

Flavonoid Structure-Activity Studies Identify 6-Prenylchrysin and Tectochrysin as Potent and Specific Inhibitors of Breast Cancer Resistance Protein ABCG2

Abdelhakim Ahmed-Belkacem,¹ Alexandre Pozza,¹ Francisco Muñoz-Martínez,² Susan E. Bates,³ Santiago Castanys,² Francisco Gamarro,² Attilio Di Pietro,¹ and José M. Pérez-Victoria^{1,2}

¹Institut de Biologie et Chimie des Protéines, Centre National de la Recherche Scientifique-Université Claude Bernard de Lyon, BioSciences Lyon-Gerland, Lyon, France; ²Instituto de Parasitología y Biomedicina "Lopez-Neyra," Consejo Superior de Investigaciones Científicas, Granada, Spain; and ³Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland

Abstract

Overexpression of breast cancer resistance protein ABCG2 confers multidrug resistance in cancer cells. The GF120918-sensitive drug efflux activity of human wild-type (R482) ABCG2-transfected cells was used for rational screening of inhibitory flavonoids and establishment of structure-activity relationships. Flavones were found more efficient than flavonols, isoflavones, and flavanones. Differentially substituted flavone derivatives indicated positive OH effects at position 5, in contrast to positions 3 and 7. A methoxy at position 7 was slightly positive in tectochrysin, whereas a strong positive effect was produced by prenylation at position 6. The potency of 6-prenylchrysin was comparable with that of GF120918 (IC₅₀ = 0.3 μmol/L). Both 6-prenylchrysin and tectochrysin seemed specific for ABCG2 because no interaction was detected with either P-glycoprotein or MRP1. The ABCG2 resistance profile *in vitro* is altered by mutation at amino acid 482. The R482T mutation limited the effect of prenylation on ABCG2 inhibition. Whereas GF120918 strongly inhibited the ATPase activity of wild-type ABCG2, neither 6-prenylchrysin nor tectochrysin altered the activity. In contrast, all three inhibitors stimulated the ATPase activity of mutant ABCG2. 6-Prenylchrysin at 0.5 μmol/L efficiently sensitized the growth of wild-type ABCG2-transfected cells to mitoxantrone, whereas higher concentrations were required for the mutant ones. In contrast, 1 μmol/L tectochrysin was sufficient to fully sensitize mutant ABCG2-transfected cells, whereas higher concentrations were required for the wild-type ones. Both flavones exhibited a lower intrinsic cytotoxicity than GF120918 and were apparently not transported by ABCG2. 6-Prenylchrysin and tectochrysin therefore constitute new and promising inhibitors for the reversal of ABCG2-mediated drug transport. (Cancer Res 2005; 65(11): 4852-60)

Introduction

Intrinsic or acquired drug resistance in tumors is a main reason for the failure of anticancer chemotherapy. The multidrug resistance (MDR) cell phenotype has classically referred to tumors that are refractory to chemically unrelated

cytotoxic agents, as due to their active outward transport. Several ATP-binding cassette (ABC) transporters are involved in MDR, including P-glycoprotein (MDR1/ABCB1; ref. 1), the multidrug resistance protein (MRP1/ABCC1; ref. 2), and the more recently discovered breast cancer resistance protein (ABCG2/BCRP/MXR/ABCP; refs. 3–5). These transporters are located in the plasma membrane and extrude a variety of drugs by using the energy of ATP hydrolysis. ABCG2 is a “half transporter” that homodimerizes to form an active transporter (6), but it has also been detected as tetramers or higher forms of oligomerization (7). The spectrum of ABCG2-effluxed anticancer drugs includes mitoxantrone, camptothecin derivatives, methotrexate, and anthracyclines (8). The precise substrate profile depends on a hotspot mutation at position 482 such that methotrexate is only transported by wild-type ABCG2 (R482) and anthracyclines and rhodamine 123 only by mutant ABCG2 (R482T or R482G), whereas Hoechst33342 and mitoxantrone are transported by all variants (9). Although single-nucleotide polymorphisms have been found at several residues in ABCG2, only arginine has been identified at position 482 in clinical isolates (10). Although the substrate spectrum is highly overlapping with P-glycoprotein, this is not the case for inhibitors, which may explain the failure of attempts to circumvent clinical MDR with P-glycoprotein inhibitors (11). The wide tissue distribution of ABCG2 also supports its role in controlling the permeability of important pharmacologic barriers (8). Therefore, ABCG2 inhibitors are of high interest, not only as reversal agents of clinical drug resistance, but also as agents that might improve the pharmacokinetics of substrate chemotherapeutic drugs by enhancing their oral bioavailability, plasma half-lives, and brain and fetal penetration. Recently, we and others have exhaustively studied natural and hemisynthetic flavonoids as modulators of P-glycoprotein (12–15), MRP1 (16–18), and related parasite (19, 20) and yeast (21) multidrug transporters. Flavonoids constitute a group of interesting polyphenolic compounds with a wide distribution in plants (22). As ABCG2 has been described to confer resistance to flavopiridol (23), a flavonoid-like cell cycle inhibitor currently in clinical trials, we analyzed the ability of flavonoids to modulate the transporter activity and found a potent inhibitory activity for some flavonol derivatives (24). Several publications then appeared, describing the interaction of a number of natural flavonoids inducing either inhibition of drug efflux in drug-selected ABCG2-overexpressing cells (25, 26) or reversal of multidrug resistance in transfected cells (27), but no correlation between flavonoid structure and activity was evident. Our aim was to establish structure-activity relationships for the inhibition of mitoxantrone efflux by wild-type ABCG2-transfected cells by screening a number of structurally related derivatives. The present

Note: A. Di Pietro and J.M. Pérez-Victoria are equal senior investigators in this study.

Requests for reprints: Attilio Di Pietro, Institut de Biologie et Chimie des Protéines, UMR5086 Centre National de la Recherche Scientifique-Université Claude Bernard de Lyon, IFR128 BioSciences Lyon-Gerland, 7 Passage du Vercors, 69367 Lyon Cedex 07, France. Phone: 33-4-72722629; Fax: 33-4-72722604; E-mail: a.dipietro@ibcp.fr.
©2005 American Association for Cancer Research.

results showed that the hydrophobic flavone 6-prenylchrysin was a potent inhibitor, 15-fold more efficient than chrysin. Interestingly, the R482T mutation limited this prenylation-dependent effect, whereas the inhibitory potency of tectochrysin was even increased, especially towards rhodamine transport. Both 6-prenylchrysin and tectochrysin were able to sensitize ABCG2-transfected cells to mitoxantrone, with low intrinsic cytotoxicity and no significant interaction with either P-glycoprotein or MRP1. These compounds therefore might be considered as potent and specific ABCG2 modulators with potential clinical interest.

Materials and Methods

Chemical compounds. Mitoxantrone and rhodamine 6G were purchased from Sigma (L'Isle d'Abeau, Chesnes, France), and nonprenylated flavonoids from Extrasynthèse (Genay, France). Prenylated derivatives of chrysin were obtained as previously described (15, 28). GF120918 was provided by GlaxoSmithKline (Madrid, Spain).

