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Methoxy Stilbenes as Potent, Specific, Untransported, and Noncytotoxic Inhibitors of Breast Cancer Resistance Protein

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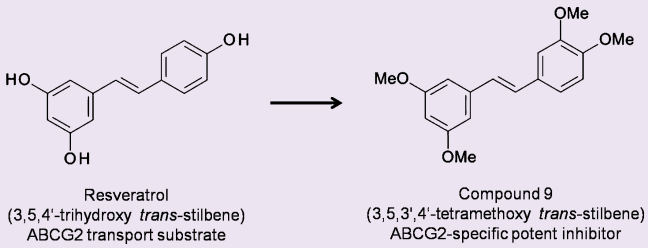
ABSTRACT: The ABCG2 multidrug transporter is known to confer cancer cell multidrug resistance by causing the efflux of anticancer drugs; therefore, selective inhibitors have the potential to improve chemotherapeutic treatments. Here, various methoxy derivatives of resveratrol are shown to be potent inhibitors of mitoxantrone efflux by ABCG2: among a series of 11 derivatives, compound **9** (3,5,3′,4′-tetramethoxy *trans*-stilbene) had an IC_{50} of 0.16 μM and showed a maximal inhibition of 75%, as measured by flow cytometry. It was not transported, as shown by HPLC fractionation and mass spectrometry titration and the lack of any cross-resistance in cell survival experiments. Compound **9** had a very low intrinsic cytotoxicity and was able to chemosensitize the growth of resistant *ABCG2*-transfected HEK293 cells at submicromolar concentrations. Drug-efflux inhibition was specific for ABCG2 since very low effects were observed with ABCB1 and ABCC1. The action mechanism of compound **9** was different from that of GF120918, which produced a complete and partly competitive but not ABCG2-specific inhibition, since ABCB1 was even more strongly inhibited. The two inhibitors also displayed different effects on the ABCG2 vanadate-sensitive ATPase activity, suggesting that they either bound to distinct sites or induced different conformational changes. Mitoxantrone efflux was fully inhibited by combining low concentrations of compound **9** with either GF120918 or a transport substrate such as prazosin or nilotinib. We conclude that methoxy derivatives of stilbene are good candidates for investigating future *in vivo* modulation of ABCG2 drug-efflux activity.



The ability of cancer cells to acquire resistance to anticancer drugs, a process termed "multidrug resistance (MDR) phenotype", is a main obstacle to chemotherapeutic treatments. The most frequent MDR mechanism is related to the overexpression of multidrug ABC ("ATP-binding cassette") transporters within plasma membranes that alters the efficiency of chemotherapeutics by lowering their intracellular concentration.[1]

Among the main ABC transporters found to be overexpressed in resistant cancer cells, ABCG2, also called ABCP for its abundance in placenta,[2] BCRP ("breast cancer resistance protein"),[3] or MXR ("mitoxantrone resistance protein"),[4] is the most recently discovered. Earlier studies revealed the existence of ABCB1/MDR1/P-glycoprotein and ABCC1/MRP1 ("multidrug resistance protein 1). The clinical relevance of ABCG2 was demonstrated in acute myeloid leukemia,[5,6] and it is clearly involved in drug bioavailability at protection barriers.[7,8] ABCG2, which transports sulfated steroids,[9] pheophorbide a,[10] and other porphyrins[11] as physiological substrates, is also able to catalyze the efflux of a large panel of drugs including topotecan, irinotecan, mitoxantrone, prazosin, and methotrexate. Other pharmacologically important compounds are also transported, including the polyphenol resveratrol,[12] which has been reported to provide both antioxidant, chemopreventive, antiinflammatory, antiaging, cardioprotective, and neuroprotective activities.[13,14]

Known inhibitors of ABCG2 belong to various classes of compounds. Nonselective, dual inhibitors already identified as ABCB1 inhibitors include GF120918/elacridar,[15] taxoids,[16] and XR9576/tariquidar.[17] Other inhibitors, such as tyrosine kinase inhibitors,[18] cyclosporin A,[19] and curcumin,[20] are transported and also interact with ABCC1. A few selective ABCG2 inhibitors have been identified, including fumitremorgin C from *Aspergillus fumigatus*,[21] which was however highly

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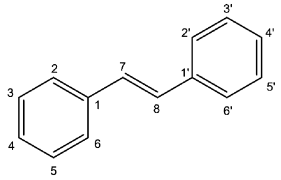
Table 1. Structures of Methoxy *trans*-Stilbenes



compound	OMe number	C2	C3	C4	C5	C3′	C4′	C5′
1	1	-	-	-	-	-	OMe[a]	-
	or	-	-	OMe[a]	-	-	-	-
2	2	-	OMe	-	OMe	-	-	-
3	3	OMe	OMe	-	-	-	OMe	-
4	3	-	OMe	-	OMe	-	OMe	-
5	3	-	OMe	OMe	-	-	OMe	-
6	4	OMe	OMe	-	OMe	-	OMe	-
7	4	-	OMe	OMe	OMe	-	OMe	-
8	4	-	OMe	-	OMe	OMe	-	OMe
9	4	-	OMe	-	OMe	OMe	OMe	-
10	5	-	OMe	OMe	OMe	OMe	-	OMe
11	5	OMe	OMe	-	OMe	OMe	-	OMe

[a]Because of the symmetry of compound **1** and for reasons of comparability, here are shown two alternative structures; the exact numbering should be 4.

