Modulation of P-Glycoprotein-Mediated Multidrug Resistance by Flavonoid Derivatives and Analogues

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Flavonoid derivatives were synthesized and tested for their ability to modulate P-glycoprotein (Pgp)-mediated multidrug resistance (MDR) in vitro. These compounds belong to various flavonoid subclasses, namely: chromones, azaisoflavones, and aurones. Among the investigated compounds, three showed potent reversing activity. 2-(4-Methylpiperazin-1-ylcarbonyl)-5-hydroxychromone (**4a**), 5,7-dimethoxy-3-phenyl-4-quinolone (**5**), and 4,6-dimethoxyaurone (**6**) potentiated daunorubicin cytotoxicity on resistant K562 cells. They were also able to increase the intracellular accumulation of rhodamine-123, a fluorescent molecule which acts as a probe of P-glycoprotein-mediated MDR. This suggests that these compounds act, at least in part, by inhibiting P-glycoprotein activity. The most active compound, 5-hydroxy-2-(4-methylpiperazin-1-ylcarbonyl)chromone (**4a**) was found to be a powerful reversal agent, more potent than cyclosporin A, used as the reference molecule. No effect was observed on MRP transport nor on cell proliferation. Little apoptosis was induced on K562S cells with **4a** compared to K562R, probably due to the extrusion of the compound by Pgp.

Introduction

The treatment of cancer with anticancer drugs is frequently impaired by the intrinsic or acquired resistance of tumor cells. Broad-spectrum resistance to structurally and mechanistically diverse anticancer agents constitutes the multidrug resistance (MDR) phenotype.[1] The overexpression of a particular class of transmembrane glycoprotein, encoded by a small family of *mdr* genes, is a common mechanism by which tumor cells acquire MDR.[2] The best known are P-glycoprotein (Pgp),[3] and, to a lesser extent, the multidrug resistance-associated protein (MRP).[4] Pgp which features ATPase activity decreases the intracellular drug concentration below its active threshold by rapidly pumping the anticancer drug out of the MDR-tumor cells.[5] Various attempts are being made to develop noncytotoxic compounds which will interfere with the P-gp function, thus allowing intracellular accumulation of the cytotoxic drug. In this context, several molecules including the calcium channel blocker verapamil, the calmodulin inhibitor trifluoroperazine, and cyclosporin have been used to reverse multidrug resistance.[6] However, the clinical application of these agents have met with limited success mainly because of adverse effects when used at concentrations required to overcome multidrug resistance.[7] Therefore, new potent and noncytotoxic reversal agents with minimal adverse effects are strongly hoped for.

In this context, flavonoids were investigated as a new class of MDR modulators (chemical structures are shown in Figure 1). Chalcones, flavones, and flavonols have been demonstrated to possess MDR reversing activity through high affinity binding with P-gp.[8−10] Isoflavones have been described as inactive on Pgp-mediated MDR.[11] Finally, aurones have never been investigated as reversal agents. The only data available regarding aurones, in the field of MDR, is their binding affinity with the C-terminal nucleotide-binding domain (NBD2) of Pgp.[12] Studies of structure−activity relationships made on chalcones, flavones, and flavonols have concluded that a hydroxyl group on position 5 (position 6′ in chalcones) is important. 5-OH methylation leads to slightly less active compounds. Because of the chelating effect induced by the adjacent carbonyl group, the hydroxyl loses its acidic properties and therefore does not affect the activity which can be decreased by the presence of acidic groups.[6] Hydroxylation on position 7 (position 4′ in chalcones) was deleterious for activity, probably due to the acidic group influence, whereas methoxylation was slightly beneficial. The 2,3 double bond (the α,β-double bound in chalcones) and the

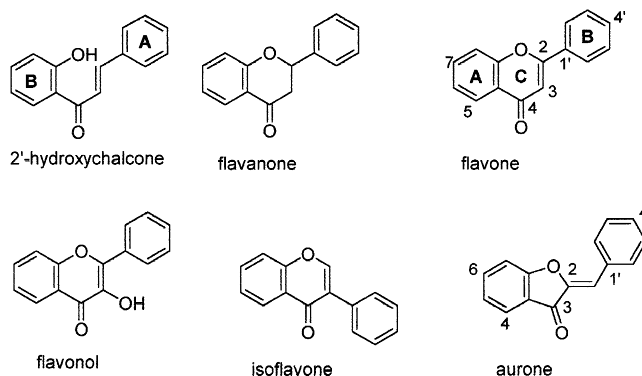

Figure 1. Chemical structure of flavonoid subclasses.

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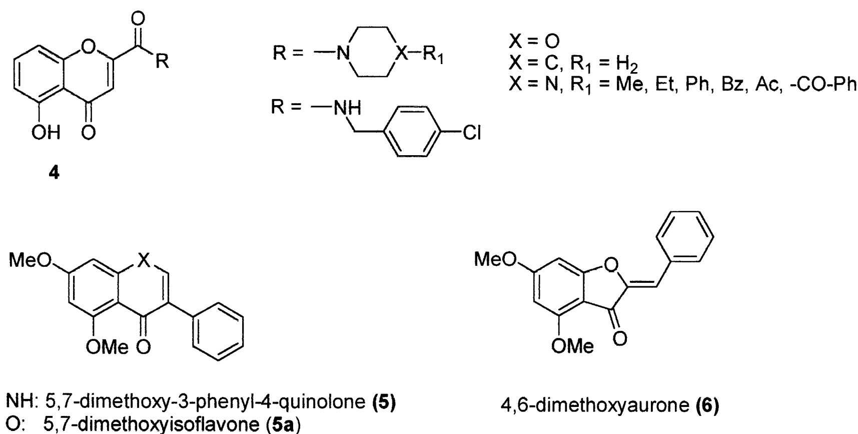
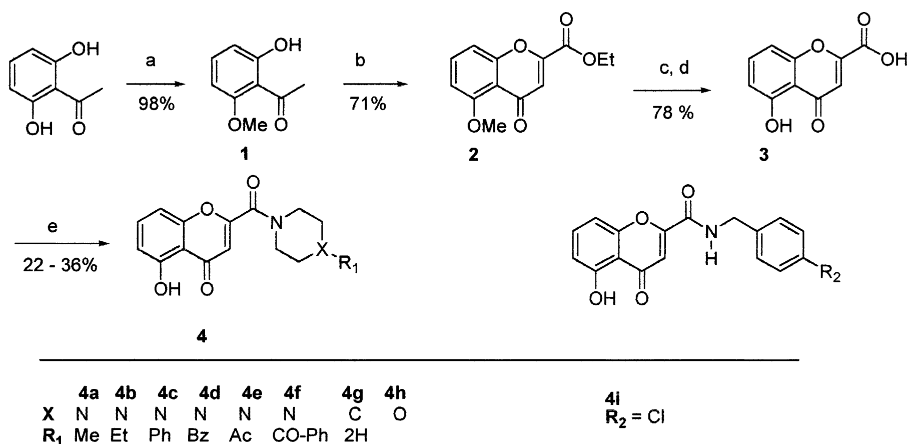


