C-Isoprenylation of Flavonoids Enhances Binding Affinity toward P-Glycoprotein and Modulation of Cancer Cell Chemoresistance

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Previous studies have shown that flavones bind to P-glycoprotein (Pgp) with higher affinity than isoflavones, flavanones, and glycosylated derivatives. In the present work, a series of *C*- or *O*-substituted hydrophobic derivatives of chrysin were synthesized to further investigate structural requirements of the A ring toward Pgp modulation. Increasing hydrophobicity at either position 6, 8, or 7 increased the affinity of in vitro binding to a purified cytosolic domain of Pgp, but only benzyl and 3,3-dimethylallyl *C*-substitution produced a high maximal quenching of the protein intrinsic fluorescence. Inhibition of membrane Pgp within leukemic cells, characterized by intracellular drug accumulation, was specifically produced by isoprenylated derivatives, with 8-(3,3-dimethylallyl)chrysin being even more efficient than the commonly used cyclosporin A.

Introduction

Multidrug resistance (MDR) of cancer cells is often correlated with the overexpression of P-glycoprotein (Pgp), an ABC-type plasma membrane transporter that rejects chemotherapeutic drugs out of the cell by using ATP hydrolysis as an energetic source.[1,2] Tumor cells expressing Pgp are resistant to a number of major cytotoxic agents, including anthracyclines, vinca alcaloids, taxanes, and epipodophyllotoxins. Therefore, it is of high priority to develop molecules that can inhibit Pgp activity.

Pgp is able to bind a number of compounds, but most of them are transported.[3] Hydroxylated steroids such as cortisol, dexamethasone, aldosterone, or corticosterone can be effluxed by Pgp,[4,5] whereas more hydrophobic ones such as progesterone and antiprogestin RU 486 are not transported and behave as efficient modulators of cellular MDR by inhibiting anticancer drug efflux.[4,6,7] Unfortunately, it appears very difficult to use such compounds at a therapeutic level because of their hormonal consequences.

Flavonoids have also been reported as modulators of Pgp.[8−10] We recently showed that flavones and flavonols bind more strongly to Pgp cytosolic site than isoflavones, flavanones, and glycosylated derivatives.[11] Moreover, the hydroxylation at position 5, in addition to ketone at position 4, is essential for the ability of these modulators to mimic the adenine moiety of ATP. We also showed that such flavonoids are able to interact with both ATP and steroid binding sites within the cytosolic domain of the transporter. Since most Pgp effectors are rather hydrophobic, it appeared of interest to test the activity of lipophilic flavonoids substituted by either methyl, isopropyl, benzyl, or isoprenyl groups. Isoprenylated flavonoids are natural compounds which constitute a minor class of plant secondary metabolites recently reviewed.[12] As starting material, we have chosen the simplest 5,7-dihydroxyflavone named chrysin. In this paper, we describe the effects of ring A alkylation on the interaction of flavonoids with Pgp. Mono- and disubstituted chrysin derivatives were prepared to study the effects of hydrophobicity and substituent position on the affinity of binding to the transporter and on related inhibition leading to intracellular drug accumulation.

Results and Discussion

Although *O*-alkylated flavonoids are of easier access than *C*-alkylated ones, the latter compounds are expected to be more advantageous because phenolic hydroxyls remain free and can play a role in the interaction. There are two main methods to obtain *C*-prenylated flavonoids. The first one[13] consists of a sigmatropic rearrangement of *O*-prenylated compounds which gives a high specificity in the prenylation position. The second one is a direct *C*-alkylation process in alkali medium.[14] This method may afford a mixture of isomers but is a one-step reaction because no protecting group is required. As a general alkylation process we retained the latter method since it affords the highest diversity of isomers in a single experiment (Scheme 1). All alkylations were performed in a solution of aqueous tetramethylammonium hydroxide/MeOH in the presence of tetraethylammonium iodide. *C*-Methylation and *C*-isopropylation of chrysin were performed under rather forced conditions, by refluxing chrysin with 5 equiv of methyl iodide or isopropyl bromide, respectively. In each case, mixtures of *C*-monoalkylated (**1**, **4**) and *O*- plus *C*-polyalkylated isomers (**3**, **6**, **7**) were obtained. Both

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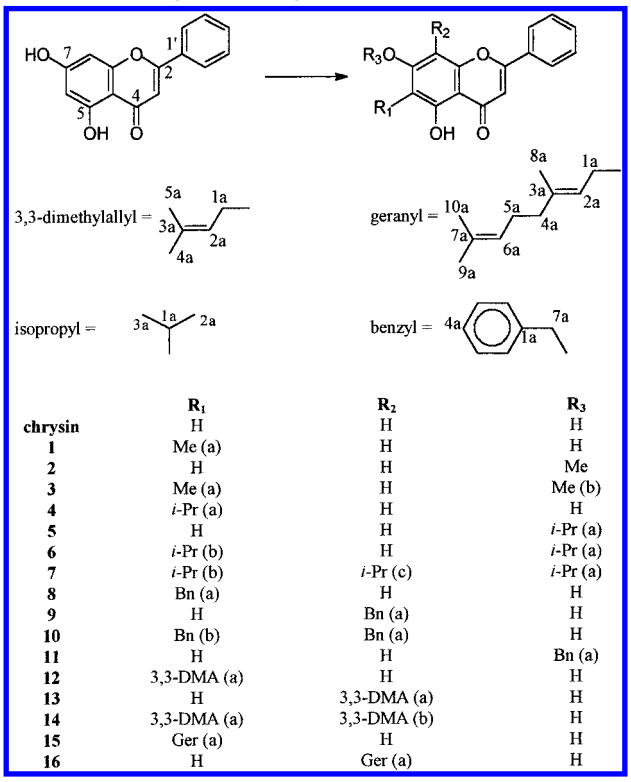
Scheme 1. Alkylated Chrysin Derivatives



