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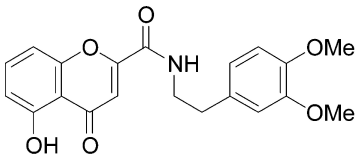

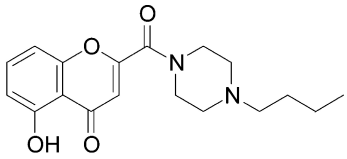

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Piperazinobenzopyranones and Phenalkylaminobenzopyranones: Potent Inhibitors of Breast Cancer Resistance Protein (ABCG2)

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In continuing research that led us to identify chromanone derivatives (*J. Med. Chem.* **2003**, *46*, 2125) as P-glycoprotein inhibitors, we obtained analogues able to modulate multidrug resistance (MDR) mediated by the breast cancer resistance protein (BCRP). The linkage of 5-hydroxybenzopyran-4-one to piperazines or phenalkylamines affords highly potent inhibitors of BCRP. By using sensitive (HCT116) and resistant colon cancer cells expressing BCRP, we evaluated the effect of 14 benzopyranone (chromone) derivatives on the accumulation and the cytotoxic effect of the anticancer drug, mitoxantrone. At 10 μM, three compounds increased both intracellular accumulation and cytotoxicity of mitoxantrone in HCT116/R cells with a comparable rate as fumitremorgin C and Gleevec used as reference inhibitors. The most potent molecules **5b** and **5c** are still active at 1 μM, whereas FTC shows weak inhibition. These molecules do not induce cell death as shown by the cell cycle distribution study, which makes them potential candidates for in vivo studies.

Introduction

The ATP-binding cassette (ABC) transporters are a large group of membrane proteins found in various tissues. To date, 49 human transporters have been described and are more or less implicated in multidrug resistance (MDR).[1] P-glycoprotein (PGP, ABCB1 in the HUGO nomenclature), the product of the multidrug resistance gene (mdr1), is the most described in the literature and has been associated with poor outcome especially in acute myeloid leukaemia (AML).[2] The last protein described, breast cancer resistance protein (BCRP, ABCG2) as a member of the ATP-binding cassette is associated with chemotherapy resistance in AML.[3] This protein is also found in the intestinal epithelium, liver canaliculi, the placenta trophoblast, and capillary, especially in brain endothelial cells.[4] BCRP consists of 655 amino acids with a molecular mass of 72.1 kDa. Opposite to ABCB1, ABCG2 is a half-membrane transporter that contains one ATP-binding site and six transmembrane domains.[5] As well as PGP, BCRP is able to cause cross resistance against various molecules, such as doxorubicin, topotecan, SN38, mitoxantrone, methotrexate, flavopiridol, zidovudine, and lamivudine.[6−10] It is interesting to highlight that BCRP is able to transport PGP substrates and is not inhibited by PGP chemosensitizers such as calcium channel blockers, cardiovascular drugs, and immunosuppressors.[11] In this context and in order to overcome BCRP-mediated multidrug resistance, BCRP-specific inhibitors are highly needed.

Several molecules have been recently reported in the past 5 years. Such inhibitors include the antifungal agent, fumitremorgin C (FTC),[12,13] taxanes,[14] estrogen antagonists, and agonists.[15] Houghton and co-workers have reported the tyrosine kinase inhibitor, imatinib mesylate (Gleevec),[16] as a potent inhibitor of BCRP, but the study was challenged by Burger et al.[17] In our present study and on the basis of Houghton's results, we found that Gleevec is a potent inhibitor of BCRP overexpressed in colon cancer cells. Two independent and very recent studies have reported the potential of flavones and benzoflavones in the inhibition of BCRP.[18−20]

In our program oriented to the discovery of MDR modulators,[21,22] we recently reported a series of molecules consisting of a benzopyranone unit linked to N-substituted piperazines and discovered that *N*-methylpiperazine derivative **A** (Figure 1) was a powerful inhibitor of P-glycoprotein.[23] When tested on BCRP, the same molecule did not show any inhibition activity. This result led us to operate structural modifications on the piperazine region in order to obtain specific BCRP inhibitors. Here, we targeted the synthesis of N-substituted derivatives of piperazinobenzopyranone (structure **B**) and phenalkylaminobenzopyranone (structure **C**) (Figure 1) and compared them to fumitremorgin C (FTC) and Gleevec (STI 571).[12−14] The synthesized compounds were evaluated by two cellular tests that measure the effect on the intracellular accumulation and cytotoxicity of mitoxantrone.[24,25]

Chemistry

The synthesis of compounds disclosed in this study was easily accomplished starting from acid **1** for which the synthesis was already reported (Scheme 1).[23] Piperazine derivatives were obtained by coupling acid **1** with *N*-Boc-piperazine in the presence of EDC as a coupling agent to afford **2**. The Boc group was removed by treatment with trifluoroacetic acid (TFAA), and the