Cell cultures. The human fibroblast HEK-293 lines transfected with either wild-type or mutant ABCG2 or the empty vector were obtained as described (29). The human breast cancer cell lines MDA-MB-231 stably transfected by either *pcDNA3* or *pcDNA3-ABCG2* encoding for the T482 mutant transporter (4), were kindly provided by Dr. Douglas Ross (University of Maryland School of Medicine, Baltimore, MD). As this ABCG2-transfected cell line showed a heterogeneous profile of rhodamine 6G efflux by flow cytometry, cell line subcloning was done by the limited dilution method. The NIH-3T3 drug-sensitive parental cell line and the NIH-3T3 MDR-G185 cell line transfected with human *MDR1-G185* (30) were provided by Dr. I. Pastan (National Cancer Institute, NIH, Bethesda, MD). The 2008 human ovarian carcinoma cell line transfected with the plasmid pCMV-Neo and the 2008-MRP1 cell line transfected with the same plasmid but containing the cDNA of human MRP1 (31) were provided by Dr. P. Borst (Division of Molecular Biology, The Netherlands Cancer Institute, Amsterdam, the Netherlands). All cells were cultured in DMEM glutamax II (Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and drug supplemented in some cases with either 500 µg/mL G418 (MDA-MB-231 and HEK-293) or 60 ng/mL of colchicine (NIH-3T3 MDR-G185).

Flow cytometry. HEK-293 cells were exposed to 5 µmol/L mitoxantrone for 30 minutes at 37°C in the presence or absence of various concentrations of flavonoids added as DMSO solutions (0.5% final concentration), washed in PBS, and incubated in substrate-free medium with the same flavonoid concentration for 1 hour. When indicated, HEK-293 or MDA-MB-231 cells were exposed to either 0.5 µg/mL rhodamine 6G or 2 µmol/L Hoechst 33342. Intracellular drug fluorescence was monitored with a FACscan flow cytometer (Becton Dickinson, Mountain View, CA) for mitoxantrone and rhodamine 6G, or a FACSvantage SE Option Diva apparatus equipped with an UV laser for Hoechst 33342. The maximal fluorescence (100%) was the difference between mean fluorescence of control cells and either R482 or T482 ABCG2-transfected cells, incubated with substrate but without inhibitor. The highest flavonoid concentration tested did not significantly modify the fluorescence of control cells. Cells without drug were included as an autofluorescence control.

P-glycoprotein and MRP1 transport activities. The flavones tectochrysin and 6-prenylchrysin were tested for their ability to interfere with P-glycoprotein- and MRP1-mediated drug efflux using a microplate assay, based on the screening method described by Sarver et al. (32) with minor modifications. Briefly, the day before the experiment, parental drug-sensitive cells and *MDR1*- or *MRP1*-transfected cells were plated at a density of 2×10^4 cells per well in 96-well plates (NUNC, Roskilde, Denmark). After 24 hours, when cells were forming a 90% confluent monolayer in all the wells, the original medium was removed and the cells were incubated at 37°C in the presence of increasing concentrations of flavone in the cell culture medium, with the following P-glycoprotein and MRP1 fluorescent substrates and incubation times: either 10 µmol/L daunomycin, 3 µmol/L rhodamine 6G, or 3 µmol/L bodipy-vinblastine for

2 hours, or 1 µmol/L calcein-AM for 20 minutes. Values of P-glycoprotein substrate accumulation in the presence of flavone were normalized to those obtained in the presence of 3 µmol/L GF120918, which were considered as 100% inhibition of MDR1-mediated transport. On the other hand, the flavone-induced accumulation of MRP1 substrates was normalized to the accumulation values observed in the parental counterpart of MRP1-expressing cells (100% drug accumulation). After the incubation step in the presence of the appropriate fluorescent probes, the cell monolayers were washed twice with cell culture medium to remove any trace of extracellular fluorescence. Finally, 50 µmol/L of lysis buffer [20 mmol/L Tris-HCl (pH 7.4) and 0.2% SDS] were added to each well and incubated at 4°C overnight in the dark. The accumulated intracellular fluorescence due to each fluorophore was measured in a microplate spectrofluorometer "SpectraMax Gemini EM" (Molecular Devices, Sunnyvale, CA) using the SoftmaxPro 4.3 software.

Generation of recombinant baculoviruses and membrane preparation. The *pcDNA3* plasmid containing T482 ABCG2 cDNA, provided by Dr. D. Ross, was used for subcloning into the *pTriEx-4-Neo* plasmid (Novagen, VWR, Fontenay-sous-Bois, France). Recombinant baculoviruses, carrying human T482 ABCG2 cDNA, were generated with Bacvector transfection kits (Novagen), according to manufacturer instructions. Briefly, Sf9 cells were cotransfected with BacVector-3000 Triple Cut Virus DNA and either *pTriEx* or *pTriEx-ABCG2(T482)* to obtain recombinant baculoviruses. Sf9 cells (10^6 /mL) were infected with recombinant viruses at an infection multiplicity of 20 for 1 hour. After a 72-hour culture at 27°C, cells were harvested by centrifugation at $500 \times g$ for 10 minutes and the membranes were prepared as described (33). Sf9 membranes containing overexpressed wild-type R482 ABCG2 (reference SB-MXR-Sf9-ATPase) were purchased from Solvo Biotechnology (Szeged, Hungary).

ATPase activity assay. Membranes from Sf9 insect cells (2.5 µg protein) were incubated for 90 minutes at 37°C in 50 mmol/L HEPES/KOH (pH 8) with 5 mmol/L ATP, 5 mmol/L MgCl₂, 500 µmol/L EGTA, 5 mmol/L sodium azide, 1 mmol/L ouabain, and an ATP-regenerating system (4 mmol/L phosphoenolpyruvate and 60 µg/mL pyruvate kinase) in 50 µL, final volume. All effectors (mitoxantrone, flavones, and GF120918) were added as DMSO solutions (1% final concentration). The reactions were stopped by adding 1 mL of 20 mmol/L H₂SO₄, and samples were immediately transferred on ice. P_i release was measured by a colorimetric method (34).

Cytotoxicity assay. Exponentially growing HEK293-R482 and HEK293-T482 cells, respectively transfected with wild-type and mutant ABCG2 vectors, and the corresponding control cells were trypsinized and plated (2×10^3 cells per 200 µL per well) in 96-well microplates and allowed to attach for 24 hours at 37°C under 5% CO₂. A 200-µL sample of drug solution, diluted in medium with 0.5% DMSO, final concentration, was added and cells were incubated for 48 hours at 37°C under 5% CO₂. MDA-MB-231-(T482)ABCG2 cells and respective control cells were used as 10^3 cells per 200 µL per well and incubated for 96 hours. Cytotoxicity was evaluated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (35).

Statistical analysis. Sets of data were compared with a Student's *t* test. Differences were considered either statistically significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, or not significant, NS. All statistical tests were done using the Sigma Stat software for Windows.

Results

Rational screening of inhibitory flavonoids on ABCG2-mediated drug efflux in transfected cells. HEK-293 cells transfected with wild-type (R482) ABCG2 actively effluxed mitoxantrone and Hoechst 33342, whereas mutant (T482) ABCG2-transfected cells (either HEK-293 or MDA-MB-231 lines) also showed efflux of rhodamine 6G (data not shown). Mean fluorescence analysis indicated a 7- and 12-fold higher intracellular mitoxantrone accumulation in control HEK-293 cells compared with the wild-type and mutant ABCG2-transfected cells, respectively.

Comparison of representatives of different classes of flavonoids, each containing three OH groups (two at positions 5 and 7, cf. Fig. 1A), indicated that all of them were able to inhibit wild-type (R482) ABCG2-mediated mitoxantrone efflux (***) with varying efficiencies (Fig. 1B). The flavone apigenin, with OH at position 4', was more efficient ($IC_{50} = 16 \mu\text{mol/L}$) than the flavonol galangin, with OH at position 3 ($19 \mu\text{mol/L}$, *) and the isoflavone genistein, with ring B branched to ring C at position 3 instead of 2 ($24 \mu\text{mol/L}$, **). The least efficient compound was the flavanone naringenin, with its reduced 2,3-bond ($37 \mu\text{mol/L}$, **). A similar preference for flavones was observed for mutant (T482) ABCG2 when rhodamine 6G was the substrate with MDA-MB-231 transfected cells (data not shown).