Figure 2. Structure of studied molecules: piperazinylchromones **4**, 3-phenyl-4-quinolone **5**, and aurone **6**.

Scheme 1[a]



[a] Reagents: (a) MeI, K_2CO_3, acetone, reflux, 4 h; (b) diethyl oxalate, NaOEt, EtOH, 100 °C, 10 h (c) $NaHCO_3$ (20% in H_2O), 80 °C, 3 h; (d) HBr/AcOH, 100 °C, 2 h; (e) *N*-alkylpiperazine, *N*-acylpiperazine, piperidine, morpholine, or benzylamine, EDC, Et_3N, THF, 24 h.

carbonyl group are also essential for MDR modulation.[13−15] On another hand, a parameter shared by many MDR modulators is the presence of at least one basic nitrogen. A survey on MDR modulators concluded that this last criterion was frequently a piperazine unit.[6,16−19]

On the basis of these data from literature, we wanted to report the synthesis and MDR modulation of a variety of flavonoid derivatives, namely piperazinylcarbonylchromones, 3-phenyl-4-quinolones (azaisoflavone), and aurones (Figure 2). Piperazinylcarbonylchromones (compounds **4**) were designed by combining a chromone unit (5-hydroxy-4-chromanone containing all structural elements identified among flavonoids) with a piperazine unit, so as to obtain a synergistic effect on the MDR modulation. 3-Phenyl-4-quinolone (azaisoflavone, **5**) was investigated as an analogue of inactive isoflavones, to check the importance of nitrogen. Finally we report the effect of 4,6-dimethoxyaurone (**6**) on MDR modulation for the first time.

Chemistry

The synthesis of azaisoflavone **5**, isoflavone **5a**, and aurone **6** (Figure 2) investigated in this study has been already reported; therefore, it will not discussed here.[20,21] The synthetic strategy chosen for the preparation of piperazinylcarbonylchromones and analogues (**4**) required the preparation of 2-carboxy-5-hydroxychromone **3**.[22] Chromone **3** was obtained in four steps starting from 2,6-dihydroxyacetophenone (Scheme 1). Treatment of the latter with methyl iodide gave 2-hydroxy-6-methoxyacetophenone (**1**).[23] Condensation of **1** with diethyl oxalate in the presence of sodium ethoxide in EtOH and then acidic cyclization afforded ester **2**.[22,24] Saponification of the ester with $NaHCO_3$ followed by a demethylation with HBr in AcOH provided 2-carboxy-5-hydroxychromone (**3**).[25] The target amides **4a**−**i** were synthesized by reacting acid **3** with either a piperazine derivative, piperidine, morpholine, or a benzylamine using EDC as a coupling agent. *N*-Benzoylpiperazine, is not commercially available; therefore, it was prepared according to a recently reported method.[26]

Results and Discussion

In this study, we investigated compounds **4a**−**i**, **5**, **5a**, and **6** (Figure 2 and Scheme 1). Although isoflavones are inactive on Pgp-mediated MDR, we included isoflavone **5a** with the aim to compare it with azaisoflavone **5**. These compounds were meant to be tested in vitro to assess their ability to restore intracellular accumulation of daunorubicin (DNR). Since DNR cytotoxicity experiments cannot assess the effect of the tested compounds on Pgp transport activity, we started our screening by an assay of Pgp transport function, using rhodamine-123. The latter is a fluorescent dye and probe of Pgp transport activity. After this, the most active compounds on the transport activity were selected and tested for their ability to restore intracellular accumulation of DNR.

Table 1. Mean of Rhodamine-123 Fluorescence, in Arbitrary Unit (a.u.), in K562 Cells with or without Expression of PGP Protein[a]

compound (in K562R)	rhodamine-123 accumulation at selected concentrations of compound[b–d]			
	1 μM	0.1 μM	0.01 μM	1 nM
4a	207	138	134	76
4b	85	47	44	40
4c	82	57	40	39
4d	136	95	72	45
4e	38	37	43	41
4f	42	47	45	38
4g	45	44	46	43
4h	42	44	41	39
4i	46	45	42	50
5	82	55	43	36
5a	49	46	54	40
6	249	203	178	40
cyclosporin A	102	87	80	72

[a] Controls (without modulator): K562S, 200; K562R, 40. [b] An arbitrary unit (au) which represents the fluorescence intensity at 590 nm. [c] Results are the mean of at least 3 independent experiments. [d] Interexperimental variations were inferior to 25%.