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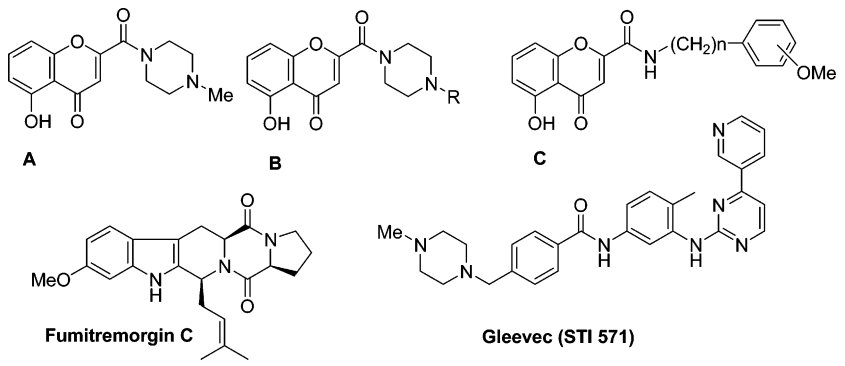
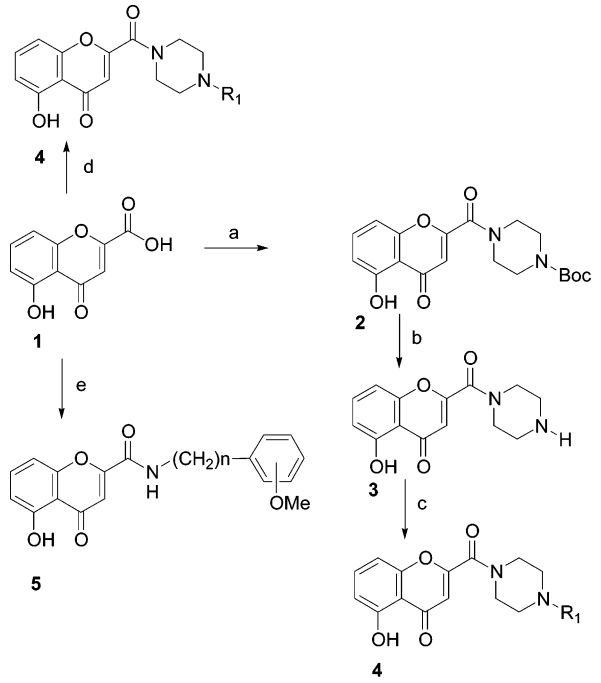


Figure 1.

Scheme 1[a]



[a] (a) *N*-Boc-piperazine, EDC, THF, 24 h, 19%; (b) TFAA, CH_2Cl_2, 30 °C, 15 h, 26%; (c) haloalkanes, K_2CO_3, DMF, 60 °C, 14−36%; (d) N-substituted piperazine, EDC, THF, 24 h, 10−16%; (e) Ar−$(CH_2)_n$−NH_2, EDC, THF, 24 h, 17−70%.

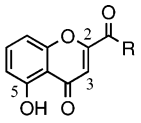
amine **3** was reacted with haloalkanes in the presence of K_2CO_3 to provide target compounds **4**. When N-substituted piperazines were commercially available, then the synthesis was achieved in one step by condensing **1** with these N-substituted piperazines. Phenalkylaminobenzopyranones **5** were obtained by coupling of acid **1** with phenalkylamines in the presence of EDC.

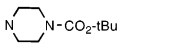
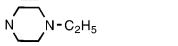
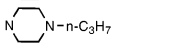
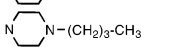
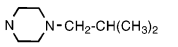
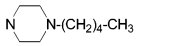
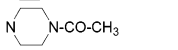
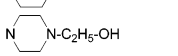
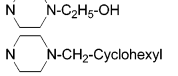
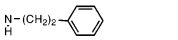
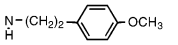
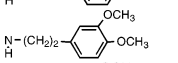
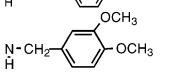
Results and Discussion

BCRP was reported as an effective transporter of several anticancer drugs and was shown to confer resistance to mitoxantrone in the MCF7 cell line.[6] Moreover, BCRP was overexpressed in several mitoxantrone-resistant cell lines.[25,26] On the basis of these observations, we used human carcinoma cell lines HCT116 sensitive to the cytotoxic effect of mitoxantrone (HCT116/S) and HCT116 transfected with BCRP mRNA, which will confer resistance to mitoxantrone (HCT116/R).

A preliminary screening using an in vitro test to assess the ability of a set of representative compounds to restore intracellular accumulation of mitoxantrone allowed us to retain two series of benzopyran-4-one derivatives: benzopyranone linked to N-substituted piperazines and benzopyranone linked to a phenalkylamine unit.

Table 1. Mean of Intracellular (HCT116-Resistant Cells) Mitoxantrone Fluorescence Intensity in the Presence of the Modulator at 10 μM



Compound	R	Fluorescence intensity[a]
Fumitremorgin C		315±4
Gleevec		334±3
2		179±4
3		86±3
4a		108±4
4b		104±2
4c		223±5
4d		92±2
4e		225±5
4f		112±4
4g		72±3
4h		92±3
5a		282±5
5b		320±5
5c		315±5
5d		197±2

[a] Mitoxantrone accumulation (fluorescence intensity in arbitrary units a.u.). Fluorescence intensity (a.u.) for controls (without modulator): HCT116/S, 371; HCT116/R, 97.