	R_1	R_2	R_3
chrysin	H	H	H
1	Me (a)	H	H
2	H	H	Me
3	Me (a)	H	Me (b)
4	*i*-Pr (a)	H	H
5	H	H	*i*-Pr (a)
6	*i*-Pr (b)	H	*i*-Pr (a)
7	*i*-Pr (b)	*i*-Pr (c)	*i*-Pr (a)
8	Bn (a)	H	H
9	H	Bn (a)	H
10	Bn (b)	Bn (a)	H
11	H	H	Bn (a)
12	3,3-DMA (a)	H	H
13	H	3,3-DMA (a)	H
14	3,3-DMA (a)	3,3-DMA (b)	H
15	Ger (a)	H	H
16	H	Ger (a)	H

C-benzylation and *C*-isoprenylation of chrysin required, as expected, smoother conditions (1 equiv of benzyl chloride, prenyl bromide, or geranyl bromide). While mono-*C*-benzylflavones (**8**, **9**) as well as di-*C*-benzylchrysin (**10**) were produced after 90 min reaction at room temperature, the isoprenylated flavones (**12**−**16**) were obtained under microwave irradiation, which allowed us first to improve yields and second to decrease the reaction times to a few minutes.[15,16] Microwave-induced synthesis was not used for *C*-benzylation because of degradation processes. *O*-Alkylations were realized in DMF at room temperature in the presence of K_2CO_3 and 2-bromopropane or benzyl chloride as alkylating reagent, yielding *O*-isopropyl- (**5**) and *O*-benzylchrysin (**11**), respectively. The reaction mixtures were submitted to several chromatographic stages yielding pure alkylated chrysin derivatives. The compounds were identified on the basis of their spectroscopic data. The alkylation position was determined by comparing the ^{1}H and ^{13}C NMR chemical shifts with those of the literature[13−14,17] and by the ^{1}H−^{13}C heteronuclear long-range correlations obtained in the 2D-NMR maps.

The binding of chrysin derivatives to the purified C-terminal cytosolic domain, NBD2, of Pgp was directly measured by quenching of tryptophan-related intrinsic fluorescence, as described previously for unsubstituted flavonoids[11] or halogenated chalcones.[18] Table 1 shows that the increase in hydrophobicity of chrysin by alkylation with either methyl, isopropyl, benzyl, 3,3-dimethylallyl, or geranyl substituents was correlated with an increase in affinity for in vitro binding to the Pgp cytosolic domain, as monitored by a lowering in K_D values. This effect was observed among compounds monoalkylated at either position 6 (**1** < **4**, **8**, **12** < **15**), position 8 (**9** < **13** < **16**), or position 7 (**2** < **5** < **11**). A similar situation was encountered in the case of di- or trisubstituted derivatives (**3** < **6** < **7**, **10**, **14**). In fact, the K_D value measured for the most hydrophobic compound **16** was more than 500-fold lower than that of chrysin. It can be anticipated that alkylation strengthened the flavonoid interaction at the modulatory site that binds hydrophobic steroids.

However, only mono-*C*-substituted benzyl (**8**, **9**) or 3,3-dimethylallyl (**12**, **13**) derivatives displayed a high maximal quenching (ΔF_{max}) comparable to that observed with chrysin. Indeed relatively low values were produced by 6- or 8-*C*-monosubstituted methyl (**1**), isopropyl (**4**), or geranyl (**15**, **16**) derivatives, as well as by 6,8-*C*-disubstituted compounds (**10**, **14**), indicating a different modification of the tryptophan environment. A more complex situation occurred in the case of 7-*O*-monosubstituted derivatives since both very low (**11**) or moderate values (**2**, **5**) were observed. A high maximal quenching might be indicative of ATP site overlapping that would be lowered by most hydrophobic substitutions, possibly due to a shift toward the steroid-interacting region.

Only isoprenylated derivatives were able to inhibit Pgp-mediated daunomycin efflux from leukemic K562/R7 cells, leading to enhanced intracellular accumulation of the drug (Table 1, right column). Whereas chrysin produced a limited inhibition of the daunomycin efflux (20% as compared to cyclosporin A), a much higher effect was observed with dimethylallyl substitution at position 6 (**12**) or even higher at position 8 (**13**). As a matter of fact, the effect of 8-dimethylallylchrysin (**13**) was stronger than that of cyclosporin A, which is considered to be one of the most potent modulators available. The difference observed between the two positions of dimethylallyl substitution might indicate some difference in binding orientation. The inhibition was not further increased by increasing the hydrophobicity of the substituent(s). This might indicate that such compounds (**14**−**16**) could be hydrophobically trapped by the membrane and exhibit lower availability for Pgp. On the other hand, it was surprising that the mono-*C*-benzyl derivatives (**8**, **9**) did not produce any modulating effect, suggesting that they do not reach the Pgp target. Either penetration through, or retention by, the membrane or interaction with other more reactive targets might be responsible for this apparent lack of efficiency in Pgp-overexpressing leukemic cells.

It is important to mention that the modulating effects of *C*-isoprenylated chrysin derivatives were observed at concentrations which were not toxic for the cells, suggesting that these compounds should be investigated in vivo as potential Pgp modulators in tumor cells. Additional works are in progress to further characterize the inhibitory flavonoid-binding site on Pgp at the molecular and cellular levels, with the aim to design even more potent and specific inhibitors.

Experimental Section

General Chemistry Methods. NMR spectra were recorded on DPX 300 (300 MHz for ^{1}H, 75 MHz for ^{13}C) instruments. Solvents were used as internal references ($CDCl_3$, DMSO-d_6, acetone-d_6 and methanol-d_4). EI-MS were obtained at 70 eV using a Micromass ZAB2-SEQ spectrometer. The ionization current and the chamber temperature were 200 μA and 200 °C, respectively. FAB- and CI-positive MS were recorded on a Finnigan MAT 95 XL spectrometer. Elemental analyses were performed by the Analytical Department of Marseille University, St. Jérôme, France. UV spectra were recorded in ethanol using a Shimadzu UV−vis spectrophotometer model 1240.