1. Rhodamine-123 Accumulation Assay. Rhodamine-123 is a mitochondrial membrane potential-sensitive fluorescent probe used for the functional assay of Pgp-mediated cell resistance.[27,28] The reversing properties were tested at four concentrations (10^{-6} M, 10^{-7} M, 10^{-8} M, and 10^{-9} M) on K562S (sensitive) and K562R (adriamycin resistant) cells. Cyclosporin A, one of the MDR modulators used clinically which acts on Pgp transport activity, was used as a reference. As shown in Table 1, the results are expressed by the mean of rhodamine-123 fluorescence on cells expressing Pgp (arbitrary unit, a.u). At 1 μM, except for the isoflavone **5a** and benzylchromone **4i**, all compounds showed a good activity, although the maximum activity was observed for **4a**, **4d**, and **6**. At 0.01 μM, compounds **4a** and **6** were still strongly active and even more active than cyclosporin A. Finally at 10 nM, *N*-methylpiperazinylcarbonylchromone (**4a**) was as active as cyclosporin A. These compounds are Pgp specific since MRP transport activity evaluation by using the calcein-AM method did not show any inhibitory activity (data not shown).[29,30]

2. Effect on Cell Proliferation and Necrosis. 2.1. Cell Cycle Assay. To complete our study, the effect of each compound on the cell cycle distribution of the different phases was investigated. K562S and K562R cell distribution in G0/G1, S, and G2+M phases was analyzed at a 10^{-6} M concentration of each compound. We concluded that there was no significant difference between controls and cells treated with various modulators.[31] On the basis of the rhodamine-123 accumulation assay and the lack of cell cycle modification, we selected **4a**, **5**, and **6** for further experiments.

2.2. Cell Division, Apoptosis, and Necrosis Assay. The balance between cell proliferation and drug-induced cell death by apoptosis or necrosis plays a major role in determining the response to chemotherapy. To check the balance factor, a triple labeling using PKH67, annexin biotin (streptavidin AMCA), and propidium iodide were used to simultaneously follow cell division, apoptosis, and necrosis, respectively. PKH is a green fluorescent membrane-intercalating dye which has the advantage that its fluorescence intensity decreases proportionally to the number of cell divisions, and labeling with PKH67 does not alter cell cycle distribution nor cell growth.[32,33]

Mean PKH67 fluorescence intensity on K562S and K562R treated with **4a**, **5**, or **6** was evaluated for 2 days. The mean PKH67 fluorescence ratio (day0/day2) of controls and cells treated with one of the compounds revealed that there was no difference between controls and treated cells. However, we found that the three compounds were able to induce little apoptosis, ranging from 30% to 35% for necrosis, and from 4% to 10% for apoptosis, especially at 10^{-6} M after 2 days in K562S and at a lesser degree on K562R.[31] No cell division effect was observed, which correlates to cell cycle results.

3. Cell Cytotoxicity Assay Using TOTO-3 Dye Incorporation. Compounds **4a**, **5**, and **6**, the most active identified by the rhodamine-123 accumulation assay, were tested for their ability to potentiate DNR cytotoxicity on K562S and K562R cells, which were shown to overexpress P-glycoprotein. Flow cytometry, a highly sensitive method, was used to detect the effect of modulators on MDR cells in heterogeneous cell population; potency of the compounds was compared with that of cyclosporin A, an established Pgp inhibitor. The dose–response curves which show the percentage of viable cells (which correspond to cells with low level of TOTO-3 fluorescence) are reported in Figures 3 and 4.[34,35] At different concentrations, compounds **4a** and **6** are much more active than cyclosporin A. Figures 3 and 4 indicate that K562S viability was reduced to 20% when treated with DNR and cyclosporin A, **4a**, or **6** compared to K562R cells which was less reduced (viability at 40%). Quinolone **5** induced a lower decrease of cellular viability (65%) for both K562S and K562R cells. Finally, the ability of these compounds to induce necrosis in sensitive and resistant cells was evaluated, and no significant difference between cyclosporin A and **4a**, **5**, or **6** was found. As an example and as shown in Figure 5, a mixture of DNR and cyclosporin A at 10^{-6} M induced 78% of necrosis in K562S cells, where **4a** induced 82% as detected by TOTO-3. At the same concentration, DNR and cyclosporin A induced 58% of necrosis in K562R cells whereas a mixture of DNR and **4a** induced 61% of necrosis.

Structure–Activity Relationships

The structures investigated in this study were structurally modified flavonoids, and they were chosen to answer structural criteria identified among MDR reversal agents. In the chromones series (compounds **4**), the accumulation assay showed that at 10^{-6} M, compounds **4a–d** enhanced rhodamine-123 accumulation in which **4a** was the most active. This higher activity was confirmed at lower concentrations (10^{-8} M). At nanomolar concentrations, **4a** was still active, as well as cyclosporin A. It seems that the nature of the substituent on the piperazine ring was important. From these data, we can conclude that the basicity of the alkylated nitrogen was involved in rhodamine-123 accumulation. The stronger activity of **4d** compared to **4c** might be due to the basicity of nitrogen. The nitrogen in **4c** is less basic because its lone pair is delocalized to the phenyl ring., We synthesized *N*-acetyl and *N*-benzoyl analogues (compounds **4e** and **4f**) to confirm the importance of the basicity factor, when both nitrogen

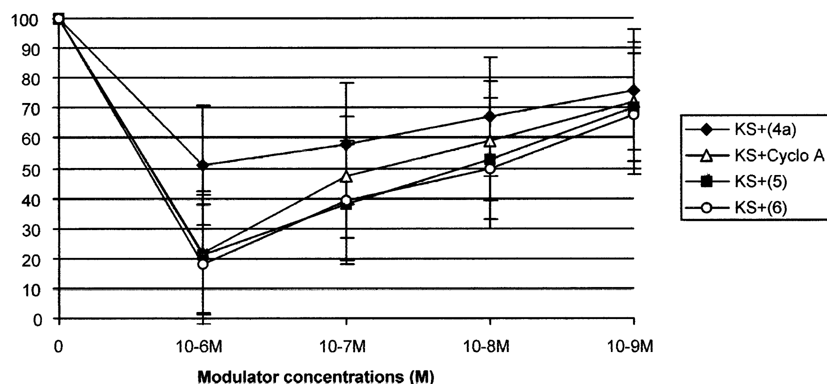


Figure 3. Viability of K562S controls and treatment with cyclosporin A, **4a**, **5**, or **6** at different concentrations, after 72 h.