Structure-activity relationships identifying the flavones 6-prenylchrysin and tectochrysin as potent and specific inhibitors. Because the flavone apigenin seemed the most effective

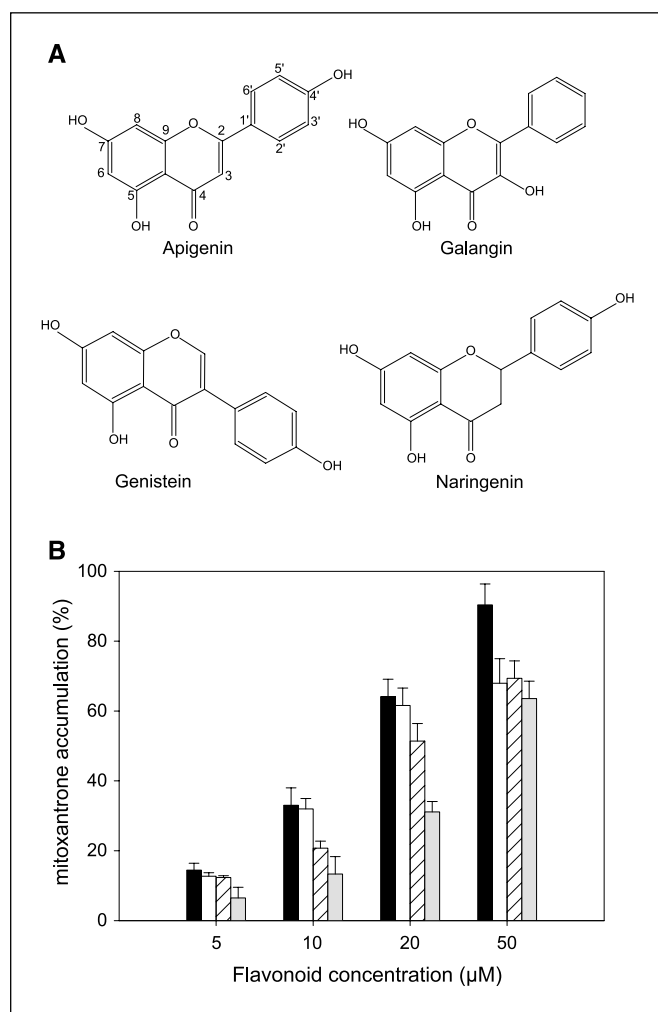


Figure 1. Inhibition by different classes of flavonoids of mitoxantrone efflux in ABCG2-transfected cells. A, structure of representative three OH-containing compounds from different flavonoid classes: apigenin (flavone), galangin (flavonol), genistein (isoflavone), and naringenin (flavanone). B, inhibition of R482 ABCG2-mediated mitoxantrone efflux by the above four compounds. Intracellular mitoxantrone accumulation into HEK-293 cells transfected by either R482 ABCG2 or empty vector (used as the control of maximal accumulation), was monitored by flow cytometry. Cells were incubated in presence of the indicated concentrations of either apigenin (dark columns), galangin (white columns), genistein (dashed columns), or naringenin (grey columns). The data are expressed as % of the control (empty vector). Columns, means of three independent determinations; bars, \pm SD.

inhibitor of ABCG2, a series of flavones were screened to evaluate the effects produced by OH number and position and hydrophobic substituents (Fig. 2A). IC_{50} values were determined from plots of percent mitoxantrone accumulation as a function of inhibitor concentration, as illustrated in Fig. 3. Several OH groups exhibited clearly negative effects on the inhibition of drug efflux: (i) at position 3 in 3-OH flavone compared with unsubstituted flavone ($IC_{50} = 8.1 \pm 1.9$ versus $2.8 \pm 0.6 \mu\text{mol/L}$, **), consistent with the slightly lower efficiency of galangin compared with apigenin in Fig. 1B; (ii) at position 7 in 7-OH flavone compared with flavone (7.1 ± 0.3 versus $2.8 \pm 0.6 \mu\text{mol/L}$, **); and (iii) at position 4' in apigenin compared with chrysin (16 versus $4.6 \mu\text{mol/L}$, **). In contrast, OH at position 5 produced a positive effect in chrysin compared with 7-OH flavone (4.6 ± 0.5 versus $7.1 \pm 0.3 \mu\text{mol/L}$, **).

C-isoprenylation at either position 6 or 8 markedly increased the inhibition potency (***) the highest effect being produced by prenyl (=3,3-dimethylallyl, cf. Fig. 2A) at position 6, with a 15-fold increased affinity comparatively to chrysin (0.29 ± 0.06 versus $4.6 \pm 0.5 \mu\text{mol/L}$). The inhibitory potency of 6-prenylchrysin was equivalent to GF120918. Slightly lower effects were produced by either the 1,1-dimethylallyl isomer substituent at the same position (**), or prenyl at position 8 (*). The effect did not depend only on hydrophobicity because more hydrophobic 6,8-digeranyl and 6-farnesyl derivatives were much less efficient (**).

The above structure-activity relationships are summarized in Fig. 2B, highlighting both positive and negative substituents for inhibition. Apparently as potent as well-known GF120918, the best flavone identified in this work was 6-prenylchrysin, with an OH at position 5 and a prenyl at position 6 on ring A, a 2,3-double bond on ring C and no OH on ring B. Mutant (T482) ABCG2 exhibited the same preference for the class of flavonoid (i.e., flavones) and similar dependence on OH position (negative at positions 3 and 7 but positive at position 5, **). However, the main difference was the effect of prenylation: in the case of 6-prenylchrysin, it was 12-fold lower for T482 than R482 (3.6 ± 1.9 versus $0.29 \pm 0.06 \mu\text{mol/L}$, respectively; **, cf. Fig. 3A); similar differences were observed for the other prenylated derivatives. Interestingly, a similar distinction was observed for the hydrophobic conventional inhibitor GF120918 (6.9 ± 2.6 versus $0.31 \pm 0.14 \mu\text{mol/L}$, for T482 and R482, respectively, **), as illustrated in Fig. 3C. In contrast, the interaction with tectochrysin was even slightly increased (Fig. 3B), which made it one of the best flavone inhibitors for T482 ABCG2 ($IC_{50} = 1.9 \pm 0.3 \mu\text{mol/L}$). Notably, the inhibitory potency of tectochrysin was further increased when using rhodamine 6G as the substrate ($IC_{50} = 0.32 \pm 0.04 \mu\text{mol/L}$, data not shown, **).

The specificity of 6-prenylchrysin and tectochrysin for ABCG2 relatively to P-glycoprotein and MRP1 was analyzed by assaying the ability of increasing concentrations of these compounds to inhibit the efflux of daunomycin, calcein-AM, and bodipy-vinblastine which are substrates of both transporters, in addition to rhodamine 6G that is selectively transported by P-glycoprotein. Table 1 shows that tectochrysin, up to $200 \mu\text{mol/L}$, and 6-prenylchrysin, up to $25 \mu\text{mol/L}$, exhibited a limited effect against both P-glycoprotein and MRP1 transport activities in MDR1- and MRP1-transfected cells respectively.