The assay, realized at 10 μM, shows that compounds **4c**, **4e**, and **5a**−**c** induce a significant increase of mitoxantrone accumulation in HTC116/R cells and derivatives **5b** and **5c** are comparable to FTC and Gleevec (Table 1). On the basis of these results, we selected the most active compounds from each series: **4c** (piperazine series) and **5a**−**5c** (phenalkylamine series) and evaluated them at lower concentrations (1

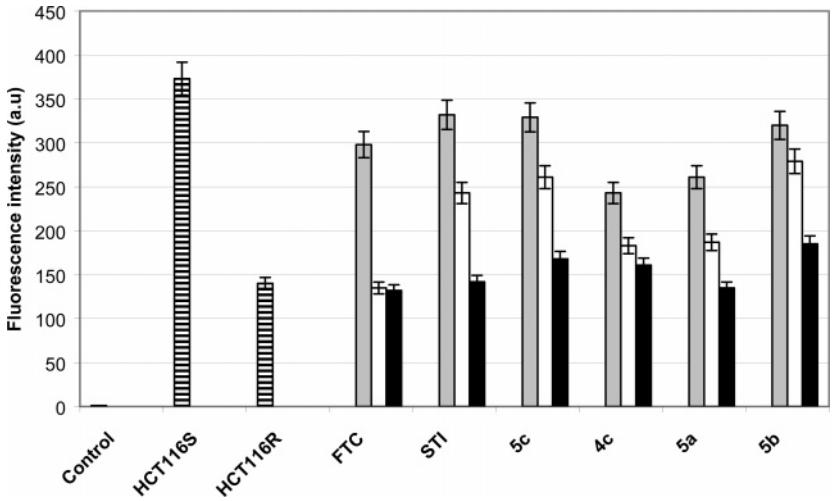


Figure 2. Effect of compounds **5a**−**c** and **4c** on the accumulation of mitoxantrone in HCT116-resistant (HCT116/R) cells. Fumitremorgin C and STI (Gleevec) were used as reference inhibitors of BCRP transport activity: (hatched bar) cells without modulator, (gray bar) 10 μM, (□) 1 μM, (■) 0.1 μM. The results are expressed by the mean intensity of mitoxantrone fluorescence (arbitrary unit, a.u.).

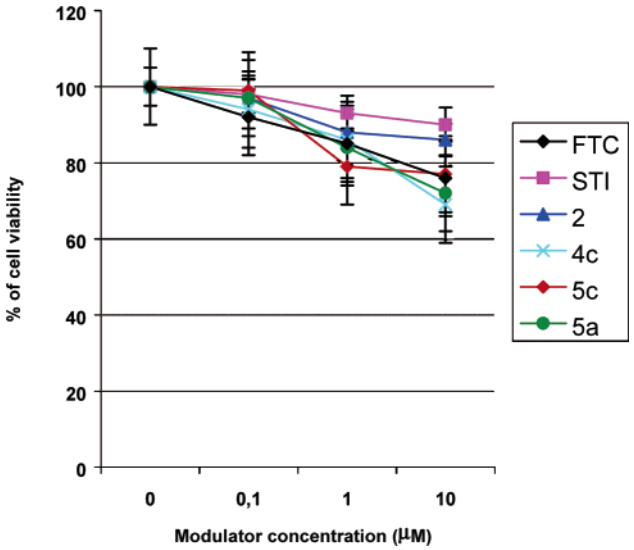


Figure 3. Viability of HCT116/S cells after treatment with compounds **2**, **4c**, **5c**, and **5a**.

and 0.1 μM) (Figure 2). At 1 μM, compounds **5b** and **5c** induced accumulation of mitoxantrone with a higher rate than FTC, and **5b** was the most active, even slightly more active than Gleevec.

To investigate the cytotoxicity of active molecules, we selected active compounds representing each series (**2**, **4c**, **5a**, and **5c**) and checked the effect of each compound on the cell cycle distribution. Thus, HCT116/S cells were treated with each compound at three different concentrations (10, 1, and 0.1 μM) followed by measuring cell cycle distribution in G0/G1, S, and G2/M phases. As indicated in Figure 3, at 10 μM there was about 60% viable cells, indicating the absence of significant cytotoxicity.

On the basis of the mitoxantrone accumulation assay and the lack of cell cycle perturbation, we selected compounds **4c** and **5a**−**c** and evaluated their ability to potentiate cytotoxicity of mitoxantrone in HCT116/R cells. Flow cytometry (FC), a highly sensitive method, was used to detect the effect of modulators. The balance between cell proliferation and drug-induced cell death by apoptosis plays a major role in determining response to chemotherapy. To check the balance factor, a double labeling using annexin V-FITC and propidium iodide (PI) was used to simultaneously follow cell division and apoptosis. An example of results obtained from FC is shown in Figure 4a. The percentage of viable, apoptotic, and dead cells for all compounds at three concentrations is shown in Figure 4b. At 10 μM, piperazine derivative **4c** allows us to obtain the same percentage of apoptotic (27%) and dead (22%) cells as FTC used as reference (Figure 4b). At the same concentration (10 μM), phenethylamine derivative **5b** induced 42% of apoptotic cells and 8% of dead cells. Compounds **5a** and **5c** induce approximatively the same percentage as Gleevec.