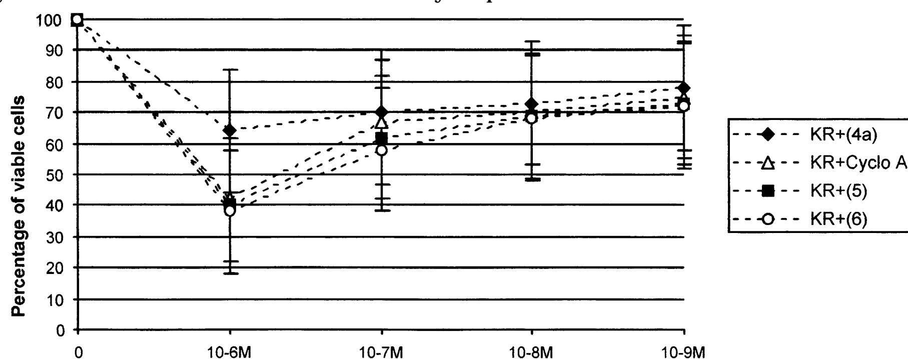


Figure 4. Viability of K562R control and treated with cyclosporin A, **4a**, **5**, or **6** at different concentrations, after 72 h.

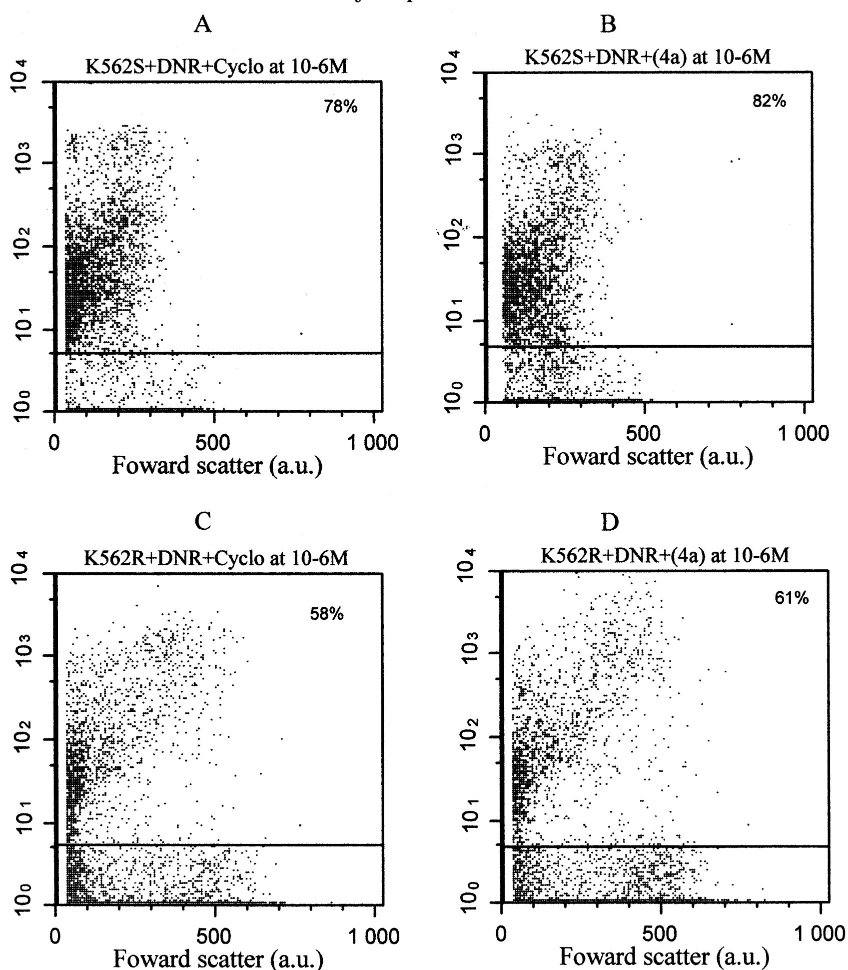


Figure 5. Viability of K562S and K562R, controls, and treatment with cyclosporin A or **4a**, at 10^{-6} M after 72 h, as measured by CMF using TOTO-3 dye. Two populations were observed: viable cells with a low level of TOTO-3 fluorescence, and necrotic cells with a high level of TOTO-3 fluorescence. (A) K562S cells treated with DNR and cyclosporin A. (B) K562S cell treated with DNR and **4a**. (C) K562R cells treated with DNR and cyclosporin A. (D) K562R cells treated with DNR and **4a**.

atoms are not basic, or compounds **4g** and **4h**, in which the nitrogen was replaced with a carbon (**4g**) or with an oxygen (**4h**). In all cases, the rhodamine-123 accumulation assay did not lead to any significant activity (Table 1).

Substitution of the piperazine group with *p*-chlorobenzylamine group (**4i**) led to a significantly less active compound. Due to insolubility problems, *p*-chlorobenzylamine derivative was the only compound tested among a series of benzylamines. We also synthesized analogues of **4a**–**d** when the piperazine unit was linked to the chromone via a methylene groupe (data not shown). Unfortunately, these compounds were insoluble in all systems used, therefore they were not tested.

On the basis of these data, we synthesized and tested azaisoflavone **5** (3-phenyl-4-quinolone) and compared it to the parent isoflavone **5a**. Isoflavones were shown to have no effect on rhodamine-123 accumulation.[11] We hypothesized that the substitution of the intracyclic oxygen by a nitrogen would confer one of the structural features. As shown in both assays (accumulation and cytotoxicity), quinolone **5** was strongly active compared to isoflavone. This stronger activity could not only be attributed to the quinolone nitrogen atom (weakly basic), but also to its ability of forming hydrogen bonds on the active site of Pgp. Finally, we tested 4,6-dimethoxyaurone (**6**) which isastructural isomer of flavone. Aurones are natural compounds, frequently found in vegetables, especially in fruit and flowers in which they contribute to coloration.[36] Methoxylation at positions 4 and 6 was chosen, because, according to the numbering system and the biosynthesis of aurones, positions 4 and 6 are equivalent to positions 5 and 7 in flavones. Aurone **6** was found to be as active as *N*-methylpiperazinylcarbonylchromone (**4a**) when using the rhodamine-123 accumulation assay. This strong activity, even in the absence of any nitrogen atom, is quite interesting. It may indicate the importance of the aurone 3D structure, because its isomer, 5,7-dimethoxyflavone, is almost inactive.[37] Therefore, it is conceivable that the introduction of any nitrogen atom would enhance the activity. For example, azaaurones, in which the intracyclic oxygen is replaced by nitrogen, are interesting.