Effects of 6-prenylchrysin, tectochrysin, and GF120918 on the ATPase activity of wild-type (R482) and mutant (T482) ABCG2. Modification of the ATPase activity of ABC transporters by a compound is a well-established way to prove its direct interaction. We therefore investigated the effects produced by flavones on ATP hydrolysis in insect cell membranes enriched with

Figure 2. Flavonoid structure-activity relationships for inhibition of ABCG2 transport activity. **A**, effects of flavone substitution on ABCG2-mediated mitoxantrone efflux. IC₅₀ values were determined by flow cytometry, as in Fig. 1B, upon incubation of either R482 or T482 ABCG2-transfected HEK-293 cells in the presence of 5 μmol/L mitoxantrone and various concentrations of flavone derivatives. Values are the means ± SD of three independent experiments. The structure of C-isoprenylation substituents (*bottom*). **B**, summarizing diagram of flavonoid structure-activity relationships towards drug-efflux inhibition: favorable (*thick arrows*) and unfavorable substitutions (*thin-crossed arrows*).

Inhibitor substitution	IC ₅₀ (μM)	
	BCRP-R482	BCRP-T482
Flavone	2.8 ± 0.6	1.7 ± 0.4
3-OH-flavone	8.1 ± 1.9	4.9 ± 0.1
7-OH-flavone	7.1 ± 0.3	13.9 ± 1.51
Chrysin (5, 7-diOH-flavone)	4.6 ± 0.5	4.5 ± 0.8
Tectochrysin (5-OH, 7-OCH ₃ -flavone)	3.0 ± 0.9	1.9 ± 0.3
6-Prenylchrysin	0.29 ± 0.06	3.6 ± 1.9
6-(1,1-Dimethylallyl)chrysin	0.78 ± 0.15	>10
8-Prenylchrysin	0.89 ± 0.31	>10
8-(1,1-Dimethylallyl)chrysin	1.4 ± 0.5	>10
6-Geranylchrysin	1.0 ± 0.4	ND
6-Farnesylchrysin	>10	ND
6,8-Digeranylchrysin	2.1 ± 0.5	ND
GF120918	0.31 ± 0.14	6.9 ± 2.6

prenyl

1,1-dimethylallyl

geranyl

farnesyl

B

human R482 or T482 ABCG2. The functionality of the recombinant transporter was previously controlled by flow cytometry, with rhodamine 6G in the case of mutant ABCG2, on insect cells collected after 48 hours of infection of which drug-efflux activity was fully sensitive to GF120918 (data not shown). The ATPase activity of mutant ABCG2-enriched membranes from insect cells was 21.7 ± 1.2 nmol ATP hydrolyzed \times min⁻¹ \times mg protein⁻¹; it was inhibited by vanadate with high affinity (IC₅₀ around 4 μmol/L) and conversely stimulated up to $43 \pm 2\%$ at 20 μmol/L mitoxantrone (Fig. 4). Interestingly, GF120918 at 5 μmol/L, as well as the different chrysin derivatives (chrysin, tectochrysin, and 6-prenylchrysin), produced a similar stimulation as mitoxantrone. In sharp contrast, the ATPase activity of wild-type ABCG2 was insensitive to both mitoxantrone and chrysin derivatives, whereas GF120918 produced a strong inhibition. As a control, the glycosylated flavonoid rutin, which did not alter rhodamine 6G efflux by mutant ABCG2-transfected cells, did not modify the ATPase activity of the corresponding mutant membranes (data not shown).

6-Prenyl-chrysin and tectochrysin as apparently nontransported modulators that potently revert resistance to mitoxantrone. Because chrysin and derivatives stimulated the ATPase activity of mutant ABCG2 in the same way as the substrate mitoxantrone, the possibility that these compounds might be

transported was investigated by determining the ABCG2-induced cross-resistance to flavones. Tectochrysin and 6-prenylchrysin exhibited a relatively low cytotoxicity in both wild-type and mutant ABCG2-transfected cells (Fig. 5A), with respective IC₅₀ values of 15.2 ± 1.9 and 16.2 ± 2.3 μmol/L for tectochrysin (*b*) and 11.2 ± 1.3 μmol/L and 9.0 ± 1.6 μmol/L for 6-prenylchrysin (*a*). Interestingly, cytotoxicity was not significantly different in control cells (15.9 ± 2.1 and 8.5 ± 1.2 μmol/L, respectively, NS), indicating that ABCG2-transfected cells were not cross-resistant to either tectochrysin or 6-prenylchrysin. No cross-resistance was observed for GF120918 in the MDA-MB-231 cells (NS), either, where the cytotoxicity was higher with IC₅₀ values of 2.9 ± 0.9 μmol/L for mutant ABCG2-transfected cells and 4.5 ± 1.3 μmol/L for control cells (data not shown).

The ABCG2-transfected cells exhibited a relative resistance to mitoxantrone of 10.3-fold for wild-type R482 and 30-fold for mutant T482, by reference to control cells (transfected with empty vector), with respective IC₅₀ values of 1.03 ± 0.54 and 3.0 ± 0.5 μmol/L, compared with 0.10 ± 0.07 μmol/L (Table 2). In the case of wild-type ABCG2-transfected cells, mitoxantrone resistance was almost or completely reverted by 0.5 μmol/L 6-prenylchrysin, 2 μmol/L tectochrysin, or 1 μmol/L GF120918. In contrast, the most efficient inhibitor in the case of mutant ABCG2-transfected cells was tectochrysin, which fully reverted mitoxantrone resistance at

1 $\mu\text{mol/L}$, with an already significant effect at 0.2 $\mu\text{mol/L}$ (data not shown), whereas 6-prenylchrysin and GF120918 were less efficient in these mutant cells, requiring at least a 2 $\mu\text{mol/L}$ concentration to reach complete reversion. Figure 5B summarizes the reversing effect produced by either 6-prenylchrysin (a and d), tectochrysin (b and e), or GF120918 (c and f) at 1 $\mu\text{mol/L}$ on both wild-type R482 (a-c) and mutant T482 (d-f) *ABCG2*-transfected cells. The enhanced mitoxantrone resistance in the cells transfected with T482 is compatible with the previously noted gain-of-function in

mitoxantrone transport in these cells (29). It seems that 6-prenylchrysin produced a more complete reversion in wild-type than in mutant *ABCG2*, in contrast to tectochrysin that was more efficient against mutant *ABCG2*. The differential effect between wild-type and mutant was also observed with GF120918. In contrast, the above concentrations of 6-prenylchrysin and tectochrysin did not sensitize control cells to mitoxantrone. Previous results with mutant *ABCG2*-transfected MDA-MB-231 cells showed that unsubstituted chrysin also completely reverted mitoxantrone resistance at 5 $\mu\text{mol/L}$ (data not shown).

