Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Investigation of Chalcones as Selective Inhibitors of the Breast Cancer Resistance Protein: Critical Role of Methoxylation in both Inhibition Potency and Cytotoxicity

Glaucio Valdameri,[†,‡,∥] Charlotte Gauthier,[†,∥] Raphaël Terreux,[§] Rémy Kachadourian,[#] Brian J. Day,[#] Sheila M. B. Winnischofer,[‡] Maria E. M. Rocha,[‡] Véronique Frachet,[⊥] Xavier Ronot,[⊥] Attilio Di Pietro,*[,†,∥] and Ahcène Boumendjel*[,⊗,∥]

[†]Equipe Labellisée Ligue 2012, BMSSI UMR 5086 CNRS/Université Lyon 1, Institut de Biologie et Chimie des Protéines, Lyon, France

[‡]Department of Biochemistry and Molecular Biology, Federal University of Paraná, Curitiba, PR, Brazil

[§]Equipe Bioinformatique: structures et interactions, BMSSI UMR 5086 CNRS/Université Lyon 1, Institut de Biologie et Chimie des Protéines, Lyon, France

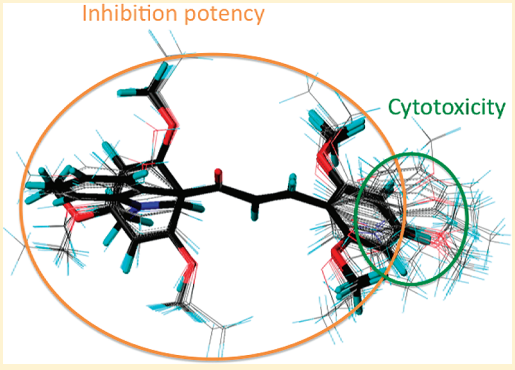
[#]Department of Medicine, National Jewish Health, Denver Colorado 80206, United States

[⊥]AGing Imaging Modeling, FRE 3405, Université Joseph Fourier, CNRS, EPHE, Faculté de Médecine, La Tronche, France

[⊗]Université Joseph Fourier—Grenoble/CNRS, UMR 5063, Département de Pharmacochimie Moléculaire, Grenoble, France

Supporting Information

ABSTRACT: ABCG2 plays a major role in anticancer-drug efflux and related tumor multidrug resistance. Potent and selective ABCG2 inhibitors with low cytotoxicity were investigated among a series of 44 chalcones and analogues (1,3-diarylpropenones), by evaluating their inhibitory effect on the transport of mitoxantrone, a known ABCG2 substrate. Six compounds producing complete inhibition with IC_{50} values below 0.5 μM and high selectivity for ABCG2 were identified. The number and position of methoxy substituents appeared to be critical for both inhibition and cytotoxicity. The best compounds, with potent inhibition and low toxicity, contained an *N*-methyl-1-indolyl (compound **38**) or a 6′-hydroxyl-2′,4′-dimethoxy-1-phenyl (compound **27**) moiety (A-ring) and two methoxy groups at positions 2 and 6 of the 3-phenyl moiety (B-ring). Methoxy substitution contributed to inhibition at positions 3 and 5, but had a negative effect at position 4. Finally, methoxy groups at positions 3, 4, and 5 of the B-ring markedly increased cytotoxicity and, therefore, should be avoided.



INTRODUCTION

Anticancer agents constitute a large therapeutic arsenal used in clinics. Although their efficacy is dependent on the cancer evolution stage and location, most anticancer drugs suffer from cell resistance to the treatment. This resistance can be related to several mechanisms leading to subsequent loss of drug efficacy. One of the well-established mechanisms is associated with overexpression of ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp/ABCB1), multidrug resistance protein 1 (MRP1/ABCC1), and breast cancer resistance protein (BCRP/ABCG2),[1] which bind and extrude the anticancer drugs out of the cells, creating drug resistance.

ABCG2 was simultaneously discovered in three laboratories and is therefore known as ABCP for its abundance in placenta,[2] BCRP for its discovery in breast cancer,[3] and MXR for its resistance to mitoxantrone.[4] ABCG2 is considered to be a privileged target for drug discovery, with the aim of conceiving and developing inhibitors of its efflux activity that can be combined with anticancer drugs for increasing their intracellular concentration. Several types of ABCG2 inhibitors have been reported, but very few representatives have shown promising results in preclinical trials.[5,6]

Pursuing our efforts toward the identification of potent and selective ABCG2 inhibitors, derived from the naturally occurring flavonoids,[7−9] we investigated chalcones (1,3-diarylpropenones) and analogues such as 1-indolyl-3-phenylpropenones and 1-phenyl-3-indolylpropenones (Figure 1).

Chalcones were early shown to bind to P-gp[10,11] and inhibit its transport activity.[12] Different substituents on the A-ring appeared to be required for inhibiting ABCG2,[13] but the inhibition was rather low due to inadequate substitutions in both rings A and B.[13] Very recently, the inhibition was found to be decreased by replacing the A-ring with a naphthyl moiety, or

Received: December 6, 2011
Published: March 26, 2012

   dx.doi.org/10.1021/jm2016528 | *J. Med. Chem.* 2012, 55, 3193−3200

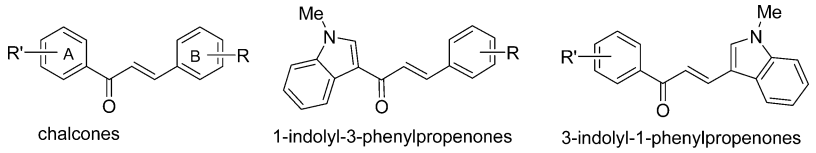
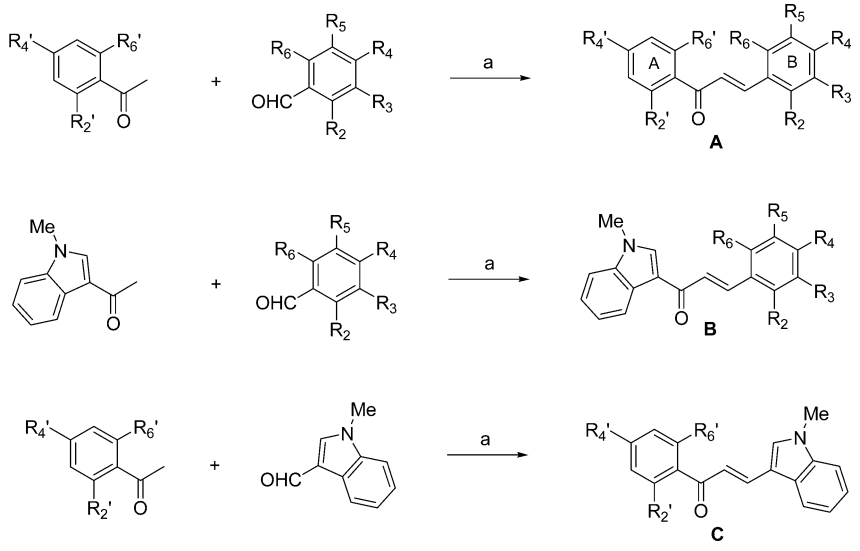


Figure 1. General structures of targeted chalcones and analogues.