More interesting still, the three most active compounds (**4a**, **5**, and **6**) potentiate DNR cytotoxicity as much as cyclosporin A (Figures 3, 4, and 5). Apoptosis induced by compounds **4a**, **5**, and **6** on K562S and, at a lesser degree on K562R, which may lead to an extrusion of the compounds by Pgp, so this activity might be in competition with anticancer drugs.

In conclusion, according to both accumulation and cytoxicity data, we found that 5-hydroxychromone linked to a piperazine unit constituted a class of highly active compounds as MDR modulators. We also reported for the first time MDR modulation by one aurone and one 3-phenyl-4-quinolone. These data opens the way for investigation in a new potential pharmacophores in the field of MDR modulators. The relatively short and easy synthesis of these molecules make them potential candidates for further development.

Experimental Section

General Chemistry Methods. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-200 instrument (200 MHz for ^{1}H, 50 MHz for ^{13}C). Chemical shifts were reported as δ values (ppm) relative to Me_4Si as an internal standard. EI and DCI mass spectra were recorded on a Fisons Trio 1000 instrument. Elemental analyses were performed by the Analytical Department of CNRS, Vernaison, France. Thin-layer chromatography (TLC) was carried out using E. Merck silica gel F-254 plates (thickness 0.25 mm). Flash chromatography was carried out using Merck silica gel 60, 200–400 mesh. All solvents were distilled prior to use. Chemicals and reagents were obtained either form Aldrich or ACROS companies and used without modification.

2-Hydroxy-6-methoxyacetophenone (1). The title compound was prepared according to Lau et al.:[23] mp 58–62 °C (lit. 57–58 °C); ^{1}H NMR ($CDCl_3$): δ 13.21 (s, OH); 7.34 (t, 1H, J = 8.3 Hz, H4′); 6.58 (d, 1H, J = 8.3 Hz, H5′); 6.39 (d, 1H, J = 8.3 Hz, H3′); 3.90 (s, 3H, OCH_3); 2.68 (s, 3H, H2). ^{13}C NMR ($CDCl_3$): δ 205.0 (C_1); 164.6 ($C_{6'}$); 161.5 ($C_{2'}$); 136.0 ($C_{4'}$); 111.3 ($C_{1'}$); 110.7 ($C_{5'}$); 101.1 ($C_{3'}$); 55.6 (OCH_3); 33.6 (C_2). MS (EI, *m*/*z*) 166 (M^+). Anal. ($C_9H_{10}O_3$) C, H.

2-Ethyloxy-5-methoxychromone (2). A solution of **1** (10.68 g, 64.33 mmol) in ethyl oxalate (35 mL, 250 mmol) was added to a freshly prepared solution of EtONa in EtOH (prepared by addition of 8.87 g of sodium to 100 mL of absolute EtOH). The mixture was stirred at 100 °C for 10 h, after which, the mixture was cooled to room temperature, ethanol was evaporated, and water was added. The solution was acidified with HCl (2 M) and extracted with EtOAc. The organic layer was separated, washed with brine, dried (Na_2SO_4), and concentrated. The resulting residue was dissolved in ethanol (50 mL) and heated at 100 °C for 15 min; concentrated HCl (5 mL) was added, and the solution was stirred at 100 °C for 1 h. Ethanol was evaporated, and the residue was kept in the refrigerator (4 °C) overnight and yielded 11.32 g (71%) of **2**, as brown crystals: mp 125–126 °C (crystallization from MeOH:CH_2Cl_2. lit. 130–131° C);[24] ^{1}H NMR ($CDCl_3$): δ 7.88 (t, 1H, J = 8.4 Hz, H_7); 7.41 (d, 1H, J = 8.4 Hz, H_8); 7.26 (s, 1H, H_3); 7.12 (d, 1H, J = 8.1 Hz, H_6); 4.71 (q, 2H, J = 7.1 Hz, OCH_2CH_3); 4.25 (s, 3H, OCH_3); 1.70 (t, 3H, J = 7,1 Hz, OCH_2CH_3). ^{13}C NMR ($CDCl_3$): δ 177.7 (C_4); 160.2 (CO_2Et); 159.5 (C_2); 157.7 (C_5); 150.1 (C_9); 134.5 (C_7); 116 (C_3); 114 (C_{10}); 110.2 (C_6); 106.7 (C_8); 62.6 ($CO_2CH_2CH_3$); 56.2 (OCH_3); 13.8 ($CO_2CH_2CH_3$). MS (DCI, *m*/*z*) 249 (M + H)$^+$. Anal. ($C_{13}H_{12}O_5$) C, H.

2-Carboxy-5-hydroxychromone (3). Ester **2** (5 g, 20.16 mmol) was dissolved in a solution of $NaHCO_3$ (100 mL, 20% in H_2O) and heated at 80 °C for 3 h. After cooling, the solution was acidified with concentrated HCl and extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4, and evaporated to yield acid as a beige powder (90%). The latter was dissolved in 40 mL of HBr:AcOH (1:2) and heated at 100 °C for 2 h. After being cooled to room temperature, the solution was extracted with CH_2Cl_2. The organic layer was washed with H_2O, brine, dried over Na_2SO_4, and evaporated to yield 3.61 g (87%) of **3** as a brown solid: mp 253–255 °C (lit. 264 °C);[37] ^{1}H NMR ($CDCl_3$): δ 12.2 (s, 1H, OH); 7.75 (t, 1H, J = 8.5 Hz, H_7); 7.15 (d, 1H, J = 8.7 Hz, H_8); 6.95 (s, 1H, H_3); 6.85 (d, 1H, J = 8.3 Hz, H_6). ^{13}C NMR ($CDCl_3$): δ 184.1 (C_4), 174.0 (CO_2H); 161.6 (C_2); 160.4 (C_5); 154.8 (C_9); 136.6 (C_7); 113.6 (C_3); 111.9 (C_6); 108 (C_8); 106.9 (C_{10}). MS (DCI, *m*/*z*) 207 (M + H)$^+$. Anal. ($C_{10}H_6O_5$) C, H.