Table 1. Effects of Chrysin Alkylation on the Binding to Pgp Cytosolic Domain and on the Inhibition of Daunomycin Cellular Efflux

	substituents					binding to Pgp[b]		
flavone	R_1	R_2	R_3	hydrophobicity index[a]	log P[a]	ΔF_{max} (%)	K_D (μM)	relative daunomycin accumulation[c]
chrysin	H	H	H	0.358	5.70	94.6 ± 3.1	13.1 ± 0.9	0.20
1	Me	H	H	0.512	5.85	46.6 ± 7.2	3.10 ± 0.97	0.20
2	H	H	Me	0.719	6.06	68.1 ± 9.9	6.3 ± 4.3	0.23
3	Me	H	Me	0.842	6.18	57.3 ± 3.3	1.30 ± 0.35	0.30
4	*i*-Pr	H	H	0.608	5.95	39.0 ± 1.6	0.21 ± 0.06	0.24
5	H	H	*i*-Pr	0.898	6.24	53.2 ± 4.7	1.32 ± 0.34	0.30
6	*i*-Pr	H	*i*-Pr	1.248	6.60	44.5 ± 2.9	0.28 ± 0.18	0.29
7	*i*-Pr	*i*-Pr	*i*-Pr	1.479	6.84	38.4 ± 1.1	0.033 ± 0.008	0.29
8	Bn	H	H	0.754	6.10	72.4 ± 1.4	0.34 ± 0.04	0.09
9	H	Bn	H	0.754	6.10	99.1 ± 3.8	0.99 ± 0.14	0.25
10	Bn	Bn	H	1.060	6.41	40.9 ± 1.9	0.036 ± 0.017	0.39
11	H	H	Bn	1.076	6.42	29.3 ± 2.3	0.068 ± 0.044	0.27
12	3,3-DMA	H	H	0.812	6.15	83.4 ± 1.5	0.30 ± 0.03	0.83
13	H	3,3-DMA	H	0.853	6.20	81.0 ± 1.9	0.28 ± 0.04	1.52
14	3,3-DMA	3,3-DMA	H	1.296	6.56	21.5 ± 0.5	0.015 ± 0.003	0.84
15	Ger	H	H	1.288	6.64	48.2 ± 1.1	0.045 ± 0.005	0.81
16	H	Ger	H	1.350	6.71	67.0 ± 2.1	0.025 ± 0.005	0.90

[a] Hydrophobicity indexes were established using the function $R_M = \log(1/R_f - 1)$ after TLC on C18 reverse-phase silica gel 20- × 20-cm plates in methanol:water (8:2). Log P values were estimated by extrapolation from the curve log $P = f(R_M)$ established with data obtained for quercetine and platanetine according to previous results.[22] [b] Direct binding to Pgp recombinant cytosolic domain was measured by quenching of intrinsic fluorescence. Data analysis with the Grafit program allowed the determination of dissociation constant and percent maximal quenching of the intrinsic fluorescence. [c] Pgp-positive leukemic K562/R7 cells were incubated with daunomycin and assayed by flow cytometry for intracellular remaining fluorescent drug in the absence or presence of cyclosporin A. Results of daunomycin-efflux inhibition, leading to its intracellular accumulation, are expressed as a ratio of the effect obtained with 10 μM chrysin derivatives to the effect of cyclosporin A (taken as a reference).

TLC was carried out using Merck silica gel Si60 F254 20- × 20-cm plastic sheets and RP-18 F254s 20- × 20-cm aluminum sheets and HPTLC on DIOL-F254s 10- × 10-cm sheets. Analytical HPLC was carried out on a Thermo Separation Products system equipped with a P-4000 quaternary gradient pump system and a UV-6000LP photodiode array detector using analytical 250- × 4.6-mm columns packed with Merck Lichrospher 100Si 5 μm, Merck Lichrospher 100RP18 5 μm or Merck Lichrospher 100DIOL 5 μm. Flash chromatography was carried out using Merck silica gel 60, 70−230 mesh, or Pharmacia Sephadex LH-20. MPLC were carried out using Merck silica gel 15−25 μm, Lichroprep 100DIOL 40−63 μm, or Lichroprep 100RP18 25−40 μm with UV detection at 254 and 366 nm. Microwave irradiation were realized in a Bluewind 1797 microwave oven at 120 and 240 W by successive periods of 1 and 2.5 min.

Methylated Chrysin Derivatives 1−3. To 1 g of chrysin (3.92 mmol) dissolved in 10 mL of methanol and 15 mL of 10% aqueous tetramethylammonium hydroxide was added 1 g of tetraethylammonium iodide (3.89 mmol; 1 equiv). After homogenization, 1.22 mL of methyl iodide (19.65 mmol; 5 equiv) was added dropwise to the solution. The latter was refluxed for 2 h. After return to room temperature, the mixture was diluted with water, acidified with HCl (6 N), and extracted with ethyl acetate. The organic layer was then concentrated to dryness affording a brown residue; the latter was chromatographed on silica gel using chloroform:acetic acid (95:5) as mobile phase and was submitted to a DIOL MPLC using first a gradient of chloroform in hexane (0 to 100% in 180 min) and then a gradient of methanol in chloroform (0 to 50% in 60 min) affording pure compounds **1** (47.4 mg; yield 4.5%) and **3** (138.7 mg; yield 12.4%). Compound **2** was obtained by methylation of chrysin using anhydrous K_2CO_3 and dimethyl sulfate in acetone. Its spectroscopic data were in accordance with those of literature.[17]