Scheme 1[a]



[a](a) KOH (50% in H_2O), EtOH, 60 °C.

by B-ring methoxylation, but no effect on cytotoxicity was reported.[14] Interestingly, replacement of one phenyl with an indolyl moiety was shown to significantly increase the chalcone activity,[15] in agreement with its presence in other types of potent and specific ABCG2 inhibitors.[16−18]

The aim of this work was to shed light on structural requirements responsible not only for selective inhibition of ABCG2 but also for cytotoxicity. The results led to the identification of selective chalcones and chalcone-like compounds with both high potency and low cytotoxicity, making them good candidates for future in vivo preclinical evaluation.

CHEMISTRY

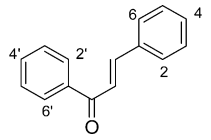
The investigated chalcones and analogues were synthesized according to the classical method illustrated in Scheme 1. For chalcones (upper reaction A), the conveniently substituted acetophenones and benzaldehydes were reacted together in the presence of KOH in a mixture of water and ethanol. Some methoxylated or ethoxylated chalcones required the preparation of methoxylated acetophenones, ethoxylated acetophenones, and/or ethoxylated benzaldehydes (when not commercially available). In such a case, they were obtained by methylation or ethylation of hydroxylated acetophenones and benzaldehydes, by using methyl iodide or ethyl iodide in the presence of K_2CO_3 in acetone.[19] For 1-indolyl-3-phenylpropenones (middle reaction B), the same synthesis conditions as for reaction A were used, starting from 3-acetyl-*N*-methylindol and a derivative of benzaldehyde.[20] The 3-indolyl-1-phenylpropenones (bottom reaction C) were also synthesized under the same conditions, starting from an acetophenone derivative and *N*-methylindolyl-3-carboxaldehyde.[20] Finally, the synthesis of 1,3-diindolylpropenone (**44**; Table 2) was performed starting from 3-acetyl-*N*-methylindole and *N*-methylindolyl-3-carboxaldehyde in the presence of KOH in a mixture of EtOH/H_2O.[20]

BIOLOGICAL EVALUATION, SAR, AND DISCUSSION

The substituents to be introduced on the chalcone scaffold were chosen among those frequently found in naturally occurring compounds, such as methoxy (OMe) and hydroxyl (OH) groups often present at positions 2′, 4′, and 6′ of the A-ring and 2, 3, 4, 5, and 6 of the B-ring.[21] Furthermore, a primary screening of diversely substituted chalcones concluded that the OR substituents are quite promising in providing the most active inhibitors and relevant structure−activity relationship (unpublished results). By contrast, substitution of the B-ring by halogens gave variable results: a single Cl at either position 2 or 3 was found to increase inhibition of mitoxantrone efflux,[13] in contrast to the negative effect produced at position 4, whereas all substitutions at either position 2, 3, or 4 were negative to inhibition of ABCG2-mediated efflux.[14] Interestingly, substitution at position 2 by Cl, or by F to a lower extent, was recently found to promote glutathione biosynthesis.[22] Taking into account such structural elements, we synthesized and evaluated a series of 34 chalcones (Table 1) and 10 chalcone-like analogues containing an indolyl moiety (Table 2). The chalcones and analogues were evaluated by flow cytometry for their ability to restore, through ABCG2 inhibition, the intracellular accumulation of mitoxantrone, a well-known transported anticancer agent, in an *ABCG2*-transfected human fibroblast HEK293 cell line, using GF120918 as a control for complete inhibition.

dx.doi.org/10.1021/jm2016528 | *J. Med. Chem.* 2012, 55, 3193−3200

Table 1. Inhibition of Mitoxantrone Efflux by Substituted Chalcones in *ABCG2*-Transfected Cells



									ABCG2 inhibition[a] (%)		
compd[b]	2′	4′	6′	2	3	4	5	6	at 2 μM	at 10 μM	class
1	OMe		OMe						2.0 ± 0.61	2.6 ± 2.4	3
2	OMe		OMe	OMe					−0.9 ± 8.2	0.7 ± 7.7	3
3	OMe		OMe		OMe				−6.8 ± 9.4	1.9 ± 11.3	3
4	OMe		OMe	OMe				OMe	2.5 ± 13.6	6.7 ± 13.1	3
5	OMe		OMe	OMe		OMe		OMe	−0.9 ± 10.5	18.3 ± 15.6	3
6	OMe		OMe			OMe	OMe	OMe	2.7 ± 13.9	9.4 ± 13.3	3
7	OMe		OMe		OMe	OMe		OMe	2.7 ± 12.2	18.9 ± 18.0	3
8	OMe		OMe		OMe	OMe	OMe		13.0 ± 14.4	40.4 ± 19.6	3
9	OEt		OEt	OMe		OMe		OMe	22.1 ± 0.13	55.1 ± 11.7	3
10	OEt		OEt	OMe				OMe	1.3 ± 10.7	17.4 ± 12.7	3
11	OEt		OEt	OMe		OMe			21.2 ± 11.7	45.4 ± 13.3	3
12	OMe	OMe				OMe		OMe	42.6 ± 18.4	55.3 ± 13.4	2
13	OMe	OMe	OMe	OMe		OMe		OMe	16.7 ± 6.6	71.8 ± 11.4	3
14	OMe	OMe	OMe	OMe		OMe			11.9 ± 14.3	54.3 ± 17.4	3
15	OMe	OMe	OMe	OMe				OMe	11.5 ± 15.7	15.7 ± 9.4	3
16	OEt	OEt	OEt	OEt		OEt		OEt	37.0 ± 15.7	55.0 ± 11.2	2
17				OMe				OMe	0.02 ± 9.81	26.7 ± 10.2	3
18	OH	OMe			OMe		OMe		56.4 ± 13.7	63.2 ± 9.6	2
19	OH	OMe				OMe	OMe	OMe	44.7 ± 10.3	59.7 ± 9.0	2
20	OH	OMe				OMe	OMe		71.1 ± 10.3	76.5 ± 6.7	2
21	OH	OMe		OMe		OMe		OMe	45.9 ± 14.8	68.4 ± 12.4	2
22	OH	OMe					OMe	OMe	27.8 ± 15.1	48.5 ± 14.0	2
23	OH	OMe			OMe			OMe	47.5 ± 5.1	41.6 ± 23.1	2
24	OH	OMe			OMe	OMe	OMe		50.1 ± 19.8	101.2 ± 14.0	3
25	OH	OMe		OMe				OMe	58.2 ± 19.3	79.3 ± 13.8	2
26	OH	OMe				OMe		OMe	59.6 ± 11.1	54.7 ± 10.8	2
27	OMe	OMe	OH	OMe				OMe	130.7 ± 15.9	130.5 ± 12.6	**1**
28	OMe	OMe	OH		OMe		OMe		115.0 ± 16.7	117.9 ± 22.8	**1**
29	OMe	OMe	OH						67.9 ± 1.0	60.4 ± 17.2	2
30	OMe	OMe	OH	OMe					75.7 ± 8.9	93.1 ± 8.9	3
31	OMe	OMe	OH		OMe				93.0 ± 12.9	97.9 ± 2.2	**1**
32	OMe	OMe	OH	OMe		OMe		OMe	72.9 ± 6.9	90.0 ± 14.5	3
33	OMe	OMe	OH		OMe	OMe	OMe		72.5 ± 6.67	84.9 ± 10.7	2
34	OMe	OMe	OH			OMe	OMe	OMe	54.9 ± 11.1	95.0 ± 12.6	3