Amides 4. Acid **3** was dissolved in THF (2 mL/mmol), treated with EDC (1.2 equiv), and stirred at room temperature for 1 h. Benzylamine or piperazine derivative (1.5 equiv) was slowly added, followed by addition of Et_3N (1.2 equiv). After the mixture was stirred at room temperature for 24 h, water was added, and the mixture was extracted with EtOAc. The organic layer was washed with water, brine, dried over Na_2SO_4, and concentrated. The product was purified by chromatography on silica gel eluted with a gradient of cyclohexane/EtOAc (9/1) to (6/4) to yield amides **4** as yellow solids.

5-Hydroxy-2-(4-methylpiperazin-1-ylcarbonyl)chromone (4a): 22%; mp 96–98 °C; ^{1}H NMR (CD_3OD): δ 7.61 (t, 1H, J = 8.4 Hz, H_7); 7.01 (d, 1H, J = 8.4 Hz, H_8); 6.81 (d, 1H, J = 8.3 Hz, H_6); 6.49 (s, 1H, H_3); 3.72–3,60 (m, 4H, $CONCH_2$); 2.50–2,43 (m, 4H, $CONCH_2CH_2$); 2.32 (s, 3H, NCH_3). ^{13}C NMR

(CD_3OD): δ 184.6 (C_4); 162.1 (*C*ON); 160.5 (C_2); 157.5 (C_5, C_9); 137.6 (C_7); 112.9 (C_3); 111.3 (C_8); 108.6 (C_6); 55.9 and 55.2 (CON*C*H_2); 50.4 and 45.9 ($CONCH_2$*C*H_2); 43.2 (N*C*H_3). MS (DCI, *m/z*) 289 $(M + H)^+$. Anal. Calcd for $C_{15}H_{16}N_2O_4$: C, 62.49; H, 5.59; N, 9.72. Found. C, 62.02; H, 5.77; N, 9.43.

2-(4-Ethylpiperazin-1-ylcarbonyl)-5-hydroxychromone (4b): 36%; mp 112−114 °C; ^{1}H NMR ($CDCl_3$): δ 12.16 (s, 1H, OH); 7.52 (t, 1H, *J* = 8,4 Hz, H_7); 6.90 (d, 1H, *J* = 8.3 Hz, H_8); 6.78 (d, 1H, *J* = 8.3 Hz, H_6); 6.41 (s, 1H, H_3), 3.73−3.52 (m, CONC*H*$_2$); 2.55−2,49 (m, 4H, ($CONCH_2C$*H*$_2$); 2.38 (q, 2H, *J* = 7.1 Hz, NC*H*$_2CH_3$); 1.06 (t, 3H, *J* = 7.2 Hz, N−CH_2C*H*$_3$). ^{13}C NMR ($CDCl_3$): δ 182.8 (C_4); 160.8 (*C*ON); 160.2 (C_2); 159.1 (C_5); 155.8 (C_9); 136,0 (C_7); 112.1 (C_3); 111.3 (C_{10}); 110.4 (C_8); 107.2 (C_6); 52.9 and 51.2 (CON*C*H_2); 52.1 (N*C*H_2CH_3); 47.2 and 42.5 ($CONCH_2$*C*H_2); 11.7 (NCH_2*C*H_3). MS (DCI, *m/z*) 303 $(M + H)^+$. Anal. ($C_{16}H_{18}N_2O_4$) C, H, N.

5-Hydroxy-2-(4-phenylpiperazin-1-ylcarbonyl)chromone (4c): 28%; mp 159−161 °C; ^{1}H NMR ($CDCl_3$): δ 12.2 (s, 1H, OH); 7.57 (t, 1H, *J* = 8.4 Hz, H_7); 7.3 (d, 1H, *J* = 7.8 Hz, H_8); 7.26 (d, 1H, *J* = 7.6 Hz, H_6); 6.91 (m, 5H, $-C_6H_5$); 6.51 (s, 1H, H_3); 3.91−3.71 (m, 4H, CONC*H*$_2$); 3.27 (m, 4H, $CONCH_2C$*H*$_2$). ^{13}C NMR ($CDCl_3$): δ 182.8 (C_4); 160.8 (*C*ON); 160.3 (C_2); 158.7 (C_5); 155.7 (C_9); 150.9 ($C_{1'}$); 136.1 (C_7); 129.5 ($C_{3'}$, $C_{5'}$); 121 ($C_{4'}$); 116.7 ($C_{2'}$, $C_{6'}$); 112.2 (C_3); 111.2 (C_{10}); 110.6 (C_8); 107.1 (C_6); 50.0 and 49.7 (CON*C*H_2); 46.9 and 42.5 ($CONCH_2$*C*H_2). MS (DCI, *m/z*) 351 $(M + H)^+$. Anal. ($C_{20}H_{18}N_2O_4$) C, H, N.