Isopropylated Chrysin Derivatives 4, 6, 7. To 1 g of chrysin (3.92 mmol) dissolved in 8 mL of methanol and 12 mL of 10% aqueous tetramethylammonium hydroxide was added 1 g of tetraethylammonium iodide (3.89 mmol; 1 equiv). After homogenization, 1.85 mL of isopropyl bromide (19.65 mmol; 5 equiv) was added dropwise to the solution. The latter was refluxed for 48 h. After return to room temperature, the mixture was diluted with water, acidified with HCl (6 N), and extracted with ethyl acetate. The organic layer was then concentrated to dryness affording a brown residue; the latter was chromatographed on silica gel using a gradient of ethyl acetate in hexane (0 to 100% in 300 min) affording pure compounds **4** (29.8 mg; yield 0.85%), **6** (124 mg; yield 11.1%) and **7** (12.8 mg; yield 2.5%).

***O*-Isopropylated Chrysin Derivative 5.** To 100 mg of chrysin (0.392 mmol), 81 mg K_2CO_3 (0.590 mmol; 1.5 equiv) and 12.6 mg of tetrabutylammonium bromide (0.0392 mmol; 0.1 equiv) in 10 mL of dimethylformamide was added 46 μL of isopropyl bromide (0.49 mmol; 1.25 equiv). After 8 days at room temperature under stirring, the mixture was diluted with water, acidified with HCl (6 N), and extracted with ethyl acetate. The organic layer was then concentrated to dryness affording a brown residue; the latter was chromatographed on silica gel using hexane:ethyl acetate (95:5 to 0:100 in 150 min) as mobile phase affording pure compound **5** (68.8 mg; yield 59.1%).

***C*-Benzylated Chrysin Derivatives 8−10.** To 1 g of chrysin (3.92 mmol) dissolved in 10 mL of methanol and 20 mL of 10% aqueous tetramethylammonium hydroxide was added 1 g of tetraethylammonium iodide (3.89 mmol; 1 equiv). After homogenization, 0.45 mL of benzyl chloride (3.93 mmol; 1 equiv) was added dropwise to the solution. After 90 min at room temperature, the mixture was diluted with water, acidified with HCl (6 N), and extracted with ethyl acetate. The organic layer was then concentrated to dryness affording a brown residue; the latter was chromatographed on silica gel using a gradient of ethyl acetate in hexane (10 to 100% in 350 min) affording two fractions, A and B. Fraction A was pure compound **10** (36.1 mg; yield 2.1%). Fraction B was then submitted to a DIOL MPLC using first a gradient of chloroform in hexane (0 to 100% in 230 min) and then a gradient of methanol in chloroform (0 to 50% in 60 min) affording two fractions, C and D. Fraction C was pure compound **8** (75.4 mg; yield 5.6%). Fraction D was submitted to another DIOL MPLC using first a gradient of chloroform in hexane (0 to 100% in 300 min) affording pure compound **9** (56.3 mg; yield 4.2%).

***O*-Benzylated Chrysin Derivative 11.** To 100 mg of chrysin (0.39 mmol), 81 mg K_2CO_3 (0.590 mmol, 1.5 equiv) and 12.6 mg of tetrabutylammonium bromide (0.039 mmol; 0.1 equiv) dissolved in 4 mL of dimethylformamide was added 56 μL of benzyl chloride (0.49 mmol; 1.25 equiv) in a 25-mL PTFE flask. The latter was submitted to 8 successive 2.5-min microwave irradiations at 240 W (pause time between 2 irradiation periods to return to 25 °C). The mixture was diluted with water, acidified with HCl (6 N), and extracted with ethyl

acetate. The organic layer was then concentrated to dryness affording a brown residue; the latter was chromatographed on silica gel using hexane:ethyl acetate (95:5 to 6:4 in 180 min) as mobile phase affording pure compound **11** (60.5 mg; yield 44.7%).

Prenylated Chrysin Derivatives 12−14. To 1 g of chrysin (3.92 mmol) dissolved in 10 mL of methanol and 20 mL of 10% aqueous tetramethylammonium hydroxide was added 1 g of tetraethylammonium iodide (3.89 mmol; 1equiv). After homogenization, 450 μL of 3,3-dimethylallyl bromide (3.91 mmol; 1 equiv) was added dropwise to the solution. The latter was submitted to 10 successive 1-min microwave irradiations at 120 W (pause time between 2 irradiation periods to return to 25 °C). The mixture was diluted with water, acidified with HCl (6 N), and extracted with ethyl acetate. The organic layer was then concentrated to dryness affording a brown residue; the latter was chromatographed on silica gel using hexane:ethyl acetate (8:2) as mobile phase affording two fractions, A and B. Fraction A was pure compound **14** (35.5 mg; yield 2.3%). Fraction B (269 mg) was submitted to a DIOL MPLC using a gradient of *i*-PrOH in hexane (5 to 40% in 150 min) as mobile phase, which afforded pure compounds **12** (90.2 mg; yield 7.2%) and **13** (139.6 mg; yield 11%).

Geranylated Chrysin Derivatives 15, 16. To a solution of 1 g of chrysin (3.92 mmol) in 10 mL of methanol and 20 mL of 10% aqueous tetramethylammonium hydroxide was added 1 g of tetraethylammonium iodide (3.89 mmol; 1 equiv). After homogenization, 800 μL of geranyl bromide (3.92 mmol; 1 equiv) was added dropwise to the solution. The latter was submitted to 6 successive 1-min microwave irradiations at 120 W (pause time between 2 irradiation periods to return to 25 °C). The mixture was diluted with water, acidified with HCl (6 N), and extracted with ethyl acetate. The organic layer was then concentrated to dryness affording a brown residue, which was chromatographed on Sephadex LH-20 using acetone as mobile phase and DIOL MPLC with hexane:$CHCl_3$ (70:30 to 0:100 in 70 min) affording pure compounds **15** (320 mg; 0.8197 mmol; yield 20.91%) and **16** (150.5 mg; 0.3855 mmol; yield 9.83%).