[a]The percent inhibition on ABCG2-mediated mitoxantrone transport was determined by flow cytometry as described under Experimental Section, and GF120918 (5 μM) was used as a control (100 ± 9.7%). Data are the mean ± SD of at least three independent experiments. [b]The synthesis, physical characteristics, and structural evidence were previously reported[19] and are given here in the Supporting Information.

The inhibitory activity against ABCG2-mediated mitoxantrone efflux is reported in Tables 1 and 2, expressed as percent inhibition observed at 2 and 10 μM concentrations of each compound, relative to 5 μM GF120918 taken as a reference inhibitor. For facilitating comparison and interpretation of the results, we ranked the studied compounds in three different classes, according to their apparent affinity and maximal extent of inhibition:

Class 1 included high-affinity inhibitors (IC_{50} < 1 μM) producing a complete inhibition at both 2 and 10 μM, similarly to recently reported chromones.[23]

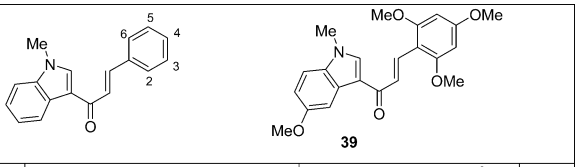
Class 2 comprised high-affinity inhibitors producing an incomplete maximal inhibition (limited to 50−75%), similarly to hydrophobic flavones[7] and methoxy-*trans*-stilbenes.[24]

Class 3 was composed of low-affinity inhibitors. The following compounds displayed especially low, if any, inhibition: **1**−**7**, **10**, **15**, **17**, **42**, and **43**.

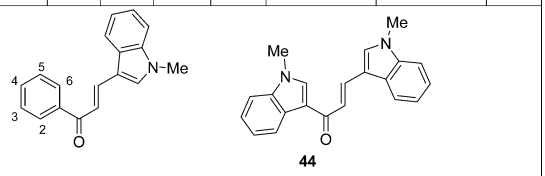
As shown in Table 1, chalcones in which the A-ring (1-phenyl moiety) was substituted by OMe at positions 2′ and 6′ displayed either a low inhibition (class 3 chalcone **8**) or no inhibition at all (class 3 chalcones **1**−**4** and **6**), independently of the number and positions of OMe groups on the B-ring (3-phenyl moiety). The substitution of 2′,6′-OMe groups with ethoxyls slightly improved the activity in class 3 chalcone **9** (vs **5**), but not in class 3 chalcone **10** (vs **4**). Shifting the 6′-OMe group to the 4′-position produced an increased inhibition in class 2 chalcone **12** (vs **5** and **7**). The presence of three OMe groups on the A-ring led to the moderately active class 3 chalcones **13** (vs **5**), whereas their replacement by ethoxy groups had limited, if any, effect in chalcone **16** (vs **13**).

dx.doi.org/10.1021/jm2016528 | *J. Med. Chem.* 2012, 55, 3193−3200

Table 2. Inhibition by Indolylphenylpropenones



Compound	Substituents					ABCG2 inhibition (%) [a]		Class
	2	3	4	5	6	2 (μM)	10 (μM)	
35 [b]	OMe		OMe			83.8 ± 11.4	107.5 ± 8.7	3
36 [b]		OMe	OMe	OMe		49.3 ± 15.3	70.0 ± 16.4	3
37 [b]	OMe		OMe		OMe	93.8 ± 8.8	108.8 ± 11.4	1
38 [b]	OMe				OMe	103.5 ± 20.2	113.3 ± 11.9	1
39 [b]	-	-	-	-	-	89.08 ± 7.60	111.03 ± 4.48	1



40	OMe		OMe			88.2 ± 3.3	124.7 ± 16.7	3
41			OMe			55.5 ± 3.6	89.0 ± 11.4	3
42	OMe		OMe		OMe	3.7 ± 5.7	30.0 ± 13.4	3
43	OMe				OMe	2.9 ± 15.2	17.4 ± 14.8	3
44 [b]	-	-	-	-	-	52.1 ± 14.8	98.8 ± 17.0	3

[a]The conditions were the same as in Table 1. [b]The synthesis, physical characteristics, and structural evidence were previously reported[19] and are given in the Supporting Information.