5-Hydroxy-2-(4-benzylpiperazin-1-ylcarbonyl)chromone (4d): 28%; mp 109−113 °C; ^{1}H NMR ($CDCl_3$): δ 12.1 (s, 1H, OH); 7.51 (t, 1H, *J* = 8.4 Hz, H_7); 7.30−7,26 (m, 5H, C_6H_5); 6.86 (d, 1H, *J* = 8.5 Hz, H_8); 6.78 (d, 1H, *J* = 8.3 Hz, H_6); 6.4 (s, 1H, H_3); 3.71 (ls, 2H, C*H*$_2$Ph); 3,41 (m, 4H, CONC*H*$_2$); 2.45 (m, 4H, $CONCH_2C$*H*$_2$). ^{13}C NMR ($CDCl_3$): δ 184.1 (C_4); 161.9 (*C*O−N); 161.4 (C_2); 160.1 (C_5); 156.9 (C_9); 138.4 ($C_{1'}$); 137.2 (C_7); 130.1 ($C_{2'}$, $C_{6'}$ or $C_{3'}$, $C_{5'}$); 129.4 ($C_{2'}$, $C_{6'}$ or $C_{3'}$, $C_{5'}$); 128.6 ($C_{4'}$); 113.3 (C_{10}); 112.4 (C_3); 111.5 (C_8); 108.3 (C_6); 63.8 (N*C*H_2Ph); 54.4 and 53.4 (CON*C*H_2); 48.2 and 43.6 ($CONCH_2$*C*H_2). MS (DCI, *m/z*) 365 $(M + H)^+$. Anal. Calcd for $C_{21}H_{20}N_2O_4$. C, 69.22; H, 5.53; N, 7.69. Found. C, 68.98; H, 5.69; N, 7.54.

2-(*N*-Acetylpiperazin-1-ylcarbonyl)-5-hydroxychromone (4e): 35%; amorphous; ^{1}H NMR ($CDCl_3$): δ 7.59 (t, 1H, *J* = 8.4 Hz, H_7); 6.91 (dd, 1H, J_1 = 0.8 Hz, J_2 = 8.6 Hz, H_8); 6.86 (dd, 1H, J_1 = 0.8 Hz, J_2 = 8.5 Hz, H_6); 6.52 (s, 1H, H_3); 3.77−3.59 (m, 8H, H-piperazinyl); 2.15 (s, 3H, *C*H_3CO). ^{13}C NMR ($CDCl_3$): δ 182.8 (C_4); 169.2 (N*C*OCH_3); 160.9 (C_2*C*O-piperazinyl); 160.8 (C_2); 158.3 (C_5); 155.7 (C_9); 136.2 (C_7); 112.4 (C_3); 111.3 (C_{10}); 111.0 (C_8); 107.1 (C_6); 46.7 and 45.6 (CON*C*H_2); 42.6 and 41.4 (CON*C*H_2); 21.3 (*C*H_3CO). MS (DCI, *m/z*) 317 $(M + H)^+$. Anal. ($C_{16}H_{16}N_2O_5$) C, H, N.

2-(*N*-Benzoylpiperazin-1-ylcarbonyl)-5-hydroxychromone (4f): 23%; amorphous; ^{1}H NMR ($CDCl_3$): δ 7.57 (t, 1H, *J* = 8.4 Hz, H_7); 7.45−7.40 (m, 5H, H-benzoyl); 6.90 (dd, 1H, J_1 = 0.7 Hz, J_2 = 8.6 Hz, H_8); 6.85 (dd, 1H, J_1 = 0.7 Hz, J_2 = 8.6 Hz, H_6); 6.51 (s, 1H, H_3); 3.75−3.71 (m, 8H, COC*H*$_2$). ^{13}C NMR ($CDCl_3$): δ 182.7 (C_4); 170.7 (N*C*OC_6H_5); 160.8 (C_2*C*O-piperazinyl); 160.6 (C_2); 158.3 (C_5); 155.7 (C_9); 136.2 (C_7); 134.7 ($C_{1'}$); 130.3 ($C_{4'}$); 128.7 ($C_{3'}$, $C_{5'}$); 127.1 ($C_{2'}$, $C_{6'}$); 112.4 (C_3); 111.1 (C_{10}); 111.0 (C_8); 107.1 (C_6); 46.9 and 42.7 (CON*C*H_2). MS (DCI, *m/z*) 379 $(M + H)^+$. Anal. ($C_{21}H_{18}N_2O_5$) C, H, N.

5-Hydroxy-2-(piperidin-1-ylcarbonyl)chromone (4g): 31%; mp 104−106 °C; ^{1}H NMR ($CDCl_3$): δ 7.51 (t, 1H, *J* = 8.4 Hz, H_7); 6.92 (dd, 1H, J_1 = 0.8 Hz, J_2 = 8.4 Hz, H_8); 6.84 (dd, 1H, J_1 = 0.8 Hz, J_2 = 8.3 Hz, H_6); 6.43 (s, 1H, H_3); 3.70−3.43 (m, 4H, CONC*H*$_2$); 1.90−1.65 (m, 6H, CONC*H*$_2$C*H*$_2$); ^{13}C NMR ($CDCl_3$): δ 183.0 (C_4); 160.9 (-*C*O-); 160.3 (C_2); 156.1 (C_5); 136.5 (C_9); 135.9 (C_7); 113.6 (C_{10}); 112.1 (C_3); 109.9 (C_8); 107.2 (C_6); 48.1 and 43.5 (CON*C*H_2); 26.5 and 25.3 ($CONCH_2$*C*H_2); 24.3 ($CONCH_2$*C*H_2*C*H_2). MS (DCI, *m/z*) 274 $(M + H)^+$. Anal. ($C_{15}H_{15}NO_4$) C, H, N.

5-Hydroxy-2-(morpholin-1-ylcarbonyl)chromone (4h): 25%; mp 151−153 °C; ^{1}H NMR ($CDCl_3$): δ 7.57 (t, 1H, *J* = 8.4 Hz, H_7); 6.92 (d, 1H, *J* = 8.4 Hz, H_8); 6.85 (d, 1H, *J* = 8.3 Hz, H_6); 6.49 (s, 1H, H_3); 3.79−3.75 (m, 4H, CONC*H*$_2$); 3.61−3.57 (m, 4H, $CONCH_2C$*H*$_2$). ^{13}C NMR ($CDCl_3$): δ 182.8 (C_4); 160.9 (CON); 160.0 (C_2); 158.5 (C_5); 153.0 (C_9); 136.2 (C_7); 112.4 (C_3); 110.8 (C_8); 107.2 (C_6); 66.7 ($CONCH_2$*C*H_2); 48.0 and 42.9 (CON*C*H_2). MS (DCI, *m/z*) 276 $(M + H)^+$. Anal. ($C_{14}H_{13}NO_5$) C, H, N.