6-*C*-Methylchrysin (1): ^{1}H NMR (DMSO-d_6) δ 1.99 (s, 3H, H1a), 6.59 (s, H8), 6.95 (s, H3), ca. 7.58 (m, 3H, H3′ + H4′ + H5′), 8.05 (m, 2H, H2′ + H6′), 13.06 (s, 5OH); ^{13}C NMR (DMSO-d_6) δ 7.35 (C1a), 93.12 (C8), 103.59 (C10), 105.06 (C3), 107.04 (C6), 126.33 (C2′ + C6′), 129.12 (C3′ + C5′), 130.78 (C1′), 131.91 (C4′), 155.04 (C9), 158.45 (C5), 162.36 (C7), 162.88 (C2), 181.80 (C4); UV (λ nm (log ϵ)) 272 (4.29), 319 (4.01); MS (CI+) *m*/*z* 269 [M + H]$^+$; HRMS ($C_{16}H_{13}O_4$) calcd 269.0815, found 269.0813. Anal. ($C_{16}H_{12}O_4$·0.60H_2O) C, H.

6-*C*-7-*O*-Dimethylchrysin (3): ^{1}H NMR (acetone-d_6) δ 2.06 (s, 3H, H1a), 4.00 (s, 3H, H1b), 6.82 (s, H8), 6.83 (s, H3), ca. 7.61 (m, 3H, H3′ + H4′ + H5′), 8.09 (m, 2H, H2′ + H6′), 13.00 (s, 5OH); ^{13}C NMR (acetone-d_6) δ 8.44 (C1a), 57.64 (C1b), 91.74 (C8), 106.91 (C10), 107.30 (C3), 110.17 (C6), 128.19 (C2′ + C6′), 130.98 (C3′ + C5′), 133.25 (C1′), 133.70 (C4′), 158.01 (C9), 160.22 (C5), 165.52 (C2), 165.59 (C7), 184.20 (C4); UV (λ nm (log ϵ)) 272 (4.23), 313 (3.94); MS (CI+) *m*/*z* 283 [M + H]$^+$; HRMS ($C_{17}H_{15}O_4$) calcd 283.0971, found 283.0970. Anal. ($C_{17}H_{14}O_4$) C, H.

6-*C*-Isopropylchrysin (4): ^{1}H NMR (DMSO-d_6) δ 1.28 (d, 6H, H2a + H3a, J = 7.1 Hz), 3.47 (h, H1a, J = 7.1 Hz), 6.55 (s, H8), 6.94 (s, H3), ca. 7.57 (m, 3H, H3′ + H4′ + H5′), 8.05 (m, 2H, H2′ + H6′), 10.89 (s, 7OH), 13.29 (s, 5OH); ^{13}C NMR (DMSO-d_6) δ 20.99 (C2a + C3a), 23.91 (C1a), 94.49 (C8), 104.64 (C10), 105.90 (C3), 117. 40 (C6), 127.20 (C2′ + C6′), 130.01 (C3′ + C5′), 131.61 (C1′), 132.80 (C4′), 155.91 (C9), 159.77 (C5), 163.36 (C7), 163.68 (C2), 182.94 (C4); UV (λ nm (log ϵ)) 273 (4.06), 322 (3.82); MS (CI+) *m*/*z* 297 [M + H]$^+$; HRMS ($C_{18}H_{17}O_4$) calcd 2971128, found 297.1126. Anal. ($C_{18}H_{16}O_4$·0.40hexane) C, H.

7-*O*-Isopropylchrysin (5): ^{1}H NMR (acetone-d_6) δ 1.25 (d, 6H, H2a + H3a, J = 6.0 Hz), 4.69 (h, H1a, J = 6.0 Hz), 6.19 (d, H6, J = 2.2 Hz), 6.58 (d, H8, J = 2.2 Hz), 6.68 (s, H3), ca. 7.48 (m, 3H, H3′ + H4′ + H5′), 7.95 (m, 2H, H2′ + H6′), 12.70 (s, 5OH); ^{13}C NMR (acetone-d_6) δ 23.12 (C2a + C3a), 72.53 (C1a), 95.51 (C8), 100.95 (C6), 106.96 (C10), 107.25 (C3), 128.28 (C2′ + C6′), 131.01 (C3′ + C5′), 133.23 (C1′), 133.78 (C4′), 159.88 (C9), 164.11 (C5), 165.81 (C2), 166.20 (C7), 184.18 (C4); UV (λ nm (log ϵ)) 269 (4.46), 311 (4.10); MS (CI+) *m*/*z* 297 [M + H]$^+$; HRMS ($C_{18}H_{17}O_4$) calcd 297.1128, found 297.1126. Anal. ($C_{18}H_{16}O_4$) C, H.

6-*C*-7-*O*-Diisopropylchrysin (6): ^{1}H NMR (acetone-d_6) δ 1.32 (d, 6H, H2b + H3b, J = 7.1 Hz), 1.42 (d, 6H, H2a + H3a, J = 6.0 Hz), 3.62 (h, H1b, J = 7.1 Hz), 4.85 (h, H1a, J = 6.0 Hz), 6.81 (s, H3), 6.82 (s, H8), ca. 7.60 (m, 3H, H3′ + H4′ + H5′), 8.08 (m, 2H, H2′ + H6′), 13.26 (s, 5OH); ^{13}C NMR (acetone-d_6) δ 21.62 (C2b + C3b), 23.16 (C2a + C3a), 25.25 (C1b), 72.52 (C1a), 93.53 (C8), 106.76 (C10), 107.18 (C3), 120.40 (C6), 128.19 (C2′ + C6′), 130.96 (C3′ + C5′), 133.29 (C1′), 133.67 (C4′), 158.00 (C9), 160.76 (C5), 164.21 (C7), 165.42 (C2), 184.36 (C4); UV (λ nm (log ϵ)) 274 (4.37), 318 (4.06); MS (CI+) *m*/*z* 339 [M + H]$^+$; HRMS ($C_{21}H_{23}O_4$) calcd 339.1597, found 339.1596. Anal. ($C_{21}H_{22}O_4$) C, H.