Introduction of an OH group at the 2′-position, in chalcones **18**–**26**, had an effect similar to that of the insertion of OMe by providing essentially class 2 compounds similarly to **12**. The highest inhibition was observed in **27**, **28**, and **31**, in the concomitant presence of 6′-OH and 2′,4′-diOMe groups, which constituted the optimal substitution pattern of the A-ring. This was consistent with the efficiency produced by the same substitution on the efflux of Hoechst 33342.[14] The positive role of 6′-OH in the series **27**–**34** was evident by comparison to the lack of activity of the series **1**–**8**; in contrast, it allowed a complete inhibition to be reached by contrast to the class 2 compounds **18**–**23**, **25**, and **26**. On the B-ring, both position and number of OMe groups appeared to be important: pairs of OMe at either positions 2 and 6 (in **27**) or 3 and 5 (in **28**) gave the best inhibitors, whereas a single OMe at either position 3 (in **31**) or 2 (in **30**) was better than no OMe (in **29**). In contrast, substitution at position 4 was unfavorable when comparing **32** to **27** and **33** to **28**. This explains why our compound **31**, without OMe at position 4, was 2–3-fold more potent than the 4-OMe-containing lead recently reported.[14] Chalcone **34**, also, was ranked in the less active class 3. A critical role of methoxy groups toward inhibition, depending on their number and positions, was also recently demonstrated in our group in the case of *trans*-stilbenes as specific ABCG2 inhibitors.[24]

The positive contribution of the OH at position 6′ was also observed for inhibition of ABCB1 (P-gp) by both chalcones and flavonoids.[25] However, it was not investigated, in addition to OMe at position 2′ in previous ABCG2 studies.[12,13] As shown in the present work, the role of the OH at position 6′ was not due to a hydrogen bond with the adjacent carbonyl group (by reducing free rotation around the bound linking A-ring and carbonyl group) because the presence of OMe at position 2′ (as in **27**, **28**, and **31**) induces steric hindrance, preventing such a conformation.

The activity of indolyl-containing chalcone analogues (derivatives **35**–**44**) is shown in Table 2. From this series, we obtained three highly active derivatives (class 1 compounds **37**–**39**). By comparing the latter series to chalcones, the indolyl contribution undoubtedly appeared to be crucial: it was as efficient as 2′,4′-diOMe,6′-OH-1-phenyl (A-ring), which contrasts with the negative effect observed for a naphthyl moiety.[14] On the contrary, in the 3-indolyl series (derivatives **40**–**43**), the results were generally disappointing, with compounds ranked in class 3. The great difference observed between the 1-indolyl and 3-indolyl series may point to the impact of the electronic distribution within the molecule on the inhibition activity. The last investigated compound was 1,3-diindolylpropenone (class 3, **44**): its moderate activity indicates that the presence of at least one methoxylated phenyl group (B-ring) was required. The importance of methoxy substituents for ABCG2 inhibition was previously observed in other types of compounds such as tectochrysin versus chrysin[7] and methoxy derivatives of both rotenoids[26] and *trans*-stilbene.[24]

Displaying the contribution volumes indicates relationships between compound structure and inhibitory activity, whereas the central core was kept constant (Figure 2). As expected, the positive effect of OH at position 6′ (in **27**, **28**, and **31**) was due to electrostatic contribution (red volume). This was also the case for OMe at position 5 (in **28**). By difference, the positive contributions of OMe at position 6 (in **27**, **37**, and **38**) appeared to be due to steric effect (yellow volume), as well as OMe at position 2′ (in **27**, **28**, and **31**). The negative effect of

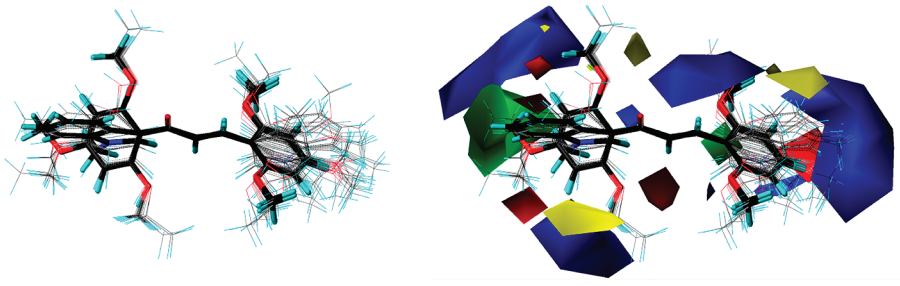


Figure 2. 3D-QSAR analyses. (Left) The 44 molecules were modeled, aligned on the central core, and inserted in the databank for a computed 3D-QSAR study. The calibration and validation coefficients were, respectively, 0.924 and 0.651. Two highly active compounds, chalcone **27** and its indolyl analogue, **38**, are displayed in capped stick, and the other molecules are represented in lines. (Right) CoMFA contribution volumes with all molecules. Volumes were plotted with 20 for positive contribution (yellow for steric and red for electrostatic fields) and 70 for negative contribution (green for steric and blue for electrostatic fields).

dx.doi.org/10.1021/jm2016528 | *J. Med. Chem.* 2012, 55, 3193–3200

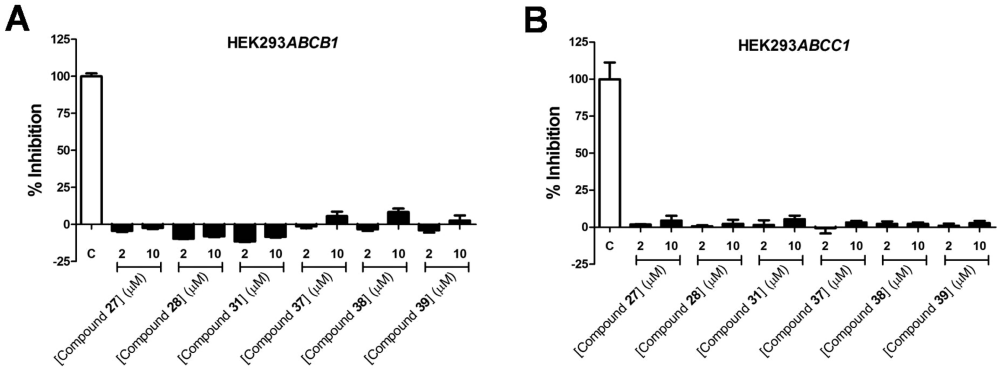


Figure 3. Inhibition specificity for ABCG2 relative to ABCB1 and ABCC1. (A) The inhibition on ABCB1-mediated mitoxantrone efflux was determined by flow cytometry as described under Experimental Section and normalized using control cells transfected by the empty vector (taken as 100% inhibition). (B) The percent inhibition on ABCC1-mediated calcein transport was determined using the same approach. Data are the mean ± SD of two independent experiments.

OMe at position 4, in **32** versus **27** and in **33** versus **28**, appeared to be electrostatic (blue volume). Surprisingly, the *N*-methyl-1-indolyl did not display particularly positive interactions, suggesting that hydrophobic interactions (not displayed here because all compounds were essentially hydrophobic) were most likely essential for the binding. In contrast, the negative effect produced by a naphthyl moiety, as recently reported,[14] might be due to steric hindrance (green volume on the left side), similarly to the *N*-methyl-3-indolyl of derivatives **40**–**44** on the right side.

