 8 of the A-ring would increase hydrophobic interactions with both the cytosolic steroid-interacting region and the mem-

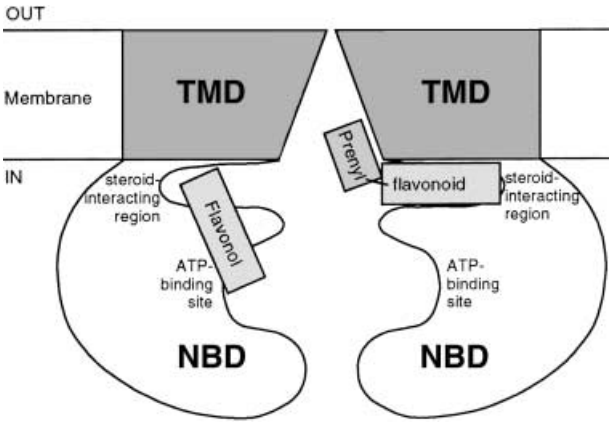


Figure 4. Schematic interaction of flavonoids with P-glycoprotein and related multidrug transporters. Flavonols, such as kaempferide, galangin or dehydrosilybin, display bifunctional interactions with NBDs, at both the ATP binding site and a vicinal sequence interacting with hydrophobic steroids such as RU486. Prenylation would strongly increase flavonoid interaction with both the NBD steroid-interacting sequence and the TMD drug binding site, while preventing overlapping of the ATP binding site.

Table 4. Inhibition of *Leishmania tropica* multidrug transporter activity, as monitored by intracellular daunomycin accumulation in drug-resistant parasites measured by flow cytometry.

Modulator	Concentration (μM)	Intracellular daunomycin accumulation	
		% of the control value*	% cyclosporin A effect†
None	–	(100)	
Cyclosporin A	75	221	(100)
Verapamil	75	270	122
Apigenin	75	133	60
	100	168	75
	150	259	117
	200	272	123
8-DMA-kaempferide	15	169	76
	25	295	133
	50	384	173
	75	389	176
Rutin	75	100	45
	100	78	35
	200	67	30
	300	67	30

* The control was performed in the absence of any modulator.
† The results are expressed with respect to the effect produced by 75 μM cyclosporin A.

Table 5. Chemosensitization to 150 μM daunomycin, as monitored by differential inhibition of parasite cell growth.

Flavonoid	Concentration (μM)	% cell growth inhibition		
		Wild-type strain	Drug-resistant strain	Differential inhibition
Chrysin	75	28.5	54.0	25.5
8-Prenyl-chrysin	20	9.3	11.4	2.1
	30	29.7	46.6	16.9
	40	37.1	71.5	34.4
8-DMA-chrysin	20	12.6	31.4	18.8
	30	25.4	87.9	62.5
Galangin	75	15.3	35.4	20.1
8-Prenyl-galangin	40	14.3	50.9	36.6
	50	31.7	79.4	47.7
8-DMA-galangin	10	0.5	31.5	31.0
	20	22.1	81.6	59.5
8-DMA-kaempferide	25	3.1	28.2	25.1
	50	22.5	78.6	56.1
Silybin	100	8.4	15.9	7.5
	200	9.7	27.8	18.1
	300	29.2	55.8	26.6
Dehydrosilybin	20	61.8	43.7	– 18.1
8-Prenyl-dehydrosilybin	2.5	5.5	34.7	29.2
	5	9.7	64.0	54.3
	10	15.2	95.3	80.1
6-Prenyl-dehydrosilybin	10	3.9	13.5	9.6
	20	0.5	41.0	40.5
	30	12.3	89.2	76.9
8-Geranyl-dehydrosilybin	10	6.6	38.8	32.2
	20	0.9	51.5	50.6
	30	61.1	94.7	33.6
6-Geranyl-dehydrosilybin	10	4.1	35.6	31.5
	20	0.7	62.3	61.6
	30	30.8	98.2	67.4

brane drug binding site of the full-length transporter. This would produce a significant shift in flavonoid positioning, in such a way that overlapping of the ATP binding site would no longer occur. Such a prenyl-flavonoid positioning appears to be efficient enough to directly inhibit drug binding and transport, while indirectly interfering with ATP hydrolysis or energy transduction. In this way, prenyl-flavonoids appear to be quite promising modulators of MDR, as mediated by P-glycoprotein in cancer cells and related ABC transporters in other species.

Since P-glycoprotein is located in the apical membrane of a number of epithelial cells, including jejunum and colon, its activity is now recognized to limit the oral absorption of drugs by mediating their secretion from blood to intestinal lumen [117, 118]. However, drug bioavailability also depends on the activity of cytochrome P450 (CYP) phase I enzymes which are expressed in the intestine [119]. Interestingly, the two enzymes share many substrates [120] and inhibitors [119], and both appear to be regulated by similar compounds [121]. This raised the question whether some diet components could act as P-glycoprotein and CYP inhibitors, and enhance drug bioavailability [122]. Increased concentration of many drugs has been demonstrated when coadministrated with grapefruit juice. However, this might be due to inhibition of either CYP enzymes, presumably by naringenin [123], or P-glycoprotein by another compound [124]. On the contrary, it has been shown that grapefruit juice significantly activates the efflux of drugs that are P-glycoprotein substrates [125]. Thus the activating effect of grapefruit juice on P-glycoprotein would partially counteract its CYP3A-inhibitory effect, which contrasts with the earlier assumption that intestinal CYP3A share common inhibitors with P-glycoprotein. In conclusion, the involvement of food components on drug bioavailability certainly deserves more attention. For example, flavonoids like naringenin may in fact inhibit CYP phase I enzymes more than P-glycoprotein. In addition, the link between CYP and P-glycoprotein activities is still a matter of debate. There are reports on independent regulation of the two enzymes [119], and increasing evidence for differential inhibition [126, 127], such as polymethoxylated

flavones from orange juice, which inhibit P-glycoprotein but not CYP3A4 [128].

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