Structure−**Activity Relationships.** On the basis of the accumulation assay, some structure−activity relationships can be drawn. In the N-substituted piperazinochromone series **2**, **3**, **4a**−**h**, our starting compound was derivative **3**, which is not substituted at the nitrogen atom of piperazine and did not exhibit any activity. N-Alkylation, i.e., ethyl **4a** and *n*-propyl **4b**, did not enhance mitoxantrone intracellular accumulation. However, alkylation by a homologue group such as *n*-butyl **4c** or *n*-pentyl **4e** led to a significant increase of accumulation, which may point to the importance of a hydrophobic chain at this region of the molecule. Interestingly, the ramifications of the *n*-butyl group to an isobutyl group **4d** or its substitution with a bulky group such as a cyclohexylmethyl **4h** led to inactive compounds, which may indicate that steric hindrance at this position is disadvantageous. When the *n*-butyl group is lengthened to a linear C-5 chain **4e**, we observed no alteration of activity. Further chain lengthening was avoided because of solubility problems, which began to be problematic with compound **4e**.

The introduction of a hydroxyl group on the *N*-ethyl substituent of the piperazine **4g** induced no gain of activity when compared to its nonhydroxylated analogue **4a**. In the same manner, oxygenated analogues such as **2** and **4f** did not provide higher mitoxantrone accumulation. Taken together, these data led us to conclude that in this series, a linear alkyl chain of four carbons brings a convenient hydrophobic degree. In addition, this chain may provide a sufficient degree of freedom, allowing the