On the basis of above results, we selected all class 1 inhibitors (**27**, **28**, **31**, and **37**–**39**) for further experimental investigations. They were first evaluated for their selectivity, under similar inhibitory conditions, on HEK293 cell lines transfected with the two other multidrug transporters, ABCB1 (P-gp) and ABCC1 (MRP1), and using HEK293 cells transfected with the empty vector (HEK293-pcDNA3.1) as a control. As shown in Figure 3, the six derivatives did not show any significant inhibition up to 10 μM, indicating a high selectivity for ABCG2 (BCRP).

Second, the IC_{50} values for ABCG2 were determined from Figure 4A and are indicated in Table 3: they were all below 0.5 μM, with compound **28** being the most potent inhibitor (IC_{50} = 0.17 μM). Finally, the cytotoxicity of each inhibitor was evaluated on both sensitive control cells and resistant ABCG2-transfected cells. As shown in Figure 4B, the six compounds induced very similar cytotoxicity profiles in both types of cell lines.

The absence of any apparent cross-resistance in ABCG2-transfected cells suggests that the chalcones were not transported by ABCG2, as this was also the case for other ABCG2-specific inhibitors such as flavones,[7] chromones,[23] and methoxystilbenes.[24] Cytotoxicities were produced at higher concentrations than for inhibition of ABCG2 transport activity. However, marked differences were observed among the different compounds: the IG_{50} value of compound **39** was 50-fold lower than the high value obtained for compounds **27** and **38**, whereas the remaining chalcones, **28**, **31**, and **37**, displayed intermediate effects (Table 3).

These results show the important role played by OMe groups at position 4 (in **39** and **37**) as well as at both positions 3 (in **31** and **28**) and 5 (in **28**) on cytotoxicity, probably due to interaction with additional target(s), different from ABCG2, which are present in both control and *ABCG2*-transfected cells. Although no cytotoxicity data were reported for the 3,4-diOMe-substituted lead reported by Juvale et al.,[14] our present results indicate that it should be at least 10–50-fold more cytotoxic than our compounds **27** and **38**, which therefore could prevent its use for in vivo experiments. The OMe substituents at positions 3 and 5 played a dual role because they also increased ABCG2 inhibition. In contrast, OMe substitutions at positions 2 and 6 were not critical for cytotoxicity, neither was the use of an 1-indolyl, instead of a 1-phenyl, moiety (in **38** versus **27**). Such marked differences in cytotoxicity were mostly responsible for the differences observed in calculating the therapeutic ratio because the IC_{50} values for ABCG2 inhibition were more constant. The two less cytotoxic compounds with IG_{50} values around 50 μM, that is, chalcone **38** and indolylphenylpropenone **27**, therefore displayed the highest values of therapeutic ratio (185 and 143, respectively), which constitutes an important parameter for considering future in vivo experiments. A much lower therapeutic ratio was obtained previously with other types of inhibitor: around 40 for 6-prenylchrysin[7] and even less for acridones,[6,27] which nevertheless displayed a significant in vivo activity.[28]

Overall, our results emphasize the differential roles of up to six OMe substituents: (i) the OMe at position 2′ of the 1-phenyl moiety greatly contributes through steric effects, in addition to OH at position 6′, to inhibition potency; (ii) the two OMe at positions 2 and 6 of the 3-phenyl moiety also greatly contribute to inhibition potency without apparent effect on cytotoxicity; (iii) the two OMe at vicinal positions 3 and 5 contribute, even more strongly, to inhibition potency but also induce additive effects on cytotoxicity; (iv) finally, the most negative role is played by OMe at position 4, which is unfavorable to inhibition, both sterically and electrostatically, and greatly increases the cytotoxicity. This is the first work describing structure–activity relationships of cytotoxicity for ABCG2 inhibitors.

In conclusion, chalcones and indolyphenylpropenones constitute promising leads as inhibitors of ABCG2. The easy chemical access to such compounds and their high activity and specificity are fundamental prerequisites for preclinical trials. The two compounds selected here with a high therapeutic ratio, **38** and **27**, without OMe at position 4, constitute good candidates to enter preclinical trials to validate their interest for further development. Our recent studies on in vivo evaluation of acridones[28] will be helpful to speed in vivo testing of the present lead chalcones.

dx.doi.org/10.1021/jm2016528 | *J. Med. Chem.* 2012, 55, 3193–3200

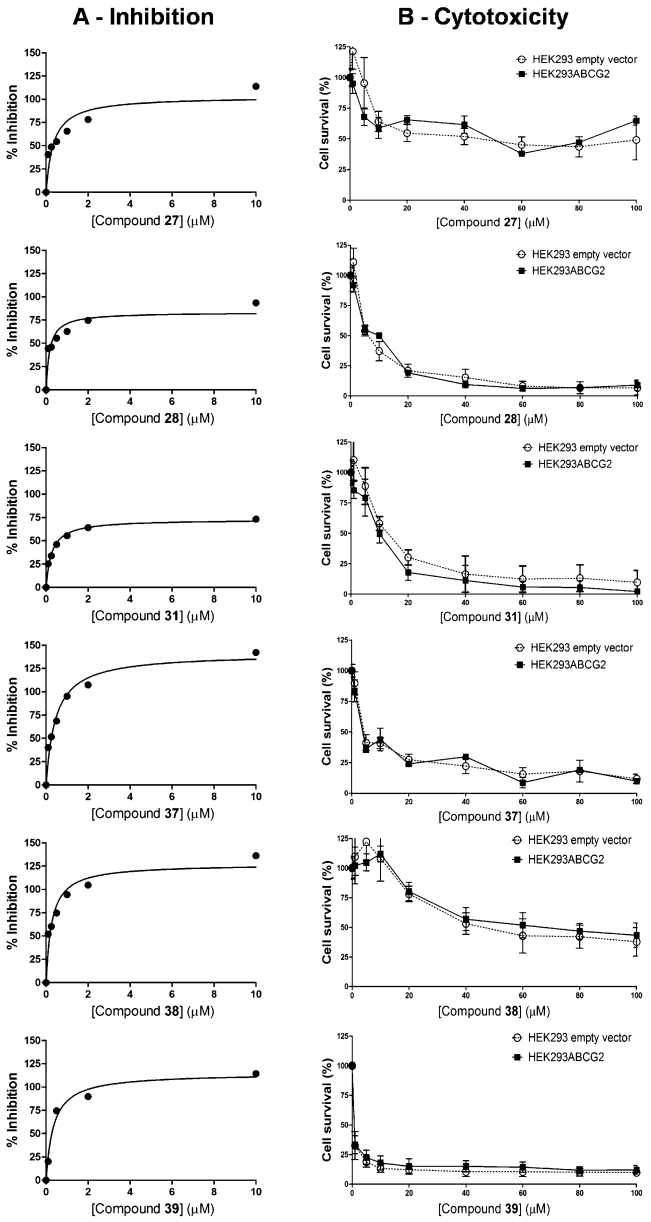


Figure 4. Inhibition of ABCG2 efflux activity and intrinsic cytotoxicity. (A) The IC_{50} values (concentrations for half-maximal inhibition) on ABCG2-mediated mitoxantrone efflux were determined by flow cytometry, as described under Experimental Section. The curves were fitted using GraphPad Prism 5 software. (B) Cell viability of *ABCG2*-transfected HEK293 cells and control cells upon treatment with compounds **27**, **28**, **31**, and **37**–**39**, up to 100 μM, for 72 h. The values represent the mean ± SD of percent cell viability with respect to the untreated control. Data correspond to at least two independent experiments performed in triplicate.