6,8-Di-*C*-7-*O*-triisopropylchrysin (7): ^{1}H NMR ($CDCl_3$) δ 1.35 (d, 6H, H2a + H3a, J = 6.2 Hz), 1.41 (d, 6H, H2b + H3b, J = 7.0 Hz), 1.48 (d, 6H, H2c + H3c, J = 7.2 Hz), 3.41 (h, H1b, J = 7.0 Hz), 3.65 (h, H1c, J = 7.2 Hz), 4.21 (h, H1a, J = 6.2 Hz), 6.70 (s, H3), ca. 7.55 (m, 3H, H3′ + H4′ + H5′), 7.95 (m, 2H, H2′ + H6′), 13.19 (br s, 5OH); ^{13}C NMR ($CDCl_3$) δ 20.03 (C2b + C3b), 21.30 (C2c + C3c), 22.34 (C2a + C3a), 25.53 (C1c), 26.28 (C1b), 77.25 (C1a), 105.89 (C3), 108.29 (C10), 119.50 (C8), 124.51 (C6), 126.45 (C2′ + C6′), 129.20 (C3′ + C5′), 131.67 (C4′), 131.81 (C1′), 154.11 (C9), 158.60 (C5), 158.98 (C7), 164.07 (C2), 183.71 (C4); UV (λ nm (log ϵ)) 279 (4.53); MS (CI+) *m*/*z* 381 [M + H]$^+$; HRMS ($C_{24}H_{29}O_4$) calcd 381.2067, found 381.2066. Anal. ($C_{24}H_{28}O_4$·0.40hexane·0.60H_2O) C, H.

6-*C*-Benzylchrysin (8): ^{1}H NMR (DMSO-d_6) δ 3.89 (s, 2H, H7a), 6.61 (s, H8), 6.97 (s, H3), ca. 7.13 (m, H4a), ca. 7.24 (m, 4H, H2a + H3a + H5a + H6a), 7.57 (m, 3H, H3′ + H4′ + H5′), 8.06 (m, 2H, H2′ + H6′), 11.05 (s, 7OH), 13.20 (s, 5OH); ^{13}C NMR (DMSO-d_6) δ 27.36 (C7a), 93.37 (C8), 103.70 (C10), 105.03 (C3), 110.79 (C6), 125.51 (C4a), 126.31 (C2′ + C6′), 127.99 (C2a + C6a), 128.16 (C3a + C5a), 129.05 (C3′ + C5′), 130.68 (C1′), 131.87 (C4′), 140.57 (C1a), 155.45 (C9), 158.63 (C5), 162.19 (C7), 162.94 (C2), 181.85 (C4); UV (λ nm (log ϵ)) 273 (4.39), 320 (4.05); MS (FAB+) *m*/*z* 345 [M + H]$^+$; HRMS ($C_{22}H_{17}O_4$) calcd 345.1128, found 345.1126. Anal. ($C_{22}H_{16}O_4$·0.80MeOH) C, H.

8-*C*-Benzylchrysin (9): ^{1}H NMR (DMSO-d_6) δ 4.12 (s, 2H, H7a), 6.38 (s, H6), 6.96 (s, H3), ca. 7.13 (m, H4a), ca. 7.24 (m, 4H, H2a + H3a + H5a + H6a), 7.57 (m, 3H, H3′ + H4′ + H5′), 7.94 (m, 2H, H2′ + H6′), 11.01 (s, 7OH), 12.83 (s, 5OH); ^{13}C NMR (DMSO-d_6) δ 27.74 (C7a), 98.50 (C6), 103.89 (C10), 104.98 (C3), 105.74 (C8), 125.74 (C4a), 126.20 (C2′ + C6′), 127.82 (C2a + C6a), 128.22 (C3a + C5a), 129.09 (C3′ + C5′), 130.80 (C1′), 131.90 (C4′), 140.43 (C1a), 154.76 (C9), 159.46 (C5), 162.18 (C7), 162.99 (C2), 182.09 (C4); UV (λ nm (log ϵ)) 274 (4.29); MS (FAB+) *m*/*z* 345 [M + H]$^+$; HRMS ($C_{22}H_{17}O_4$) calcd 345.1128, found 345.1126. Anal. ($C_{22}H_{16}O_4$·MeOH) C, H.

6,8-*C*-Dibenzylchrysin (10): ^{1}H NMR (DMSO-d_6) δ 4.04 (s, 2H, H7b), 4.27 (s, 2H, H7a), 6.99 (s, H3), ca. 7.14 (m, 2H, H4a + H4b), ca. 7.25 (m, 8H, H2a + H3a + H5a + H6a +H2b + H3b + H5b + H6b), 7.55 (m, 3H, H3′ + H4′ + H5′), 7.91 (m, 2H, H2′ + H6′), 10.16 (s, 7OH), 13.22 (s, 5OH); ^{13}C NMR (DMSO-d_6) δ 27.60 (C7b), 28.10 (C7a), 104.10 (C10), 104.97 (C3), 105.31 (C8), 111.22 (C6), 125.60 (C4b), 125.81 (C4a), 126.21 (C2′ + C6′), 127.73 (C2a + C6a), 128.05 (C2b + C6b or C3a + C5a), 128.08 (C3a + C5a or C2b + C6b), 128.26 (C3b + C5b), 129.08 (C3′ + C5′), 130.80 (C1′), 131.91 (C4′), 140.26 (C1a), 140.39 (C1b), 153.23 (C9), 157.08 (C5), 159.97 (C7), 162.99 (C2), 182.24 (C4); UV (λ nm (log ϵ)) 275 (4.16); MS (CI+) *m*/*z* 435 [M + H]$^+$; HRMS ($C_{29}H_{23}O_4$) calcd 435.1597, found 435.1596. Anal. ($C_{29}H_{22}O_4$·0.60H_2O·2hexane) C, H.