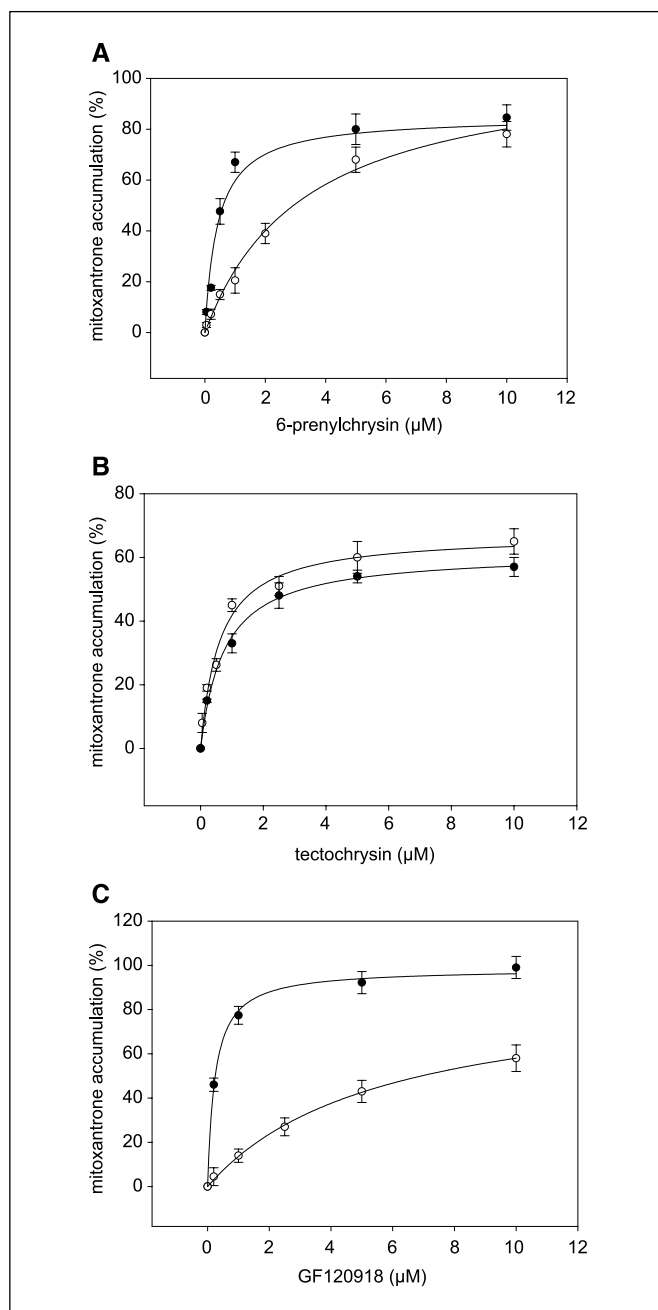


Figure 3. Concentration-dependent inhibition by 6-prenylchrysin, tectochrysin, and GF120918 on R482 or T482 *ABCG2*-mediated mitoxantrone efflux. Inhibition of R482 (●) or T482 (○) *ABCG2*-mediated mitoxantrone transport by either 6-prenylchrysin (A), tectochrysin (B), or GF120918 (C). Representative curves of three independent experiments done by flow cytometry. Point, means of triplicates; bars, \pm SD.

Discussion

The main original result of this article is that two hydrophobic flavones, 6-prenylchrysin and tectochrysin, are new, potent, and specific inhibitors of wild-type and mutant *ABCG2* drug efflux activity and therefore constitute promising candidates for future clinical trials.

6-Prenylchrysin and tectochrysin identified as potent inhibitors of *ABCG2*-mediated drug efflux through screening of flavonoids. Rational screening was facilitated by the use of *ABCG2*-transfected cell lines, with a high drug efflux capacity (monitored by 7- and 12-fold higher mitoxantrone accumulations in control cells compared with R482 and T482 *ABCG2*-transfected cells, respectively), which correlates the resistance index values of 10.3 and 30 for the two cell lines. This confirms that *ABCG2* is indeed the single main difference between the two types of cells, which contrasts with highly resistant lines (with resistance index up to 3,900-35,800 but much lower relative drug efflux capacity) selected from successive treatments with increasing drug concentrations. These stepwise selections likely produce a number of side modifications in addition to *ABCG2* overexpression, leading to questions about the accuracy of controls. This was stressed in several studies with either *ABCG2* (4, 29, 36) or P-glycoprotein (37), where additional modifications of glutathione transferase and topoisomerase II activities were reported. This might explain, at least partly, the difficulties of Zhang et al. to draw clear-cut structure-activity relationships and the high concentration of flavonoids required for complete chemosensitization (26). An additional mechanism leading to resistance to flavopiridol was indeed evidenced in *ABCG2*-expressing drug-selected cells, relative to resistance in transfected ones (29). In contrast, the differential effects observed here on both *ABCG2*-transfected cells and suited control cells (transfected with empty vector) allowed a rational two-step flavonoid screening, first identifying flavones as the favored class over flavonols, isoflavones, and flavanones, and second discriminating negative and positive roles of hydroxyl and hydrophobic substituents on drug efflux inhibition. The best inhibitors of *ABCG2* drug transport activity were found to be 6-prenylchrysin and tectochrysin. A positive effect was associated with a hydroxyl at position 5 of ring A, possibly related to conjugation with the vicinal ketone on adjacent ring C. On the contrary, a negative effect of hydroxyl at position 3 was clearly observed here, which contrasts with the potential positive role proposed by Imai et al. (27). This discrepancy might be due to the difficulty inherent in drawing quantitative structure-activity relationships from cytotoxicity experiments (27) compared with flow cytometry analysis as done here. Hydrophobic substituents seemed to increase the binding affinity at both positions 6 and 7, in agreement with the negative effects of hydrophilic hydroxyls on inhibition. Because negative effects were also produced at

Table 1. Lack of interaction of tectochrysin and 6-prenylchrysin with P-glycoprotein and MRP1

Transport substrate	Inhibition by tectochrysin (200 $\mu\text{mol/L}$)		Inhibition by 6-prenylchrysin (25 $\mu\text{mol/L}$)	
	<i>MDR1</i> cells	<i>MRP1</i> cells	<i>MDR1</i> cells	<i>MRP1</i> cells
Calcein-AM	<10%	<15%	<5%	<20%
Daunomycin	<5%	<25%	<5%	<25%
Rhodamine 6G	<6%	NT	<30%	NT
Bodipy-vinblastine	ND	<30%	ND	<11%

NOTE: Cells transfected with either the *MDR1* or *MRP1* gene were incubated with the indicated fluorescent substrates in the presence or absence of different concentrations of chrysin derivatives, and the intracellular fluorescence was measured in a microplate spectrofluorometer as detailed in Materials and Methods. Each measurement was drawn from two independent experiments performed in triplicate.
Abbreviations: NT, not transported; ND, not determined.

position 4', one may wonder about the possibility of hydrophobic substituents on ring B to further increase the inhibitory activity. The relatively simple structure of tectochrysin, a natural compound found in various plants, and its commercial availability readily allows the investigation of a series of new derivatives, such as the 6-prenyl one, to identify more potent inhibitors.

Prenylation is shown here to increase the binding affinity compared with the large chlorophenylmethylpiperidinyl substituent present in flavopiridol, which bound with lower affinity based on the high concentrations required to inhibit mitoxantrone efflux (23). The structure-activity relationships established here for ABCG2 inhibition are clearly different from those reported, on one hand, for P-glycoprotein (14) with a positive effect of 3-OH in contrast to the negative effect on ABCG2; and a much stronger effect of prenylation at position 8 over position 6 (15, 28) in contrast to the preferred position 6 in ABCG2. On the other hand, only flavonols such as quercetin (17) and dehydrosilybin (18) were able to revert multidrug resistance in MRP1 whereas prenylated derivatives were inefficient. Interestingly, a similar preference for 6-prenylation was found for the yeast Pdr5p transporter (21), which belongs to the same G class as ABCG2, suggesting that this 6-prenylation dependence of inhibition might represent a specificity for the ABCG2 multidrug transporters.