EXPERIMENTAL SECTION

Chemistry. ^{1}H and ^{13}C NMR spectra were recorded on a Brüker AC-400 instrument (400 MHz). Chemical shifts (δ) are reported in parts per million relative to Me_4Si (internal standard). Electrospray ionization (ESI) mass spectra were acquired by the Analytical Department of Grenoble University on an Esquire 300 Plus Bruker Daltonis instrument with a nanospray inlet. Combustion analyses were performed at the Analytical Department of Grenoble University, and all tested compounds have a purity of at least 95%. Thin-layer chromatography (TLC) used Merck silica gel F-254 plates (thickness = 0.25 mm). Flash chromatography used Merck silica gel 60, 200–400 mesh. Unless otherwise stated, reagents were obtained from commercial sources and were used without further purification.

Table 3. Inhibitory Activity and Cytotoxicity of the Most Potent Chalcones and Indolylphenylpropenones

compd	IC_{50} (drug efflux inhibition, μM)	IG_{50} (cytotoxicity, μM)	therapeutic ratio[a] (IG_{50}/IC_{50})
27	0.35 ± 0.14	50.0 ± 3.9	143
28	0.17 ± 0.07	6.0 ± 0.4	35
31	0.26 ± 0.04	10.0 ± 2.1	38
37	0.45 ± 0.09	4.0 ± 0.2	9
38	0.27 ± 0.08	50.0 ± 6.2	185
39	0.35 ± 0.09	1.0 ± 0.1	3

[a]The therapeutic ratio of class 1 compounds was calculated from the values of IG_{50} (concentration producing 50% inhibition of cell growth) and IC_{50} (concentration producing half-maximal inhibition of ABCG2-mediated mitoxantrone efflux).

Biology. *Materials.* Mitoxantrone, calcein-AM, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (France). All other reagents were commercial products of the highest available purity grade.

Compounds. All compounds were dissolved in DMSO and then diluted in DMEM high-glucose medium. The stock solution was stored at −20 °C and warmed to 25 °C just before use.

Cell Cultures. The human fibroblast HEK293 cell line transfected with either ABCG2 (HEK293-*ABCG2*) or the empty vector (HEK293-pcDNA3.1 cells) was obtained as previously described.[28] The human fibroblast HEK293 cell line transfected with either *ABCB1* (P-gp) or *ABCC1* (MRP1) was kindly provided by Dr. S. E. Bates (NCI, NIH, Bethesda, MD, USA). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM high glucose), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and drug supplemented in some cases with either 0.75 mg/mL G418 (HEK293-pcDNA3.1 and HEK293-*ABCG2*) or 2 mg/mL G418 (HEK293-*ABCB1*) or 5 μM etoposide (HEK293-*ABCC1*).

ABCG2- and ABCB1-Mediated Mitoxantrone Transport. The efflux assays were determined as previously reported[29] with minor modifications. Cells were seeded at a density of 1×10^5 cells/well in 24-well culture plates. After 48 h, HEK293-*ABCG2* and HEK293-*ABCB1* cells were exposed to mitoxantrone (5 μM) with or without compounds at 2 or 10 μM, and incubated at 37 °C in 5% CO_2 for 30 min. The cells were then washed with phosphate buffer saline (PBS) and, after being trypsinized and subsequently resuspended in ice-cold PBS (0.2 mL), they were kept on ice until analysis by flow cytometry. The data of intracellular drug fluorescence were acquired using a FACSCalibur flow cytometer equipped with a 635 nm red diode laser and a 670 nm bandpass filter (FL4-H) controlled by CellQuest Pro software. At least 10,000 events were collected, and the geometric mean fluorescence (GMean) for each histogram was used as the measure of fluorescence for calculation of efflux values. Cells in PBS alone yielded the Blank histogram (cell autofluorescence), whereas cells in the presence of mitoxantrone alone, or GF120918 (5 μM) and mitoxantrone, constituted the controls. In ABCG2-mediated mitoxantrone transport, the percentage of inhibition was calculated by using eq 1

$$\%\ \text{inhibition} = (C - M)/(I - M) \times 100 \quad (1)$$

where C corresponds to the intracellular fluorescence of cells in the presence of compounds and mitoxantrone, and M to the intracellular fluorescence of cells in the presence of only mitoxantrone. Here, I is the intracellular fluorescence of cells in the presence of the reference inhibitor GF120918 and mitoxantrone. In ABCB1-mediated mitoxantrone transport, the cells transfected with the empty vector (HEK293-pcDNA3.1) were used as control. The percentage of inhibition was calculated by using eq 2

dx.doi.org/10.1021/jm2016528 | J. Med. Chem. 2012, 55, 3193–3200

$$\% \text{ inhibition} = (C - M)/(C_{ev} - M) \times 100 \quad (2)$$

where C and M are defined as above. In contrast to eq 1, C_{ev} corresponds to the intracellular fluorescence of cells transfected with the empty vector in the presence of mitoxantrone.

MRP1-Mediated Calcein Transport. HEK293 cells transfected with either *ABCC1* or the empty vector were seeded at a density of 1×10^5 cells/well in 24-well culture plates. After 48 h, cells were exposed to calcein-AM (0.2 μM) with or without compounds at 2 or 10 μM and incubated at 37 °C in 5% CO_2 for 30 min. The cells were treated as described above and analyzed with a FACSCalibur flow cytometer equipped with a 488 nm argon laser and a 530 nm bandpass filter (FL1-H) controlled by CellQuest Pro software. In ABCC1-mediated calcein transport, the cells transfected with the empty vector (HEK293-pcDNA3.1) were used as a control, and the percentage of inhibition was calculated with eq 2.