7-*O*-Benzylchrysin (11): ^{1}H NMR (DMSO-d_6) δ 5.17 (s, 2H, H7a), 6.48 (d, H6, J = 2.2 Hz), 6.60 (d, H8, J = 2.2 Hz), 6.69 (s, H3), ca. 7.44 (m, 5H, H2a + H3a + H4a + H5a + H6a), 7.54 (m, 3H, H3′ + H4′ + H5′), 7.90 (m, 2H, H2′ + H6′); ^{13}C NMR (DMSO-d_6) δ 70.44 (C7a), 93.52 (C8), 98.94 (C6), 105.90 (C10 + C3), 126.29 (C2′ + C6′), 127.47 (C2a + C6a), 128.37

(C4a), 128.75 (C3a + C5a), 129.07 (C3′ + C5′), 131.29 (C1′), 131.84 (C4′), 135.72 (C1a), 157.75 (C9), 162.19 (C5), 164.02 (C2), 164.65 (C7), 182.47 (C4); UV (λ nm (log ϵ)) 269 (4.49), 309 (4.13); MS (CI+) *m/z* 345 $[M + H]^+$; HRMS ($C_{22}H_{17}O_4$) calcd 345.1128, found 345.1126. Anal. ($C_{22}H_{16}O_4$) C, H.

6-*C*-(3,3-Dimethylallyl)chrysin (12): ^{1}H NMR (DMSO-d_6) δ 1.63 (br s, H4a), 1.73 (brs, H5a), 3.23 (d, 2H1a, J = 7.2 Hz), 5.18 (br t, H2a, J = 7.2 Hz), 6.58 (s, H8), 6.96 (s, H3), 7.59 (m, 3H, H3′ + H4′ + H5′), 8.06 (m, 2H, H2′ + H6′), 10.93 (s, 7OH), 13.09 (s, 5OH); ^{13}C NMR (DMSO-d_6) δ 18.55 (C5a) 21.86 (C1a), 26.34 (C4a), 94.20 (C8), 104.62 (C10), 105.92 (C3),111.99 (C6), 122.97 (C2a), 127.23 (C2′ + C6′), 130.00 (C3′ + C5′), 131.58 (C1′ or C3a), 131.65 (C1′ or C3a), 132.80 (C4′), 156.07 (C9), 159.18 (C5), 162.95 (C7), 163.75 (C2), 182.77 (C4); UV (λ nm (log ϵ)) 273 (4.47), 320 (4.13); MS (EI) *m/e* 322 $[M]^+$; HRMS ($C_{20}H_{18}O_4$) calcd 322.1205, found 322.1208. Anal. ($C_{20}H_{18}O_4$·0.80MeOH) C, H.

8-*C*-(3,3-Dimethylallyl)chrysin (13): ^{1}H NMR (DMSO-d_6) δ 1.63 (br s, H4a), 1.75 (br s, H5a), 3.45 (d, 2H1a, J = 6.8 Hz), 5.20 (br t, H2a, J = 6.8 Hz), 6.31 (s, H6), 6.95 (s, H3), 7.59 (m, 3H, H3′ + H4′+ H5′), 8.04 (m, 2H, H2′ + H6′), 10.86 (s, 7OH), 12.76 (s, 5OH); ^{13}C NMR (DMSO-d_6) δ 18.68 (C5a), 22.19 (C1a), 26.30 (C4a), 99.38 (C6), 104.77 (C10), 105.84 (C3), 107.10 (C8), 123.26 (C2a), 127.15 (C2′ +C6′), 130.04 (C3′ + C5′), 131.92 (C1′ +C3a), 132.85 (C4′), 155.50 (C9), 159.94 (C5), 162.72 (C7), 163.95 (C2), 183.04 (C4); UV (λ nm (log ϵ)) 275 (4.41); MS (EI) *m/e* 322 $[M]^+$; HRMS ($C_{20}H_{18}O_4$) calcd 322.1205, found 322.1208. Anal. ($C_{20}H_{18}O_4$·0.20MeOH) C, H.

6,8-Di-*C*-(3,3-dimethylallyl)chrysin (14): ^{1}H NMR (DMSO-d_6) δ 1.63 (br s, H4a + H4b), 1.74 (s, H5a), 1.77 (s, H5b), ca. 3.33 (m, 2H1a, overlapping H_2O signal), 3.56 (d, 2H1b, J = 6.1 Hz), 5.15 (m, 2H, H2a + H2b), 6.96 (s, H3), 7.59 (m, 3H, H3′ + H4′ +H5′), 8.03 (m, 2H, H2′ + H6′), 9.77 (s, 7OH), 13.06 (s, 5OH); ^{13}C NMR (DMSO-d_6) δ 18.65 (C5a), 18.82 (C5b), 22.20 (C1a), 22.64 (C1b), 26.28 (C4b), 26.36 (C4a), 104.97 (C10), 105.77 (C3), 107.54 (C8), 112.61 (C6), 123.09 (C2a), 123.57 (C2b), 127.15 (C2′ +C6′), 130.01 (C3′ + C5′), 131.71 (C3a), 131.94 (C3b or C1′), 131.96 (C1′ or C3b), 132.81 (C4′), 153.55 (C9), 157.13 (C5), 160.08 (C7), 163.83 (C2), 183.16 (C4); UV (λ nm (log ϵ)) 279 (4.43), 322 (3.98); MS (EI) *m/e* 390 $[M]^+$; HRMS ($C_{25}H_{26}O_4$) calcd 390.1831, found 390.1830. Anal. ($C_{25}H_{26}O_4$·0.30MeOH) C, H.