2-[*N*-(4-Chlorobenzylaminocarbonyl)]-5-hydroxychromone (4i): 29%; mp 174−177 °C; ^{1}H NMR ($CDCl_3$): δ 7.65 (t, 1H, *J* = 8,4 Hz, H_7); 7.35 (m, 4H, $-C_6H_5$); 7.10 (d, 1H, *J* = 8.4 Hz, H_8); 6.92 (s, 1H, H_3); 6.85 (d, 1H, *J* = 8.4 Hz, H_6); 4.56 (s, 2H, C*H*$_2$Ph). ^{13}C NMR ($CDCl_3$): δ 183.4 (C_4); 161.0 (*C*ON); 158.6 (C_2); 155.3 (C_5); 155.1 (C_9); 136.2 ($C_{7'}$); 135.3 ($C_{4'}$); 134 ($C_{1'}$); 128.1−129.4 ($C_{2'}$, $C_{3'}$, $C_{5'}$, $C_{6'}$); 112.3, 112,4 (C_3, C_{10}); 111.3 (C_8); 106.9 (C_6); 43.3 ($CONCH_2$). MS (DCI, *m/z*) 330 $(M + H)^+$. Anal. ($C_{17}H_{12}ClNO_4$) C, H, Cl, N.

Cell Lines and Culture Conditions. The human myeloid leukemic K562 cell line, derived from a chronic myeloid leukemia, and its adriamycin resistant sub-line, K562/R which expresses high level of Pgp, were generously donated by Pr. J. P. Marie (Hotel Dieu, Paris). Parent resistant K562 cells were named in the text as K562S and K562R, respectively. The human cell line HL60, derived from an acute myeloid leukemia and adriamycin resistant form, HL60/R, which expressed moderate level of MRP, were donated by the M. S. Center (Division of Biology, Kansas State University). All cells were cultured at 37 °C, in a humidified atmosphere, with 5% CO_2, in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 IU/mL penicillin, and 100 ng/mL streptomycin. Chemoresistance was maintained by continuous exposure to adriamycin at 10^{-6} M for K562R and HL60/MRP.

Functional Aspect of Pgp and MRP on Cells Treated with Reversal Agents. Detection of Pgp activity using rhodamine-123 efflux. K562S/R were washed twice and suspended at a density of 10^6 cell/mL in RPMI medium. The modulator was added to K562R at various concentrations, ranging from 10^{-6} M to 10^{-9} M, for 15 min at 37 °C. For each resistant line, cyclosporin A was added at 10 μM for 15 min at 37 °C. Rhodamine-123 was diluted in order to obtain a final concentration of 0.05 μg/mL. Cells were incubated 1 h at 37 °C, then analyzed by flow cytometry (FCM). For MRP detection, calcein-AM efflux was used. HL60S/MRP were washed twice and suspended at a density of 10^6 cell/mL and then incubated with cyclosporin A for 15 min at 10 μM in order to block MRP protein. Modulator was added to HL60/MRP at various concentration ranging from 10^{-6} M to 10^{-9} M for 15 min at 37 °C. HL60S/MRP cells were incubated with calcein-AM at final concentration of 0.05 μM during 10 min at 37 °C then analyzed by FCM.

Cell Cycle. K562 and HL60 cell lines were incubated with the reversal agent at 10^{-6} M for 24 and 48 h. The cell cycle was evaluated using Becton Dickinson's Cycle Test Plus. Cells were incubated with trypsin in a spermine tetrahydrochloride detergent buffer for 10 min at room temperature. Trypsin inhibitor and ribonuclease A were added for 10 min to cells without any washing step. Finally, PI was added and incubated for 10 min, and then cells were immediately analyzed using a FACSCalibur.

Triple Labeling with PKH67, Annexin Bitotin Streptavidin AMCA, and Propidium Iodide. PKH67 labeling was performed as described previously.[29] An amount of 2×10^7 cells was suspended in 1 mL of Diluent C and stained by rapidly admixing with a working PKH67 solution prepared by diluting 20 μL of 10^{-3} M ethanolic dye stock in 1.0 mL of Diluent C (final staining concentrations: 10 μM PKH67, 5×10^6 cells/mL). PKH67 fluorescence decrease was followed during 3 days. Every day, annexinV biotin and streptavidin/AMCA were used to evaluate apoptotic cells, and PI was used to detect necrotic cells. Cells were incubated with modulator at 10^{-6} M for 24 and 48 h. Cells were pelleted and labeled with annexinV/biotin, revealed by streptavidin/AMCA and PI after 15 min at 4 °C, and finally analyzed with FACSVantage.

Cytotoxicity Assay. To avoid spectral overlapping, TO-TO-3 was chosen to follow necrosis because DNR emits a red-orange fluorescence after excitation at 488 nm. Even if both are able to label DNA and DNR, the fluorescence emission

spectrum partially overlaps the TOTO-3 excitation spectrum, and no quenching phenomenon was observed. K562S and K562R at 10^6 cells/mL were incubated with different concentrations of modulators (**4a**, **5**, **6**, and cyclosporin A) at (10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M) for 15 min at 37 °C before DNR addition. A control without DNR was made for each modulator. Cell lines were diluted at 10^5 and plated in 24-well microplates, and then DNR was added at final concentration of 10^{-6} M. After 72 h of incubation at 37 °C, TOTO-3 at 5 nM final concentration was added to each cell suspension, 5 min prior to FCM analysis.

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Supporting Information Available: The biological data (tables and figures) concerning the effect of compounds **4a**–**e**, **5**, **5a**, and **6** on cell cycle and the effect of compounds **4a**, **5**, and **6** on cell division, apoptosis, and necrosis. This material is available free of charge via the Internet at http://pubs.acs.org.

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