Cytotoxicity Assays. The cytotoxicity assays were performed using the MTT colorimetric assay,[30] based on mitochondrial dehydrogenases of viable cells that cleave the MTT, yielding purple MTT formazan which is quantified spectrophotometrically. HEK293-*ABCG2* and HEK293 cells transfected with the empty vector (HEK293-pcDNA3.1) were seeded in 96-well culture plates at a 1×10^4 cells/well density and allowed to attach for 24 h at 37 °C in 5% CO_2. The treatment was done with various concentrations of compounds in a final volume of 200 μL and allowed to grow for 72 h at 37 °C in 5% CO_2. After the appropriate treatment time, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37 °C in 5% CO_2. Then the culture medium was discarded, each well was washed with PBS, and 100 μL of DMSO was added into each well and mixed by gentle shaking for 10 min. Absorbance was measured in a microplate reader at 570 nm. Experimental conditions were set in triplicate, and control experiments were performed with DMEM high glucose containing 0.1% DMSO (v/v). Data are plotted as a percentage of reduced cell viability compared to control (untreated cells) that was taken as 100%.

Molecular Modeling. The compounds were analyzed by using the Sybyl X 1.3 molecular modeling suite software[31] and minimized by the MMFF94 force field[32] with a dielectric constant of 80 and an electrostatic cutoff of 16 Å. Minimized molecules were aligned on the central common core and put in a database; lateral chains of molecules were manually checked and aligned on a common position, and the modified conformation was minimized. The differences in internal energy between the two conformations must be <20 kcal mol^{-1} to validate the aligned conformations. Using the 44 aligned molecules, a 3D-QSAR study was initiated with the CoMFA program.[33] The grids of electrostatic and steric potential fields were computed using the C3 atom with a charge of +2 as a probe. The grid was filtered with 6 kcal mol^{-1} as the minimal variation to select probes, and validation by the leave-one-out (LOO) method was chosen.[34]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Characterization of chemical compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(A.B.) Phone: (33) 4 7663 5311. E-mail: ahcene.boumendjel@ujf-grenoble.fr. (A.D.P.) Phone: (33) 4 7272 2629. E-mail: a.dipietro@ibcp.fr.

Author Contributions

‖Both Ph.D. students (G.V. and C.G.) and senior investigators (A.D.P. and A.B.) equally contributed to the work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Drs. R. W. Robey and S. E. Bates, NCI-NIH, Bethesda, MD, USA, are acknowledged for providing HEK-293 cells transfected by either ABCB1 (MDR-19) or ABCC1, and GlaxoSmithKline is acknowledged for providing GF120918. C.G. is the recipient of a doctoral fellowship from the Ligue Nationale Contre le Cancer. G.V. is recipient of a mobility fellowship from the Brazilian CAPES (Process 2303/10-8). Financial support was provided by the CNRS and Université Lyon 1 (UMR 5086), the Ligue Nationale Contre le Cancer (Equipe labellisée Ligue 2012), and the Région Rhône-Alpes (CIBLE 2010).

■ ABBREVIATIONS USED

ABC, ATP-binding cassette; MDR, multidrug resistance; MRP1, multidrug resistance protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

■ REFERENCES

(1) Glavinas, H.; Krajcsi, P.; Cserepes, J.; Sarkadi, B. The role of ABC tansporters in drug resistance, metabolism and toxicity. *Curr. Drug Deliv.* **2004**, *1*, 27−42.

(2) Allikmets, R.; Schriml, L. M.; Hutchinson, A.; Romano-Spica, V.; Dean, M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res.* **1998**, *58*, 5337−5339.

(3) Doyle, L. A.; Yang, W.; Abruzzo, L. V.; Krogmann, T.; Gao, Y.; Rishi, A. K.; Ross, D. D. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 15665−15670.

(4) Miyake, K.; Mickley, L.; Litman, T.; Zhan, Z.; Robey, R.; Cristensen, B.; Brangi, M.; Greenberger, L.; Dean, M.; Fojo, T.; Bates, S. E. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res.* **1999**, *59*, 8−13.

(5) Ahmed-Belkacem, A.; Pozza, A.; Macalou, S.; Perez-Victoria, J. M.; Di Pietro, A. Inhibitors of cancer cell multidrug resistance mediated by breast cancer resistance protein (BCRP/ABCG2). *Anticancer Drugs* **2006**, *17*, 239−243.

(6) Boumendjel, A.; Macalou, S.; Valdameri, G.; Pozza, A.; Gauthier, C.; Arnaud, O.; Nicolle, E.; Magnard, S.; Falson, P.; Terreux, R.; Carrupt, P.-A.; Payen, L.; Di Pietro, A. Targeting the multidrug ABCG2 transporter with flavonoidic inhibitors: in vitro optimization and in vivo validation. *Curr. Med. Chem.* **2011**, *18*, 3387−3401.

(7) Ahmed-Belkacem, A.; Pozza, A.; Munoz-Martinez, F.; Bates, S. E.; Castanys, S.; Gamarro, F.; Di Pietro, A.; Pérez-Victoria, J. M. Flavonoid structure-activity studies identify 6-prenylchrysin and tectochrysin as potent and specific inhibitors of breast cancer resistance protein ABCG2. *Cancer Res.* **2005**, *65*, 4852−4860.

(8) Nicolle, E.; Boccard, J.; Guilet, D.; Dijoux, M.-G.; Zelefac, F.; Macalou, S.; Grosselin, J.; Schmidt, J.; Carrupt, P.-A.; Di Pietro, A.; Boumendjel, A. Breast cancer resistance protein (BCRP/ABCG2): new inhibitors and QSAR studies by a 3D linear solvation-energy approach. *Eur. J. Pharm. Sci.* **2009**, *38*, 39−46.

(9) Nicolle, E.; Boumendjel, A.; Macalou, S.; Genoux, E.; Ahmed-Belkacem, A.; Carrupt, P.-A.; Di Pietro, A. QSAR analysis and molecular modeling of ABCG2-specific inhibitors. *Adv. Drug Pharm. Sci.* **2009**, *61*, 34−46.

(10) Bois, F.; Beney, C.; Boumendjel, A.; Mariotte, A.-M.; Conseil, G.; Di Pietro, A. Halogenated chalcones with high-affinity binding to P-glycoprotein: potential modulators of multidrug resistance. *J. Med. Chem.* **1998**, *41*, 161−164.

(11) Bois, F.; Boumendjel, A.; Mariotte, A.-M.; Conseil, G.; Di Pietro, A. Synthesis and biological activity of 4-alkoxychalcones: potential hydrophobic modulators of P-glycoprotein-mediated multidrug resistance. *Bioorg. Med. Chem.* **1999**, *7*, 2691−2695.