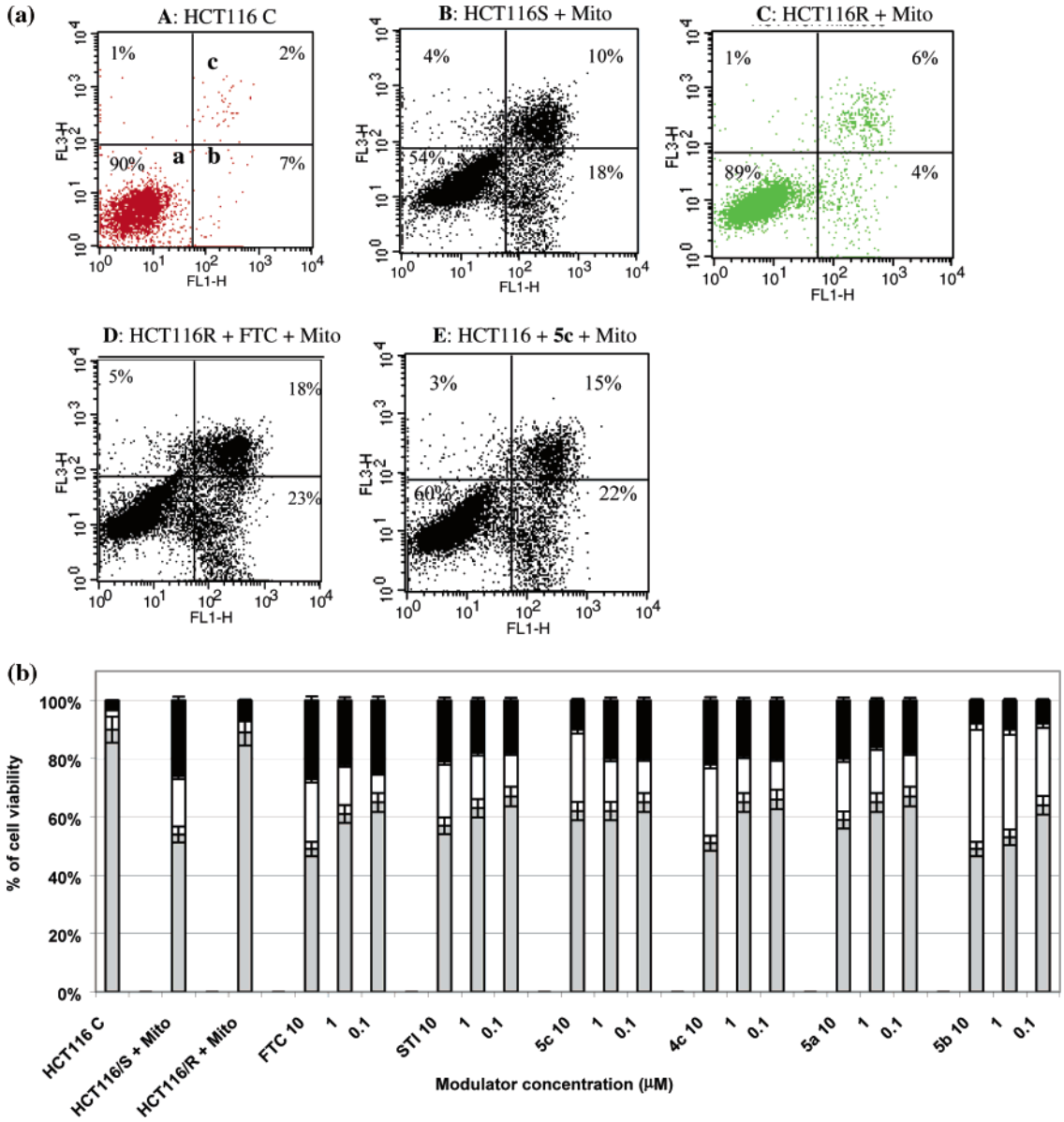


Figure 4. (a) Effect of compound **5c** (10 μM) on mitoxantrone cytotoxicity as measured by flow cytometry. Three population were observed: (a) viable cells, (b) apoptotic cells, (c) dead cells. (b) Effect of compounds **4c**, **5a**, **5b**, and **5c** on cytotoxicity of mitoxantrone in HCT116/R cells. Mitoxantrone (Mito) was evaluated at 10 μM. Compounds were evaluated at three concentrations (10, 1, and 0.1 μM): (■) dead cells; (□) apoptotic cells; (gray bar) viable cells. FTC and Gleevec (STI 571) were used as references.

molecule to adopt an extended or folded conformation that favors interactions with a hydrophobic domain of the target BCRP protein.

The second series of target molecules is characterized by the linkage of a phenalkylamino group to the chromone moiety via a carbonyl group (**5a**−**d**). Overall, the reversing activity in this series was higher than for N-substituted piperazinobenzopyranones. In particular, compounds **5a**−**c** exhibited strong activity, comparable to FTC and Gleevec.

When the activities of **5c** and **5d** were compared, it appears that a two-carbon linker (**5c**) is more contributing to the activity than a one-carbon linker (**5d**). Furthemore, deletion of one methoxy group from **5c** to provide **5b** did not alter reversing activity. However, deletion of both methoxy groups (**5a**) led to a weak loss of activity compared to **5b** and **5c**. These data emphasize the role of one or two methoxy groups, which bring lipophilicity to this part of the molecule.

Conclusion

The present investigation allowed the identification of new promising benzopyranone derivatives that inhibit BCRP (ABCG2) with the same rate as the most known inhibitors, FTC and Gleevec. We identified compounds **5b** and **5c** as being particularly active. Taken together, the interesting chemosensitizing activity and the lack of cytotoxicity make these compounds good candidates for in vivo studies. As a final remark, the most active compounds, representatives of each series (**4c**, **5b**, **5c**), share several structural features with FTC and Gleevec. The relatively short and ease of synthesis will be helpful in speeding the development of these MDR modulators.

Experimental Section

Chemistry. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-200 (200 MHz for ^{1}H, 50 MHz for ^{13}C) and Bruker AC-400 instrument (400 MHz for ^{1}H, 100 MHz for ^{13}C).

Chemical shifts are reported as δ values (ppm) relative to Me_4-Si 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 use. Chemicals and reagents were obtained either from Aldrich or ACROS and used as obtained.

2-(4-*tert*-Butyloxycarbonylpiperazin-1-ylcarbonyl)-5-hydroxychromone (2). To a solution of acid **1** (1 mmol) in anhydrous THF (15 mL/mmol) was added EDC (2 equiv) followed by adding *N*-Boc-piperazine (1.5 equiv) and triethylamine (1.2 equiv), and the solution was stirred at room temperature for 24 h. Water was added, and the pH was adjusted to 7 by adding diluted HCl (1 N). The solution was extracted with AcOEt, then washed with brine. The organic layer was separated, evaporated, and purified by chromatography using cyclohexane/AcOEt (8:2) as eluant to afford **2** as a beige powder (19%), mp 140−142 °C (cyclohexane/EtOAc). ^{1}H NMR (200 MHz, acetone-d_6): δ 7.69 (t, 1H, J = 4.2 Hz, H_7), 7.05 (d, 1H, J = 4.2 Hz, H_8), 6.82 (d, 1H, J = 4.2 Hz, H_6), 6.52 (s, 1H, H_3), 3.68 (bs, 2H, CO−N−C**H$_2$**−CH_2−N−Boc), 3.52 (bs, 2H, CO−N−C**H$_2$**−CH_2−N−Boc), 2.80 (bs, 4H, CO−N−CH_2−C**H$_2$**−N−Boc), 1.42 (s, 9H, −$C(CH_3)_3$). MS (FAB): *m/z* 374 $[M]^+$. Anal. ($C_{19}H_{22}N_2O_6$) C, H, N.

5-Hydroxy-2-piperazin-1-ylcarbonylchromone (3). Compound **2** (0.58 g, 1.5 mmol) was dissolved in CH_2Cl_2 (20 mL) and then treated with trifluoroacetic acid (0.12 mL, 1 equiv). The solution was heated at 30 °C for 15 h and then diluted with water. The aqueous phase was separated, basified with $NaHCO_3$ (8 mL), and then extracted with EtOAc. The two organic phases were combined, and solvent was evaporated to provide pure **3** as a yellow powder (26%), mp 111−113 °C (EtOAc). ^{1}H NMR ($CDCl_3$): δ 7.57 (t, 1H, J = 8.4 Hz, H_7), 6.92 (d, 1H, J = 8.7 Hz, H_8), 6. 86 (d, 1H, J = 8.3 Hz, H_6), 6.47 (s, 1H, H_3), 3.74 (bs, 2H, CO−N−CH_2−CH_2−NH), 3.51 (bs, 2H, CO−N−CH_2−CH_2−NH), 2.95 (bs, 4H, CO−N−CH_2−CH_2−NH). MS (FAB): *m/z*) 275 $[M + H]^+$. Anal. ($C_{14}H_{14}N_2O_4$) C, H, N.

