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6-Halogenochromones Bearing Tryptamine: One-Step Access to Potent and Highly Selective Inhibitors of Breast Cancer Resistance Protein

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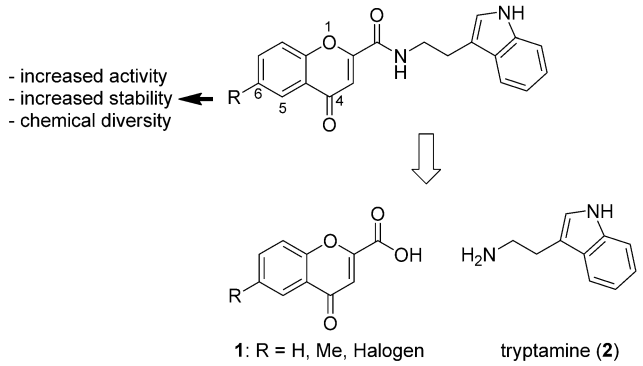
Most anticancer drugs are rendered less efficacious due to cell resistance to chemotherapy related to various mechanisms. A major mechanism is associated with the overexpression of ATP binding cassette (ABC) transporters, especially P-glycoprotein (Pgp/ABCB1), multidrug resistance-associated protein 1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2),[1] which traffic chemotherapeutic agents out of cancer cells.

ABCG2 was simultaneously discovered by three research groups and named ABCP for its abundance in placenta,[2] BCRP for its identification in breast cancer cell lines,[3] and MXR for its resistance to mitoxantrone.[4] ABCG2 constitutes an important target for the design of efflux inhibitors that would, when co-administered with an anticancer agent, give increased intracellular drug concentrations and hence greater cytotoxicity. While several types of ABCG2 inhibitors have been evaluated in vitro, very few have entered preclinical trials.[5–9]

We recently discovered that some substituted chromones are selective and potent ABCG2 inhibitors.[10] These compounds were synthesized in five steps, and the overall yields were quite low. In pursuing our efforts toward structurally simple and easily accessible specific inhibitors of BCRP, we investigated 6-halogenochromones linked to a tryptamine unit, obtained in only one step, as new potent inhibitors (Scheme 1).

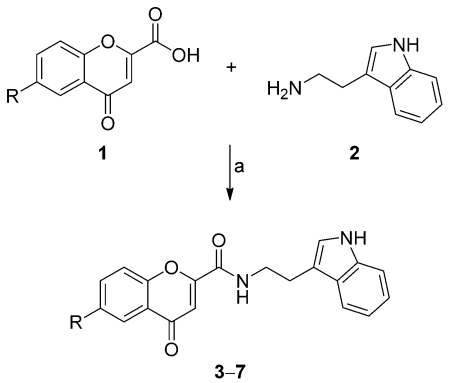
The choice of C-6 as the site of halogenation was motivated by a number of considerations: 1) the presence of a hydrophobic halogen at the C-6 position fulfills the previously identified need for a hydrophobic substituent in this part of the molecule;[10] 2) halogens, especially bromine and iodine, have a positive contribution to inhibitory activity;[11,12] 3) halogens could open interesting opportunities for the generation of further potential inhibitors, as they can be easily replaced by a number of chemical entities.



Scheme 1. Retrosynthetic rationale for the synthesis of targeted BCRP inhibitors, and the structures of the commercially available starting materials **1** and **2**.

Access to target compounds **3**–**7** was achieved in one step by coupling 6-substituted-4-oxo-4*H*-chromene-2-carboxylic acid (**1**) with tryptamine (**2**) in the presence of bis(2-oxo-3-oxazolidinyl)phosphonic chloride (BOP-Cl) as the coupling agent (Scheme 2; full details are given in the Supporting Information). 6-Iodo-4-oxo-4*H*-chromene-2-carboxylic acid (R = I) was not commercially available, but was easily obtained by hydrolysis of the commercially available corresponding ethyl ester with sodium hydrogen carbonate (20% in water) at 80 °C.

The test compounds were first screened by flow cytometry for their effects on the inhibition of mitoxantrone efflux in



Scheme 2. Synthesis of targeted inhibitors **3**–**7**. *Reagents and conditions*: a) BOP-Cl, Et_3N, DMF, RT, 24 h, 41–55%.

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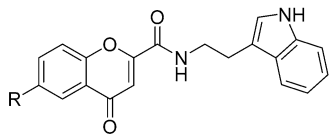
Table 1. Inhibition of mitoxantrone efflux in *ABCG2*-transfected HEK293 cells.