6-Prenylchrysin and tectochrysin behave differently from GF120918. In contrast to the strong inhibition of wild-type ABCG2 ATPase activity shown here for GF120918, which is consistent with the effects produced by both fumitremorgin C (6, 38) and Ko143 (39), 6-prenylchrysin and tectochrysin exhibited no apparent effect. This suggested a different inhibition mechanism of ABCG2-mediated drug-transport activity for chrysin derivatives by comparison to GF120918, fumitremorgin C, and Ko143. These inhibitors either bind to distinct sites on ABCG2 or induce differential effects on the coupling between ATP hydrolysis and drug transport. A combination of these different classes of ABCG2 inhibitors might therefore lead to synergistic inhibitory effects, a possibility that we are currently analyzing. On the contrary, GF120918 and flavone derivatives increased the ATPase activity of mutant T482 ABCG2, similar to the effect of the substrate mitoxantrone, which further contrasts with Ko143, that strongly inhibited in all cases (39). This indicates that the various classes of inhibitors obey overlapping but different inhibition mechanisms, which is consistent with the existence of a "large polyspecific drug-binding site" as characterized for P-glycoprotein and MRP, as well as for other non-ABC multidrug

bacterial efflux transporters (40). Concerning ABCG2, this concept was early suggested by the effects of the R482 mutation on the differential panels of transport substrates; this work shows that it also applies to specific inhibitors, as previously observed for novobiocin (29) but not for tariquidar (41). The R482 residue might, directly or indirectly, play a role in the coupling between energy supply and drug transport, and this effect would be altered upon mutation. A comparable activation or inhibition of ATP hydrolysis by modulators has been often reported for P-glycoprotein (42) and MRP1 (17, 43).

The lack of cross-resistance to tectochrysin and 6-prenylchrysin in wild-type and mutant ABCG2-transfected cells, compared with control cells, favors an apparent absence of transport. The situation was not as clear for flavopiridol that exhibited cross-resistance in highly resistant drug-selected cells (8, 23) but not in transfected ones (29); flavopiridol was found to accumulate in transfected *Xenopus laevis* oocytes in the presence of fumitremorgin C (44). A low transport of genistein was also found in polarized cells (27). In contrast, the present hydrophobic derivatives, 6-prenylchrysin

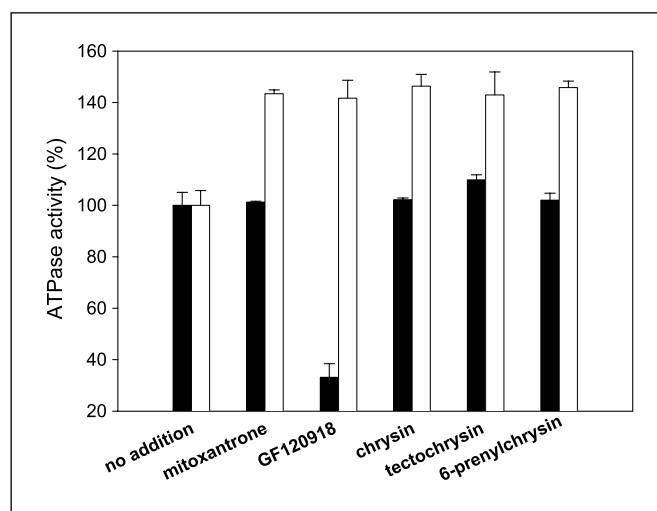


Figure 4. Modifications of the ATPase activity of R482 or T482 ABCG2 heterogeneously overexpressed in insect-cell membranes. The ATPase activity of R482 (black columns) or T482 (white columns) ABCG2 overexpressed in Sf9 insect cells was measured in the presence of either 20 $\mu\text{mol/L}$ mitoxantrone, 5 $\mu\text{mol/L}$ GF120918, or 5 μM chrysin derivative. Columns, means of three independent experiments done in triplicate; bars, \pm SD.

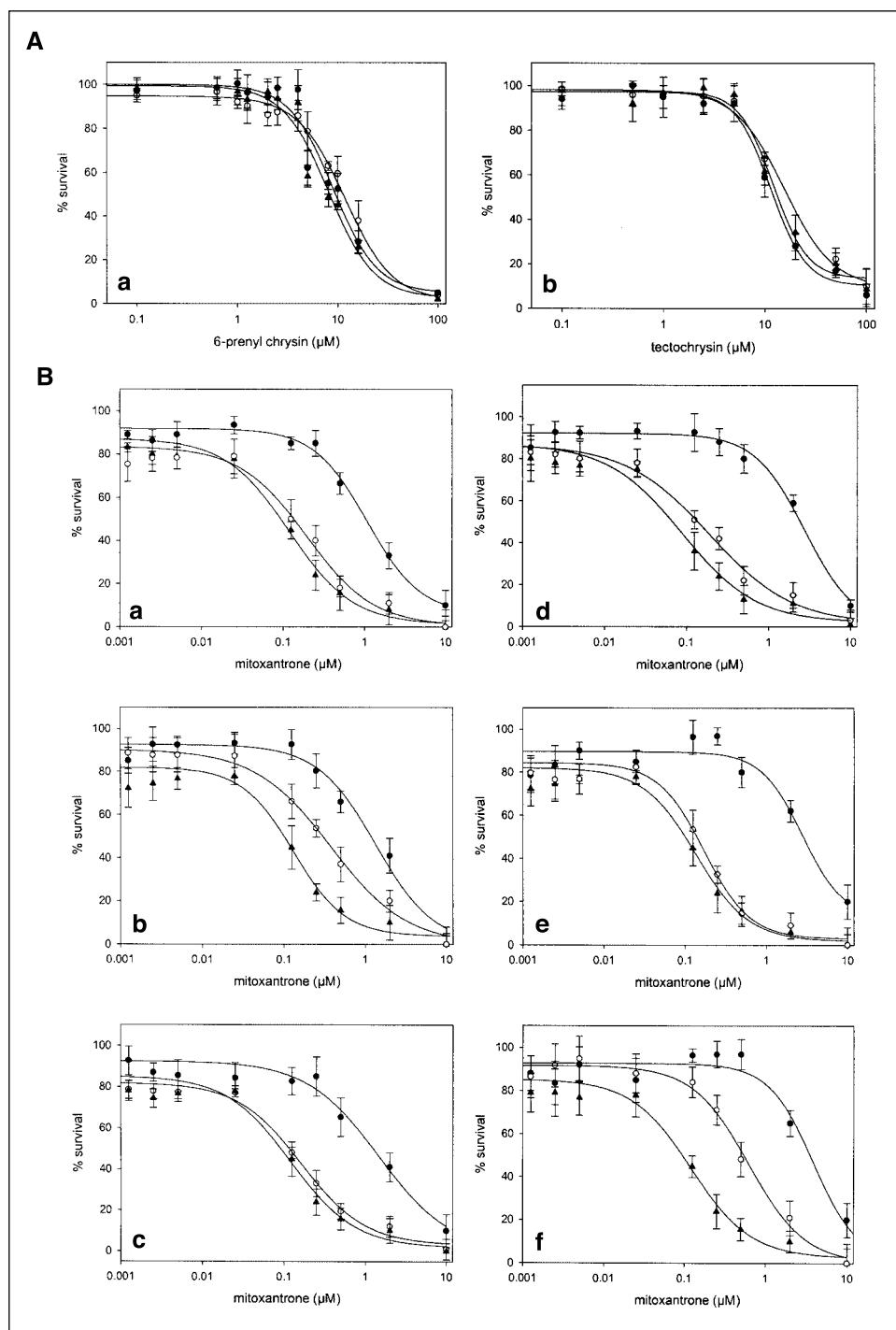


Figure 5. 6-Prenylchrysin and tectochrysin intrinsic cytotoxicity in HEK-293 cells and sensitization to mitoxantrone. *A*, intrinsic cytotoxicity. Control (▲), R482 ABCG2- (○), and T482 ABCG2- (●) transfected cells were cultured in the presence of increasing concentrations of either 6-prenylchrysin (*a*) or tectochrysin (*b*) for 48 hours. Cytotoxicity was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. *Points*, means of three independent experiments done in quadruplicate; *bars*, \pm SD. *B*, cell growth sensitization to mitoxantrone. R482 ABCG2- (*a-c*) or T482 ABCG2- (*d-f*) transfected cells were cultured with increasing concentrations of mitoxantrone in the absence (●) or the presence (○) of either 6-prenylchrysin (*a* and *d*), tectochrysin (*b* and *e*), or GF120918 (*c* and *f*) at 1 $\mu\text{mol/L}$. HEK-293 cells transfected by the empty vector (▲) cultured in presence of the same mitoxantrone concentrations were used as the control. *Points*, means of three independent experiments done in quadruplicate; *bars*, \pm SD.

and tectochrysin, exhibit a much stronger affinity than flavopiridol and genistein and, like GF120918, seem not transported; they therefore might bind to a modulatory site strongly interfering with binding of transported drugs.