6-*C*-Geranylchrysin (15): ^{1}H NMR (DMSO-d_6) δ 1.51 (br s, H10a), 1.57 (br s, H9a), 1.73 (br s, H8a), 1.93 (m, 2H4a), 1.96 (m, 2H5a), 3.23 (d, 2H1a, J = 7.0 Hz), 5.03 (br t, H6a, J = 6.6 Hz), 5.18 (br t, H2a, J = 7.0 Hz), 6.58 (s, H8), 6.95 (s, H3), 7.58 (m, 3H, H3′ + H4′+ H5′), 8.05 (dd, 2H, H2′ + H6′, J = 8.0 and 1.8 Hz), 10.93 (s, 7OH), 13.08 (s, 5OH); ^{13}C NMR (DMSO-d_6) δ 16.77 (C8a), 18.35 (C10a) 21.80 (C1a), 26.29 (C9a), 27.01 (C5a), ca. 40.00 (C4a, overlapping solvent signal) 94.18 (C8), 104.61 (C10), 105.91 (C3), 112.02 (C6), 122.77 (C2a), 124.93 (C6a), 127.21 (C2′ + C6′), 129.99 (C3′ + C5′), 131.48 (C7a), 131.64 (C1′), 132.78 (C4′), 135.10 (C3a), 156.06 (C9), 159.21 (C5), 162.97 (C7), 163.74(C2), 182.75 (C4); UV (λ nm (log ϵ)) 274 (4.42), 320 (4.12); MS (EI) *m/e* 390 $[M]^+$; HRMS ($C_{25}H_{26}O_4$) calcd 390.1831, found 390.1830. Anal. ($C_{25}H_{26}O_4$·0.90MeOH) C, H.

8-*C*-Geranylchrysin (16): ^{1}H NMR (DMSO-d_6) δ 1.42 (br s, H10a), 1.49 (br s, H9a), 1.75 (br s, H8a), 1.94 (m, 4H, 2H4a + 2H5a), 3.46 (d, 2H1a, J = 6.6 Hz), 4.94 (m, H6a), 5.19 (br t, H2a, J = 6.6 Hz), 6.32 (s, H6), 6.96 (s, H3), 7.58 (m, 3H, H3′ + H4′ + H5′), 8.04 (dd, 2H, H2′ + H6′, J = 8.2 and 1.7 Hz), 10.86 (s, 7OH), 12.76 (s, 5OH); ^{13}C NMR (DMSO-d_6) δ 15.46 (C8a), 18.26 (C10a), 22.08 (C1a), 26.18 (C9a), 26.87 (C5a), ca. 40.00 (C4a, overlapping solvent signal), 99.36 (C6), 104.79 (C10), 105.78 (C3), 107.17 (C8), 123.20 (C2a), 124.81 (C6a), 127.15 (C2′ + C6′), 129.98 (C3′ + C5′), 131.46 (C7a), 131.89 (C1′), 132.84 (C4′), 135.41 (C3a), 155.56 (C9), 159.92 (C5), 162.68 (C7), 163.95 (C2), 183.04 (C4); UV (λ nm (log ϵ)) 275 (4.39); MS (EI) *m/e* 390 $[M]^+$; HRMS ($C_{25}H_{26}O_4$) calcd 390.1831, found 390.1834. Anal. ($C_{25}H_{26}O_4$·1.5H_2O) C, H.

Biological Assays. The recombinant C-terminal cytosolic domain of Pgp, NBD2, was overexpressed and purified as described previously.[11] Fluorescence experiments were performed at 25 °C by using either a SLM-Aminco 8000C or a PTI QM-1 spectrofluorimeter. The tryptophan-specific intrinsic fluorescence of 0.5 μM recombinant domain in 1.2−2.0 mL of 20 mM potassium phosphate at pH 6.8 containing 0.5 M NaCl, 20% glycerol and 0.01% methyl 6-*O*-(*N*-heptylcarbamoyl)-α-D-glucopyranoside was scanned from 310 to 360 nm upon excitation at 295 nm, integrated and corrected for buffer contribution. The binding of chrysin and its derivatives was monitored by the quenching of intrinsic fluorescence produced by successive additions of aliquots up to 20 μM. The modifications were corrected for the inner-filter effect determined under the same conditions with 0.5 μM *N*-acetyltryptophanamide. Curve fitting of binding related to fluorescence decrease was performed with the Grafit program (Erithacus software) as previously described.[19−21]

For inhibition of Pgp-mediated drug efflux, 1 million K562/R7 human leukemic cells expressing high levels of Pgp were incubated for 1 h at 37 °C in 1 mL of RPMI 1640 medium containing a final concentration of 10 μM daunomycin, in the presence or absence of modulators. Cells were then washed three times with ice-cold phoshate buffer saline and maintained on ice until analysis by flow cytometry on a FACS-II (Becton-Dickinson Corp., Mountain View, CA). Assays were performed in duplicate, in at least three separate experiments. Cyclosporin A, a potent modulator of Pgp was used as a positive control at a final concentration of 2 μM. Control experiments showed that cells incubated with chrysin derivatives in the absence of daunomycin did not exhibit enhanced fluorescence (data not shown). The ability of chrysin derivatives to inhibit Pgp-mediated drug efflux at a concentration of 10 μM was quantified by comparing the shift in fluorescence induced by these compounds to the one obtained with cyclosporin A. In separate experiments, cell viability was tested in cells exposed to 10 μM chrysin derivatives; it was shown to be reduced by less than 5% in comparison to untreated cells (data not shown).

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