Compd	R	Inhibition[a] [%] 1 μM	5 μM	$IC_{0.5}$[b] [μM]
3	H	44±11	58±9	0.55
4	Me	28±3	46±4	–
5	F	41±3	59±3	0.60
6	Br	57±20	75±12	0.25
7	I	56±10	77±13	0.30

[a] Inhibition (%) was calculated relatively to the control agent GF120918 (5 μM), set as 100% inhibition. Inhibition (%) values were determined by flow cytometry as described in the Experimental Section. [b] Since the maximal inhibition was not complete, the affinity was estimated as the $IC_{0.5}$ value—the concentration required to cause half maximal inhibition. Data are the mean ±SD of at least three independent experiments.

Table 2. Inhibition of mitoxantrone efflux (ABCB1) and calcein efflux (MRP1) in transfected HEK293 cells.

Compd	Inhibition [%] ABCB1[a] 1 μM	5 μM	ABCC1[b] 1 μM	5 μM
3	−23.58±13.53	−31.92±20.18	3.97±3.05	3.07±3.02
6	−26.76±14.94	−33.32±21.28	4.84±3.62	5.92±4.28
7	−21.49±13.44	−34.63±20.93	0.96±0.08	5.94±4.80

[a] For *ABCB1*-transfected cells, inhibition (%) was determined using the control agent GF120918 (5 μM), set as 100% inhibition. [b] For *MRP1*-transfected cells, inhibition (%) was normalized using pcDNA3.1-transfected cells as the control. Data are the mean ±SD of at least three independent experiments.

transfected HEK293 cells overexpressing ABCG2, using GF120918, a known ABCG2 modulator, as a reference (Table 1). Lead compound **3** that lacks a substituent at C-6 (R=H) was tested for comparison. The presence of a fluoro substituent at C-6 (compound **5**) leads to an equally active inhibitor. Substitution of the fluoro with a methyl group (compound **4**), which is approximately the same size but has different electronic effects, provides a slightly less active inhibitor. As shown in Table 1, the presence of either a bromo (compound **6**) or iodo (compound **7**) atom at C-6 of the chromone moiety leads to the most active compounds in the series, bromo analogue **6** being the more potent of the two, with a slightly lower $IC_{0.5}$ value. The improved activity of compounds **6** and **7** might be due to the size and/or hydrophobicity of the halogen atom.

The next step in the evaluation process was to check the selectivity of the most active inhibitors. Indeed, ABCG2 inhibitors often lack selectivity and also target the two other well-known transporters P-gp/ABCB1 and MRP1/ABCC1. For this purpose, we evaluated compounds **3**, **6** and **7** in *P-gp- and MRP1*-transfected cells by measuring accumulation of mitoxantrone (P-gp substrate) in P-gp cells and calcein (MRP1 substrate) in MRP1 cells. As shown in Table 2, the three selected ABCG2 inhibitors did not induce any inhibitory effect against P-gp and MRP1, indicating good selectivity for ABCG2.

As mentioned above, our ultimate goal was to obtain sufficient biological data to allow the subsequent preclinical evaluation of the most promising inhibitor. Therefore, evaluation of the intrinsic cytotoxicity and the ability of these compounds to chemosensitize cancer cells toward anticancer drugs are essential. First, we evaluated the cytotoxicity of derivatives **3** and **6** in both resistant (ABCG2 overexpressing) and sensitive (HEK293-pcDNA3) cell lines. As shown in (Figure 1), the most potent ABCG2 inhibitor (compound **6**) was only toxic at high concentrations, with an IG_{50} (concentration producing 50% inhibition of cell growth) value of 60 μM, giving a therapeutic