N-Alkylation of Compound 3. General Procedure. To a solution of **3** in acetone (10 mL/mmol) was added K_2CO_3 (1.5 equiv), and the obtained solution was stirred for 30 min and then treated with alkyl halides (ethyl iodide, 1-propyl iodide, 1-butyl bromide, 1-isobutyl bromide) (1 equiv). The mixture was refluxed for 24 h and then allowed to cool to room temperature, and acetone was evaporated. The crude material was extracted twice with EtOAc, dried over Na_2SO_4, and concentrated. The target compound was obtained after purification on a chromatography column, eluting with CH_2Cl_2/MeOH (99/1).

2-(4-Ethylpiperazin-1-ylcarbonyl)-5-hydroxychromone (**4a**) and 2-(*N*-acetylpiperazin-1-ylcarbonyl)-5-hydroxychromone (**4f**) have been reported.[23]

2-(4-*n*-Propylpiperazin-1-ylcarbonyl)-5-hydroxychromone (4b). Yield 35%, yellow powder, mp 99−101 °C (cyclohexane/AcOEt). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.60 (t, 1H, J = 8.4 Hz, H_7), 6.98 (d, 1H, J = 8.4 Hz, H_8), 6.88 (d, 1H, J = 8 Hz, H_6), 6.47 (s, 1H, H_3), 3.77 (bs, 2H, CO−N**CH_2**CH_2N−), 3.57 (bs, 2H, CO−N**CH_2**CH_2N−), 2.56−2.51 (m, 4H, CO−NCH_2**CH_2**N−), 2.34 (t, 2H, J = 7.6 Hz, N−**CH_2**CH_2CH_3), 1.55 (m, 2H, N−CH_2**CH_2**CH_3), 0.95 (t, 3H, J = 7.6 Hz, N−CH_2-CH_2**CH_3**). MS (FAB): *m/z* 316 $[M]^+$. Anal. ($C_{17}H_{20}N_2O_4$) C, H, N.

2-(4-*n*-Butylpiperazin-1-ylcarbonyl)-5-hydroxychromone (4c). Yield 14%, yellow powder; mp 107−109 °C (cyclohexane/EtOAc). ^{1}H NMR ($CDCl_3$): δ 7.55 (t, 1H, J = 8.4 Hz, H_7), 6.99 (d, 1H, J = 8.3 Hz, H_8), 6.82 (d, 1H, J = 8 Hz, H_6), 3.74 (bs, 2H, $H_{2'}$ + $H_{6'}$), 3.57 (bs, 2H, $H_{2'}$, $H_{6'}$), 2.49 (bs, 4H, $H_{3',5'}$), 2.39 (t, 2H, J = 6.9 Hz, −N−CH_2−), 1.86 (m, 2H, −N−CH_2−CH_2CH_3), 1.65−1.27 (m, 2H, −CH_2−CH_3), 0.96 (t, 3H, J = 7.1 Hz, −CH_3). MS (FAB): *m/z* 331 $[M + H]^+$. Anal. ($C_{18}H_{22}N_2O_4$) C, H, N.

2-(4-Isobutylpiperazin-1-ylcarbonyl)-5-hydroxychromone (4d). Yield 36%, amorphous. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.48 (t, 1H, J = 8.4 Hz, H_7), 6.83 (d, 1H, J = 8.4 Hz, H_8), 6.74 (d, 1H, J = 8.4 Hz, H_6), 6.36 (s, 1H, H_3), 3.7−3.6 (m, 4H, CO−N**CH_2**CH_2N−), 2.45−2.35 (m, 4H, CO−NCH_2**CH_2**N−), 2.3 (m, 2H, N−**CH_2**$CH(CH_3)_2$), 1.4−1.3 (m, 2H, N−CH_2**CH**$(CH_3)_2$), 1 (d, J = 7 Hz, N−CH_2CH(**CH_3**)$_2$). MS (EI): *m/z* 330 $[M]^+$. Anal. ($C_{18}H_{22}N_2O_4$) C, H, N.