6-Prenylchrysin and tectochrysin as promising reversal agents for potential clinical trials. 6-Prenylchrysin and tectochrysin seem specific for ABCG2 among the three types of multidrug ABC transporters, because they do not significantly interfere with either P-glycoprotein or MRP1 at much higher concentrations than those that completely inhibit ABCG2 transport

activity. These results are consistent with the quite different flavonoid structure-activity relationships determined for the three transporters as discussed above. 6-Prenylchrysin and tectochrysin specificity for ABCG2 contrasts with GF120918 which is at least 40-fold more efficient on P-glycoprotein (45, 46). The fact that these two chrysin hydrophobic derivatives recognize both wild-type and mutant ABCG2 is quite important in view of a potential clinical application. 6-Prenylchrysin seems here the most powerful derivative against the wild-type transporter. Because ABCG2 is susceptible to a hotspot mutation at position 482, as observed in

Table 2. Reversion of R482 or T482 ABCG2-mediated mitoxantrone resistance

	R482 ABCG2		T482 ABCG2	
	IC ₅₀ (μmol/L)	Resistance index	IC ₅₀ (μmol/L)	Resistance index
Control	0.10 ± 0.07	(1)	0.10 ± 0.05	(1)
ABCG2	1.03 ± 0.54	10.3	3.0 ± 0.5	30
2 μmol/L 6-prenylchrysin	0.10 ± 0.04	1.0	0.20 ± 0.02	2.0
1 μmol/L 6-prenylchrysin	0.10 ± 0.04	1.0	0.27 ± 0.10	2.7
0.5 μmol/L 6-prenylchrysin	0.15 ± 0.08	1.5	0.44 ± 0.06	4.4
2 μmol/L tectochrysin	0.15 ± 0.07	1.5	ND	
1 μmol/L tectochrysin	0.28 ± 0.06	2.8	0.10 ± 0.03	1.0
2 μmol/L GF120918	0.05 ± 0.03	0.5	0.16 ± 0.06	1.6
1 μmol/L GF120918	0.08 ± 0.02	0.8	0.47 ± 0.11	4.7

NOTE: Reversion of ABCG2-mediated resistance to mitoxantrone was studied by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. The resistance index was defined as the ratio between the IC₅₀ of either R482 ABCG2 or T482 ABCG2 cells with the indicated inhibitor concentration and IC₅₀ of control cells without inhibitor. The same concentrations of inhibitors did not produce any significant effect on the control cells. Data are the means ± SD of at least three independent experiments performed in quadruplicate. All data were statistically significant ($P < 0.05$).

in vitro drug selection of cultured cells, it cannot be completely excluded that similar mutations might occur *in vivo* upon extensive chemotherapeutic treatments; in this case, a compound such as tectochrysin would be quite valuable.

Another advantage of 6-prenylchrysin and tectochrysin over GF120918 for ABCG2 inhibition is their 3- to 5-fold lower intrinsic cytotoxicity in our transfected cell lines, whereas a similar or even higher GF120918 cytotoxicity has been reported in drug-selected cell lines (45, 46). In addition, tectochrysin is a main component in a number of nutritive supplements and therefore is expected to be well tolerated. 6-Prenylchrysin and tectochrysin displayed a similarly high efficiency as GF120918 to chemosensitize transfected-cell growth to mitoxantrone, with completion at 0.5 to 1.0 μmol/L. These flavone derivatives were therefore more than one order of magnitude more potent to revert multidrug resistance, mediated by either wild-type or mutant ABCG2, than cytotoxic. Such characteristics of 6-prenylchrysin and tectochrysin make them good candidates for future clinical trials, especially because the same submicromolar concentrations were at least as efficient *in vivo* to sensitize cell growth to mitoxantrone as *in vitro* to inhibit drug efflux; this suggested the

absence of marked modification and inactivation for both chrysin derivatives.

Because the two derivatives have been proposed here to inhibit ABCG2 in a different way than GF120918, fumitremorgin C, and Ko143 (46), it would be interesting to investigate pluri-modulation with the aim to get additive or even synergistic modulatory effects.

Acknowledgments

Received 5/24/2004; revised 3/11/2005; accepted 3/22/2005.

Grant support: Centre National de la Recherche Scientifique, the Université Claude Bernard de Lyon grant UMR 5086, French-Spanish Picasso grant HF2002-0140 (interministerial agreements between A. Di Pietro and F. Gamarro), the Association pour la Recherche sur le Cancer grant ARC 3519, the Comité du Rhône de la Ligue Nationale contre le Cancer, the Spanish grant SAF-2003-04200-CO2-01 (F. Gamarro), Ligue contre le Cancer de la Creuse doctoral fellowship (A. Ahmed-Belkacem), Ligue contre le Cancer du Rhône doctoral fellowship (A. Pozza), and European Community Marie Curie fellowship grant HMPF-CF-2001-01244 (J.M. Pérez-Victoria).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Douglas D. Ross for providing the MDA-MB-231-(T482)ABCG2 and control cell lines, Dr. Ira Pastan for the NIH-3T3 MDR-G185 and control cell lines, Dr. Piet Borst for the 2008-MRP1 clone 8 and control cell lines, Robert Robey for the HEK-293-ABCG2 (R482 and T482) and pcDNA-transfected control cell lines, and GlaxoSmithKline (Madrid, Spain) for the kind gift of GF120918.

References

- Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* 1976;455:152–62.
- Cole SP, Bhardwaj G, Gerlach JH, et al. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 1992; 258:1650–4.
- Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, Dean M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res* 1998;58: 5337–9.
- Doyle LA, Yang W, Abruzzo LV, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A* 1998;95: 15665–70.
- Miyake K, Mickley L, Litman T, et al. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res* 1999;59: 8–13.
- Ozvegy C, Litman T, Szakacs G, et al. Functional characterization of the human multidrug transporter, ABCG2, expressed in insect cells. *Biochem Biophys Res Commun* 2001;285:111–7.
- Xu J, Yang Y, Bates S, Zhang JT. Characterization of oligomeric human half-transporter ATP-binding cassette G2. *J Biol Chem* 2004;279:19781–9.
- Doyle LA, Ross DD. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). *Oncogene* 2003;22:7340–58.
- Sarkadi B, Ozvegy-Laczka C, Nemet K, Varadi A. ABCG2: a transporter for all seasons. *FEBS Lett* 2004; 567:116–20.
- Honjo Y, Morisaki K, Huff LM, et al. Single-nucleotide polymorphism (SNP) analysis in the ABC half-transporter ABCG2 (MXR/BCRP/ABCP1). *Cancer Biol Ther* 2002;1:696–702.
- Bates SE, Robey R, Miyake K, Rao K, Ross DD, Litman T. The role of half-transporters in multidrug resistance. *J Bioenerg Biomembr* 2001;33:503–11.
- Castro AF, Altenberg GA. Inhibition of drug transport by genistein in multidrug-resistant cells expressing P-glycoprotein. *Biochem Pharmacol* 1997;53:89–93.
- Shapiro AB, Ling V. Effect of quercetin on Hoechst33342 transport by purified and reconstituted P-glycoprotein. *Biochem Pharmacol* 1997;53:587–96.
- Conseil G, Baubichon-Cortay H, Dayan G, Jault JM, Barron D, Di Pietro A. Flavonoids: a class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein. *Proc Natl Acad Sci U S A* 1998;95:9831–6.