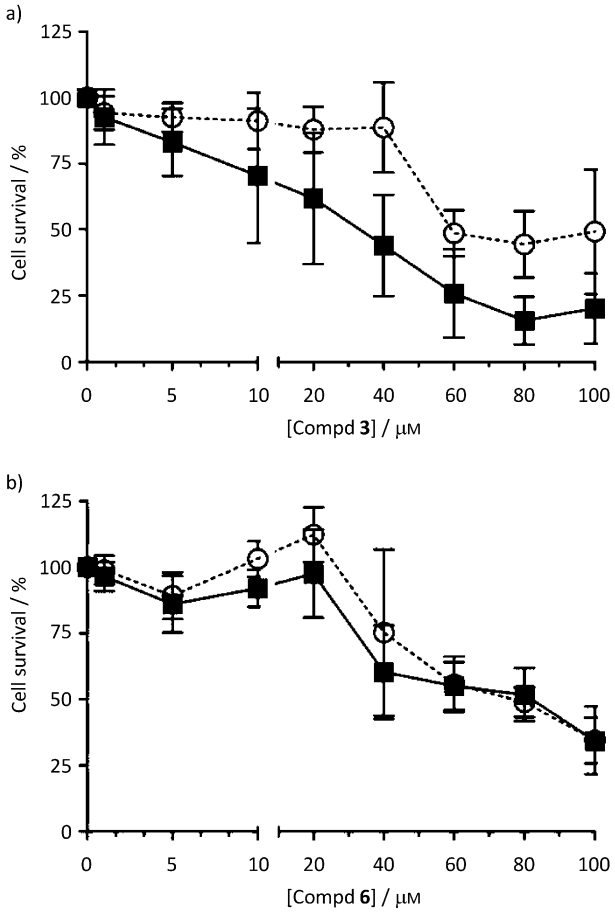


Figure 1. Cytotoxicity of a) compound **3** and b) compound **6**. Cell survival was determined by MTT assays as described in the Experimental Section. Cell viability of HEK293-*ABCG2* cells (■) and HEK293-pcDNA3.1 cells (○), upon 72 h treatment with the compounds in increasing concentrations, as indicated.

index of 240. A similar IG_{50} value was obtained with compound **3** in control cells (HEK293-pcDNA3.1).

Finally, we evaluated the ability of the inhibitors to potentiate the effect of anticancer drugs on cancer cells. Compounds **3** or **6** were co-administrated in combination with either mitoxantrone or SN-38, the active metabolite of irinotecan. The in-

hibitory activity was evaluated at two concentrations (0.2 and 1 μM) and similar profiles were obtained with mitoxantrone or SN-38, as illustrated for compound **3** in Figure 2, indicating that inhibition of BCRP-mediated drug efflux was indeed correlated to increased cytotoxicity. Similar effects were produced by compound **6** (data not shown). The addition of the chromone-derived inhibitor therefore potentiated the antiproliferative effect of the anticancer drug.

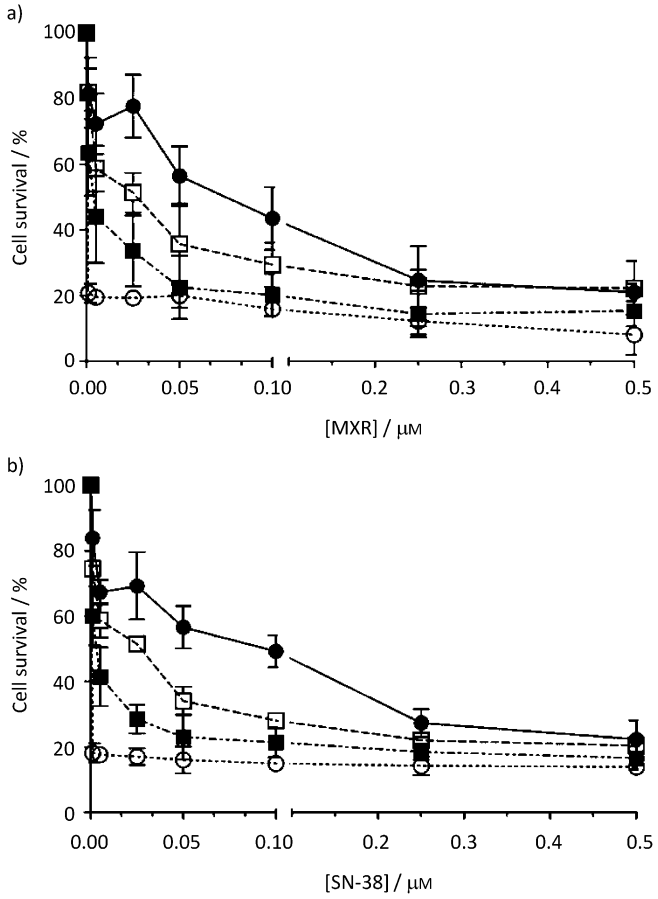


Figure 2. Sensitization to mitoxantrone and SN-38. Cell viability of HEK293-*ABCG2* cells upon co-treatment with compound **3** and a) mitoxantrone (MXR) or b) SN-38 (0–0.5 μM) for 72 h. Parallel experiments with only mitoxantrone or SN-38 were performed with HEK293-pcDNA3.1 and *ABCG2*-transfected cells. The values represent the mean ±SD of percent cell viability with respect to the untreated control. Data correspond to at least three independent experiments performed in triplicate. ABCG2: ●; ABCG2 + **3** (0.2 μM): □; ABCG2 + **3** (0.1 μM): ■; empty vector: ○.