2-(4-*n*-Pentylpiperazin-1-ylcarbonyl)-5-hydroxychromone (4e). Yield 33%, yellow powder, mp 98−100 °C (EtOAc/cyclohexane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58 (t, 1H, J = 8.4 Hz, H_7), 6.94 (d, 1H, J = 8.4 Hz, H_8), 6.86 (d, 1H, J = 8.4 Hz, H_6), 6.47 (s, 1H, H_3), 3.79 (bs, 2H, CO−N−C**H$_2$**−CH_2), 3.56 (bs, 2H, CO−N−C**H$_2$**−CH_2), 2.56−2.50 (m, 4H, CO−N−CH_2−C**H$_2$**), 2.40 (t, 2H, J = 7.6 Hz, N−C**H$_2$**−$(CH_2)_3$−CH_3), 1.51 (m, 2H, N−CH_2−C**H$_2$**−$(CH_2)_2$−CH_3), 1.32 (m, 4H, N−CH_2−CH_2−$(CH_2)_2$−CH_3), 0.92 (t, 3H, J = 7.6 Hz, N−$(CH_2)_4$−C**H$_3$**). MS (EI): *m/z* 344 $[M]^+$. Anal. ($C_{19}H_{24}N_2O_4$) C, H, N.

2-[4-(2-Hydroxyethyl)piperazin-1-ylcarbonyl]-5-hydroxychromone (4g). Yield 16%, yellow powder, mp 126−130 °C (cyclohexane/EtOAc). ^{1}H NMR ($CDCl_3$, 200 MHz): δ 12.17 (s, 1H, 5-OH), 7.55 (t, 1H, J = 8.3 Hz, H_7), 6.89 (d, 1H, J = 8.4 Hz, H_8), 6.83 (d, 1H, J = 8.3 Hz, H_6), 6.45 (s,1H, H_3), 3.76 (bs, 2H, CO−N−CH_2−), 3.65 (t, 2H, J = 5.2 Hz, CH_2−OH), 3.55 (bs, 2H, CO−N−CH_2−), 2.60 (t, 2H, J = 5.5 Hz, N−CH_2−CH_2-OH), 2.04 (bs, 4H, −CH_2−N−$(CH_2)_2$−OH). MS (EI): *m/z* 318 $[M]^+$. Anal. ($C_{16}H_{18}N_2O_5$) C, H, N.

2-(4-Cyclohexylmethylpiperazin-1-ylcarbonyl)-5-hydroxychromone (4h). Yield 10%, yellow powder, mp 118−121 °C (cyclohexane/EtOAc). ^{1}H NMR ($CDCl_3$): δ 7.56 (t, 1H, J = 8.2 Hz, H_7), 6.91 (d, 1H, J = 8.6 Hz, H_8), 6.83 (d, 1H, J = 8.5 Hz, H_6), 6.45 (s, 1H, H_3), 3.75 (bs, 2H, CO−N−C**H$_2$**), 3.52 (bs, 2H, CO−N−C**H$_2$**), 2.48 (bs, 2H, CO−N−CH_2−C**H$_2$**), 2,43 (bs, 2H, CO−N−CH_2−C**H$_2$**), 2.16 (d, 2H, J = 7.2 Hz, N−C**H$_2$**−cyclohexyl), 1.72−1.21 (m, 9H, cyclohexyl), 0.86 (m, 2H, cyclohexyl). MS (EI): *m/z* 370 $[M]^+$. Anal. ($C_{21}H_{26}N_2O_4$) C, H, N.

2-(*N*-Phenylethylaminocarbonyl)-5-hydroxychromone (5a). Yield 70%, yellow powder, mp 183−185 °C (cyclohexane/EtOAc). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 12.2 (s, 1H, OH), 7.60 (t, 1H, J = 8.4 Hz, H_7), 7.45−7.25 (m, 5H, C_6**H$_5$**), 7.10 (s, 1H, H_3), 6.92 (bs, 1H, NH), 6.84 (d, 1H, J = 8.4 Hz, H_8), 6.82 (d, 1H, J = 8.4 Hz, H_6), 3.81 (q, 2H, J = 7,2 Hz, NH−C**H$_2$**−CH_2), 3.05 (t, 2H, J = 6.8 Hz, NH−CH_2−C**H$_2$**). MS (EI): *m/z* 309 $[M]^+$. Anal. ($C_{18}H_{15}NO_4$) C, H, N.

2-[*N*-(4-Methoxyphenylethylaminocarbonyl)]-5-hydroxychromone (5b). Yield 20%, yellow powder, mp 181−183 °C (EtOAc). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.59 (t, 1H, J = 8.4 Hz, H_7), 7.19 (d, 1H, J = 8.4 Hz, H_8), 7.10 (s, 1H, H_3), 6.91 (d, 2H, J = 8.8 Hz, $H_{2',6'}$), 6.90 (d, 1H, J = 8.4 Hz, H_6), 6.86 (d, 2H, J = 8.8 Hz, $H_{2',5'}$), 3.82 (s, 3H, OCH_3), 3.73 (q, 2H, J = 6.8 Hz, N−**CH_2**CH_2), 2.92 (t, 2H, J = 6.8 Hz, N−CH_2**CH_2**). MS (EI): *m/z* 339 $[M]^+$. Anal. ($C_{19}H_{17}NO_5$) C, H, N.