15. Di Pietro A, Conseil G, Perez-Victoria JM, et al. Modulation by flavonoids of cell multidrug resistance mediated by P-glycoprotein and related ABC transporters. *Cell Mol Life Sci* 2002;59:307-22.
16. Hooijberg JH, Broxterman HJ, Heijn M, Fles DLA, Lankelma J, Pinedo HM. Modulation by (iso)flavonoids of the ATPase activity of the multidrug resistance protein. *FEBS Lett* 1997;413:344-8.
17. Leslie EM, Mao Q, Oleschuk CJ, Deeley RG, Cole SPC. Modulation of multidrug resistance protein 1 (MRP1/ABCC1) transport and ATPase activities by interaction with dietary flavonoids. *Mol Pharmacol* 2001;59:1171-80.
18. Trompier D, Baubichon-Cortay H, Chang XB, et al. Multiple flavonoid-binding sites within multidrug resistance protein MRP1. *Cell Mol Life Sci* 2003;60:2164-77.
19. Perez-Victoria JM, Chiquero MJ, Conseil G, et al. Correlation between the affinity of flavonoids binding to the cytosolic site of *Leishmania tropica* multidrug transporter and their efficiency to revert parasite resistance to daunomycin. *Biochemistry* 1999;38:1736-43.
20. Perez-Victoria JM, Di Pietro A, Barron D, Ravelo AG, Castanys S, Gamarro F. Multidrug resistance phenotype mediated by the P-glycoprotein-like transporter in *Leishmania*: a search for reversal agents. *Curr Drug Targets* 2002;3:311-33.
21. Conseil G, Decottignies A, Jault JM, et al. Prenyl-flavonoids as potent inhibitors of the Pdr5p multidrug ABC transporter from *Saccharomyces cerevisiae*. *Biochemistry* 2000;39:6910-7.
22. Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 2000;52:673-751.
23. Robey RW, Medina-Perez WY, Nishiyama K, et al. Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. *Clin Cancer Res* 2001;7:145-52.
24. Perez-Victoria JM, Pozza A, Ahmed-Belkacem A, Lazzaroni J-C, Di Pietro A. High overexpression of human breast cancer resistance protein in the plasma membrane of a mutant *Escherichia coli* and selection of flavonoid inhibitors. 4th FEBS Advanced Lecture Course "ATP-binding cassette (ABC) proteins: from genetic disease to multidrug resistance," March 1-8, 2003, Gosau (Austria).
25. Cooray HC, Janvilisri T, van Veen HW, Hladky SB, Barrand MA. Interaction of the breast cancer resistance protein with plant polyphenols. *Biochem Biophys Res Commun* 2004;317:269-75.
26. Zhang S, Yang X, Morris ME. Flavonoids are inhibitors of breast cancer resistance protein (ABCG2)-mediated transport. *Mol Pharmacol* 2004;65:1208-16.
27. Imai Y, Tsukahara S, Asada S, Sugimoto Y. Phytoestrogen/flavonoids reverse breast cancer resistance protein/ABCG2-mediated multidrug resistance. *Cancer Res* 2004;64:4346-52.
28. Comte G, Daskiewicz JB, Bayet C, et al. C-Isoprenylation of flavonoids enhances binding affinity toward P-glycoprotein and modulation of cancer cell chemoresistance. *J Med Chem* 2001;44:763-8.
29. Robey RW, Honjo Y, Morisaki K, et al. Mutations at amino-acid 482 in the ABCG2 gene affect substrate and antagonist specificity. *Br J Cancer* 2003;89:1971-8.
30. Cardarelli CO, Aksentijevich I, Pastan I, Gottesman MM. Differential effects of P-glycoprotein inhibitors on NIH3T3 cells transfected with wild-type (G185) or mutant (V185) multidrug transporters. *Cancer Res* 1995;55:1086-91.
31. Kool M, van der Linden M, de Haas M, et al. MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci U S A* 1999;96:6914-9.
32. Sarver JG, Klis WA, Byers JP, Erhardt PW. Microplate screening of the differential effects of test agents on Hoechst 33342, rhodamine 123, and rhodamine 6G accumulation in breast cancer cells that overexpress P-glycoprotein. *J Biomol Screen* 2002;7:29-34.
33. Dey S, Ramachandra M, Pastan I, Gottesman MM, Ambudkar SV. Photoaffinity labeling of human P-glycoprotein: effect of modulator interaction and ATP hydrolysis on substrate binding. *Methods Enzymol* 1998;292:318-28.
34. Van Veldhoven PP, Mannaerts GP. Inorganic and organic phosphate measurements in the nanomolar range. *Anal Biochem* 1987;161:45-8.
35. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
36. Woehlecke H, Osada H, Herrmann A, Lage H. Reversal of breast cancer resistance protein-mediated drug resistance by tryprostatin A. *Int J Cancer* 2003;107:721-8.
37. Perez-Victoria FJ, Conseil G, Munoz-Martinez F, et al. RU49953: a non-hormonal steroid derivative that potently inhibits P-glycoprotein and reverts cellular multidrug resistance. *Cell Mol Life Sci* 2003;60:526-35.
38. Robey RW, Honjo Y, van de Laar A, et al. A functional assay for detection of the mitoxantrone resistance protein, MXR (ABCG2). *Biochim Biophys Acta* 2001;1512:171-82.
39. Ozvegy-Laczka C, Koblos G, Sarkadi B, Varadi A. Single amino acid (482) variants of the ABCG2 multidrug transporter: major differences in transport capacity and substrate recognition. *Biochim Biophys Acta* 2005;1668:53-63.
40. Schumacher MA, Miller MC, Brennan RG. Structural mechanism of the simultaneous binding of two drugs to a multidrug-binding protein. *EMBO J* 2004;23:2923-30.
41. Robey RW, Steadman K, Polgar O, et al. Pheophorbide a is a specific probe for ABCG2 function and inhibition. *Cancer Res* 2004;64:1242-6.
42. Kerr KM, Sauna ZE, Ambudkar SV. Correlation between steady-state ATP hydrolysis and vanadate-induced ADP trapping in Human P-glycoprotein. Evidence for ADP release as the rate-limiting step in the catalytic cycle and its modulation by substrates. *J Biol Chem* 2001;276:8657-64.
43. Chang XB, Hou YX, Riordan JR. Stimulation of ATPase activity of purified multidrug resistance-associated protein by nucleoside diphosphates. *J Biol Chem* 1998;273:23844-8.
44. Nakanishi T, Doyle LA, Hassel B, et al. Functional characterization of human breast cancer resistance protein (BCRP, ABCG2) expressed in the oocytes of *Xenopus laevis*. *Mol Pharmacol* 2003;64:1452-62.
45. de Bruin M, Miyake K, Litman T, Robey R, Bates SE. Reversal of resistance by GF120918 in cell lines expressing the ABC half-transporter, MXR. *Cancer Lett* 1999;146:117-26.
46. Allen JD, van Loevezijn A, Lakhai JM, et al. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter *in vitro* and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther* 2002;1:417-25.