In conclusion, the present work describes the very short and easy access to potent, selective and nontoxic inhibitors of ABCG2. The most active inhibitor (compound **6**) can be prepared in multigram scale without limitation. Owing to its potency, selectivity and very low toxicity, compound **6** is being considered for in vivo evaluation in ABCG2-xenograft models with the aim of confirming its effectiveness on the sensitization of tumors to anticancer agents.[13] Furthermore, compound **6**, as well as its analogues **5** and **7**, constitute ideal starting blocks for the generation of further chemical diverse analogues with potentially improved potency against ABCG2. For example, compounds **6** and **7** are good reagents for Suzuki and Heck cross-coupling reactions with diverse boronic acid derivatives.

Experimental Section

Chemistry

Experimental protocols and characterization data for compounds **3**–**7** are given in the Supporting Information.

Biology

General: Mitoxantrone, calcein-AM and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (France). All other reagents were commercial products of the highest available purity grade. All compounds were dissolved in DMSO and then diluted in Dulbecco's modified Eagle's medium (DMEM). The stock solution was stored at −20 °C and warmed to 25 °C just before use.

Cell cultures: Human fibroblast HEK293 cell lines transfected with either ABCG2 (HEK293-*ABCG2*) or the empty vector pcDNA3.1 (HEK293-pcDNA3.1) were obtained as previously described.[14] Human fibroblast HEK293 cell lines transfected with either MDR1 or MRP1 (HEK293-*MDR1* or HEK293-*MRP1*, respectively) were kindly provided by Dr. S. E. Bates (US National Cancer Institute–National Institutes of Health, Bethesda, MD, USA). All cells were maintained in high-glucose DMEM, supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and with the selection drug (0.75 mg mL^{-1} G418 for HEK293-pcDNA3.1 and HEK293-*ABCG2*, 2 mg mL^{-1} G418 for HEK293-*MDR1*, or 5 μM etoposide for HEK293-*MRP1*. Selection drug was finally omitted, in flow cytometry experiments.

ABCG2- and ABCB1-mediated drug transport: HEK293 cells were seeded at a density of 1×10^5 cells/well into 24-well culture plates. After 48 h incubation, cells were exposed to 5 μM mitoxantrone (HEK293-*ABCG2* and HEK293-*MDR1*) for 30 min at 37 °C, in the presence or absence of test compounds at various concentrations. After cell washing with phosphate buffer saline (PBS), the cells were trypsinized. The intracellular drug fluorescence was monitored with a FACS Calibur cytometer (Becton–Dickinson). At least 10000 events were collected, for which the maximal fluorescence (100%) was the difference between geometric mean fluorescence of cells incubated with 5 μM GF120918 and without inhibitor (control). Mitoxantrone was used as an ABCB1 substrate for direct comparison with ABCG2. It is transported by ABCB1 at a lower rate than rhodamine 123 and anthracyclines but indeed constitutes a true substrate,[15] the transport of which is fully inhibited by the characteristic inhibitor GF120918. In addition, many experiments showed the same efficiency of an established ABCB1 inhibitor on various substrates.

MRP1-mediated transport: HEK293 cells transfected with either *MRP1* or the empty vector (pcDNA3.1) were seeded at a density of 1×10^5 cells/well into 24-well culture plates. After 48 h incubation, cells were exposed to 0.2 μM calcein-AM and analyzed by flow cytometry as described above. The maximal fluorescence (100%) was the difference between geometric mean fluorescence of control cells (HEK293-pcDNA3.1) and *MRP1*-transfected cells, incubated with substrate but without inhibitor.

Cytotoxicity assays: HEK293-*ABCG2* and HEK293-pcDNA3.1 cells were seeded into 96-well culture plates at a density of 1×10^4 cells/well. After overnight incubation, cells were treated with various concentrations of test compounds for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. For the sensitization experiments, after overnight incubation, cells were concomitantly treated with test compounds and increasing concentrations of mitoxantrone or SN-38 for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. In both cases, cell viability was evaluated in an MTT colorimetric assay. Control experiments were performed with high-glucose DMEM containing 0.1% of DMSO (*v*/*v*). The results are expressed as percentage of viable cells versus the control, taken as 100%.

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