2-[*N*-(3,4-Dimethoxyphenylethylaminocarbonyl)]-5-hydroxychromone (5c). Yield 50%, amorphous. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.56 (t, 1H, J = 8.4 Hz, H7), 7.12 (s, 1H, H_3), 7.00 (s, 1H, N−H), 6.90−6.83 (m, 5H, H_6, H_8, H_{phenyl}), 3.90 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.70 (q, 2H, J = 6.6 Hz, N−**CH_2**CH_2), 2.89 (t, 2H, J = 6.6 Hz, N−CH_2**CH_2**). MS (EI): *m/z* 369 $[M]^+$. Anal. ($C_{20}H_{19}NO_6$) C, H, N.

2-[*N*-(3,4-Dimethoxybenzylaminocarbonyl)]-5-hydroxychromone (5d). Yield 17%, yellow powder, mp 187−190 °C (EtOAc). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.58 (t, 1H, J = 8.4 Hz, H_7), 7.15 (s, 1H, H_3), 7.09 (s, 1H, NH), 6.95−6.85 (m, 5H, H_6, H_8, H_{phenyl}), 4.62 (d, 2H, J = 6 Hz, N**CH_2**), 3.90 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3). MS (EI): *m/z* 355 $[M]^+$. Anal. ($C_{19}H_{17}NO_6$) C, H, N.

Biology. HCT116 sensitive and resistant cells were kindly provided by Dr. Del Rio[27] (CPBS, CNRS UMR 5160, Faculté de Pharmacie, Montpellier, France). Cells were cultured in RPMI 1640 medium with 10% inactivated fetal calf serum (FCS) (Gibco BRL, Eragny, France), 2 mM l-glutamine, 100 μg/mL streptomycin, and 100 units/mL penicillin. Fumitremorgin C (FTC) was purchased from Alexis Corporation (San

Diego, CA), mitoxantrone was from Wyeth-Lederle (Paris, France), and STI571 (Gleevec) was from Novartis (Switzerland). Monoclonal antibodies anti-BCRP were purchased from Bioscience (San Diego, CA). Monoclonal mouse IgG2b used as control was from Dako (Trappes, France), and Goat F(ab′)2 antimouse IgG-FITC was from Beckman-Coulter (Roissy, France). Flow cytometry analysis was performed on a FACSCalibur (BD Biosciences, Le Pont de Claix, France) instrument.

BCRP Antigen Expression. HCT116/R and S cells were trypsinized and then washed in phosphate buffered saline (PBS). The 0.5×10^6 cells were incubated for 60 min on ice with anti-BCRP monoclonal antibody (final concentration of 2.5 mg/mL) or with a mouse IgG2b (negative control). Cells were then washed and incubated for 30 min with Goat F(ab′)2 fragment antimouse IgG-FITC. After a final wash, cells were placed on ice until flow cytometry analysis.

Mitoxantrone Accumulation Studies. Modulators were diluted in DMSO (10 mM) and then in RPMI 1640 medium to obtain a final concentration ranging from 10 to 0.1 μM. The accumulation studies were performed using flow cytometric analysis. The cells grown in flasks with about 90% confluence were trypsinized and washed with fetal bovine serum-free RPMI and resuspended in this medium to obtain a cell density of 10^6 cells/mL. The accumulation of mitoxantrone was performed by incubating 1 mL of cells with various concentrations of compound at 37 °C for 15 min, followed by addition of 3 μM of mitoxantrone. Fumitremorgin C was used as a reference modulator. After 30 min of incubation, accumulation was stopped by adding 3 mL of ice-cold PBS and centrifugation. The cells were then washed with ice-cold PBS, and the intracellular level of mitoxantrone was analyzed by flow cytometry (FC) using a FACScan flow cytometer equipped with a standard argon laser for excitation at 488 nm and a bandpass filter at 670 nm to detect mitoxantrone fluorescence.[18] Analysis of FC results was performed by CellQuest analysis software, and the accumulation of mitoxantrone was expressed in mean fluorescence intensity (in arbitrary units a.u.).

Mitoxantrone Cytotoxicity Studies. After cell attachment (24 h incubation), culture medium was replaced with fresh medium containing 1 μM mitoxantrone and various concentrations of either FTC or compound to be evaluated. After 48 h of incubation at 37 °C, cells were washed twice with PBS buffer. Cell death was assessed by the uptake of propidium iodide (2 μg/mL, Sigma) in phosphate buffered saline (PBS) into nonfixed cells and subsequent flow cytometric analysis with the FSC/FL2 profile. Apoptosis was analyzed using the Annexin V-FITC method (Vybrant apoptosis assay kit; Molecular Probes, Eugene, OR).

DNA Content Analysis. The cell cycle was evaluated with the Cycle Test kit (BD-Biosciences). Briefly, 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 without washing, and finally, PI was added and the all was incubated for 10 min, and then cells were immediately analyzed by flow cytometry.

Apoptosis Detection. To detect apoptotic and necrotic cells, the annexin V-FITC kit (Sigma, MO) was used. Briefly, 500 mL containing 5×10^5 cells treated, or not, by drugs were incubated with 5 mL of annexin V-FITC and 10 mL of propidium iodide (PI) for 10 min at room temperature, and then cell fluorescence was analyzed using a flow cytometer. Live cells show no staining by either PI or annexinV-FITC. Necrotic cells are stained by PI alone or annexin V-FITC and PI.

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Supporting Information Available: Results from elemental analysis of the synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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