(12) Liu, X.-L.; Tee, H.-W.; Go, M.-L. Functionalized chalcones as selective inhibitors of P-glycoprotein and breast cancer resistance protein. *Bioorg. Med. Chem.* **2008**, *16*, 171−180.
(13) Han, Y.; Riwanto, M.; Go, M.-L.; Ee, P. L. R. Modulation of breast cancer resistance protein (BCRP/ABCG2) by non-basic chalcone analogues. *Eur. J. Pharm. Sci.* **2008**, *35*, 30−41.
(14) Juvale, K.; Pape, V. F. S.; Wiese, M. Investigation of chalcones and benzochalcones as inhibitors of breast cancer resistance protein. *Bioorg. Med. Chem.* **2012**, *20*, 346−355.
(15) Boumendjel, A.; McLeer-Florin, A.; Champelovier, P.; Allegro, D.; Muhammad, D.; Souard, F.; Derouazi, M.; Peyrot, V.; Toussaint, B.; Boutonnat, J. A novel chalcone derivative which acts as a microtubule depolymerising agent and an inhibitor of P-gp and BCRP in *in-vitro* glioblastoma models. *BMC Cancer* **2009**, *9*, 242.
(16) Rabindran, S. K.; He, H.; Singh, M.; Brown, E.; Collins, K. I.; Annable, T.; Greenberger, L. M. Reversal of a novel multidrug resistance mechanism in human colon carcinoma cells by fumitremorgin C. *Cancer Res.* **1998**, *58*, 5850−5858.
(17) van Loevezijn, A.; Allen, J. D.; Schinkel, A. H.; Koomen, G. J. Inhibition of BCRP-mediated drug efflux by fumitremorgin-type indolyl diketopiperazines. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 29−32.
(18) Allen, J. D.; van Loevezijn, A.; Lakhai, J. M.; van der Valk, M.; van Tellingen, O.; Reid, G.; Schellens, J. H. M.; Koomen, G. J.; Schinkel, A. H. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Mol. Cancer Ther.* **2002**, *1*, 417−425.
(19) Boumendjel, A.; Boccard, J.; Carrupt, P.-A.; Nicolle, E.; Blanc, M.; Geze, A.; Choisnard, L.; Wouessidjewe, D.; Matera, E. L.; Dumontet, C. Antimitotic and antiproliferative activities of chalcones: forward structure-activity relationship. *J. Med. Chem.* **2008**, *51*, 2307−2310.
(20) Martel-Frachet, V.; Kadri, M.; Boumendjel, A.; Ronot, X. Structural requirement of arylindolylpropenones as anti-bladder carcinoma cells agents. *Bioorg. Med. Chem.* **2011**, *19*, 6143−6148.
(21) Harborne, J. B.; Williams, C. A. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481−504.
(22) Kachadourian, R.; Day, B. J.; Pugazhenti, S.; Franklin, C. C.; Genoux-Bastide, E.; Mahaffey, G.; Gauthier, C.; Di Pietro, A.; Boumendjel, A. A synthetic chalcone as a potent inducer of glutathione biosynthesis. *J. Med. Chem.* **2012**, *55*, 1382−1388.
(23) Valdameri, G.; Genoux-Bastide, E.; Peres, B.; Gauthier, C.; Guitton, J.; Terreux, R.; Winnischofer, S. M. B.; Rocha, M. E. M.; Boumendjel, A.; Di Pietro, A. Substituted chromones as highly-potent, nontoxic inhibitors, specific for the breast cancer resistance protein ABCG2. *J. Med. Chem.* **2012**, *55*, 966−970.
(24) Valdameri, G.; Pereira Rangel, L.; Sapatafora, C.; Guitton, J.; Gauthier, C.; Arnaud, O.; Ferreira-Pereira, A.; Falson, P.; Winnischofer, S. M. B.; Rocha, M. E. M.; Tringali, C.; Di Pietro, A. Methoxy stilbenes as potent, specific, untransported and noncytotoxic inhibitors of breast cancer resistance protein. *ACS Chem. Biol.* **2012**, *7*, 322−330.
(25) Boumendjel, A.; Di Pietro, A.; Dumontet, C.; Barron, D. Recent advances in the discovery of flavonoids and analogs with high-affinity binding to P-gp responsible for cancer cell multidrug resistance. *Med. Res. Rev.* **2002**, *22*, 512−529.
(26) Macalou, S.; Ahmed-Belkacem, A.; Borelli, F.; Capasso, R.; Fattorusso, E.; Taglialatela-Scafati, O.; Di Pietro, A. Non-prenylated rotenoids, a new class of potent breast cancer resistance protein inhibitors. *J. Med. Chem.* **2007**, *50*, 1933−1938.
(27) Boumendjel, A.; Macalou, S.; Ahmed-Belkacem, A.; Blanc, M.; Di Pietro, A. Acridone derivatives: design, synthesis, and inhibition of breast cancer resistance protein ABCG2. *Bioorg. Med. Chem.* **2007**, *15*, 2892−2897.
(28) Arnaud, O.; Boumendjel, A.; Gèze, A.; Honorat, M.; Matera, E. L.; Guitton, J.; Stein, W. D.; Bates, S. E.; Falson, P.; Dumontet, C.; Di Pietro, A.; Payen, L. The acridone derivative MBLI-87 sensitizes breast cancer resistance protein-expressing xenografts to irinotecan. *Eur. J. Cancer* **2011**, *47*, 640−648.
(29) Robey, R. W.; Honjo, Y.; Van de Laar, A.; Miyake, A.; Regis, J. T.; Litman, T.; Bates, S. E. A functional assay for detection of the mitoxantrone resistance protein, MXR (ABCG2). *Biochim. Biophys. Acta: Biomembranes* **2001**, *1512*, 171−182.
(30) Mosmann, T. Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cyto-toxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.
(31) SYBYL-X 1.3, Tripos International, St. Louis, MO 63144, USA.
(32) Halgren, T. A. MMFF VII. Characterization of MMFF94, MMFF94s, and other widely available force fields for conformational energies and for intermolecular-interaction energies and geometries. *J. Comput. Chem.* **1999**, *7*, 730−748.
(33) Cramer, R. D., III; DePriest, S. A.; Patterson, D. E.; Hecht, P. *The Developing Practice of Comparative Molecular Field Analysis in 3D QSAR in Drug Design: Theory, Methods and Applications*; ESCOM: Leiden, The Netherlands, 1993.
(34) Picard, R.; Cook, D. Cross-validation of regression models. *J. Am. Stat. Assoc.* **1984**, *79*, 575−583.