Table 2. Inhibitory Activity of Methoxy Stilbenes on Multidrug ABC Transporters[a]

	ABCG2-transfected HEK293 cells			*ABCB1*-transfected HEK293 cells	*ABCC1*-transfected HEK293 cells
compound	inhibition (%) 1 μM	inhibition (%) 5 μM	IC_{50} (μM)	inhibiton (%) 5 μM	
GF120918	nd	100.0	0.120 ± 0.03	100.0[b]	nd
1	none	5.1 ± 0.1		−7.3 ± 0.6	2.5 ± 0.4
2	5.5 ± 2.7	9.9 ± 3.7		−12.3 ± 0.5	−2.2 ± 0.5
3	none	6.6 ± 0.9		−14.3 ± 0.4	4.5 ± 1.6
4	13.7 ± 5.4	36.2 ± 4.6		−15.9 ± 1.0	−2.1 ± 0.1
5	11.9 ± 0.14	58.5 ± 21.6		−19.2 ± 0.3	6.4 ± 7.2
6	8.8 ± 1.9	32.5 ± 12.2		−23.5 ± 0.4	−2.4 ± 1.7
7	20.2 ± 4.9	55.7 ± 12.1		−19.8 ± 1.5	4.6 ± 4.0
8	48.8 ± 3.6	67.4 ± 8.6	0.47 ± 0.08	−12.5 ± 12.6	3.2 ± 2.9
9	65.2 ± 7.2	75.6 ± 7.9	0.16 ± 0.04	−14.2 ± 13.6	2.7 ± 1.7
10	57.1 ± 9.7	76.7 ± 10.5	0.29 ± 0.09	−9.4 ± 11.0	1.1 ± 2.6
11	12.2 ± 3.3	34.2 ± 8.5		−16.9 ± 2.3	2.3 ± 1.4

[a]Inhibition of mitoxantrone efflux in *ABCG2*-transfected cells (R482 wild-type). For *ABCG2*-transfected cells, the percent inhibition was determined using GF120918 as a control (100% inhibition). The values of (%) inhibition and IC_{50} (half-maximal inhibition) were determined by flow cytometry as described in Methods. Data are the mean ± SD of at least three independent experiments. For *ABCC1*-transfected cells, the percent inhibition was normalized using cells transfected with empty pcDNA3.1 as a control. [b]For *ABCB1*-transfected cells, the percent inhibition was determined using GF120918 as a control (100% inhibition); its IC_{50} value was 0.034 ± 0.01 μM).

neurotoxic, and Ko143,[22] one of several synthetic and less cytotoxic derivatives. Some analogues of XR9576[23] and of GF120919[24] were found to be ABCG2-specific but they also rather cytotoxic, and their *in vivo* activity was limited by their bioavailability.[25] We identified a flavonoid-binding site interacting with tectochrysin and 6-prenylchrysin, which generated up to 75% inhibition with good affinity but however again exhibited significant cytotoxicity.[26] Lower-affinity rotenoids[27] and acridone derivatives[24] bound to the same site as flavones and benzo-pyrane/furane derivatives, for which 3D models exist.[28,29]

The aim of the present study was to identify new, more potent, and less cytotoxic compounds than 6-prenylchrysin. Methoxy stilbenes were found effective for inhibition without inducing cytotoxicity, suggesting that these molecules could be used in combination with other inhibitors to block ABCG2 drug-efflux activity.

RESULTS AND DISCUSSION

Methoxy Derivatives of Stilbene As Inhibitors of ABCG2-Mediated Drug Efflux. Resveratrol, or 3,5,4′-trihydroxy-*trans*-stilbene, has been reported to be transported by ABCG2[12] and to exhibit low affinity inhibition of the efflux of other drugs.[30] Replacing the hydroxyl groups of resveratrol by methoxy groups led to more potent inhibitors, as shown by a series of 11 derivatives containing a variable number of methoxy groups at different positions on the two aromatic rings (Table 1), when analyzed by flow cytometry using a mitoxantrone-efflux assay. The results (Table 2) showed that the increased intracellular accumulation of mitoxantrone, corresponding to inhibition of mitoxantrone efflux, depended on the number of methoxy substituents, from 1 to 4, and on their positions. The highest efficiency of mitoxantrone-efflux inhibition was obtained with compound **9**, characterized by a low IC_{50} (concentration giving a half-maximal inhibition) of

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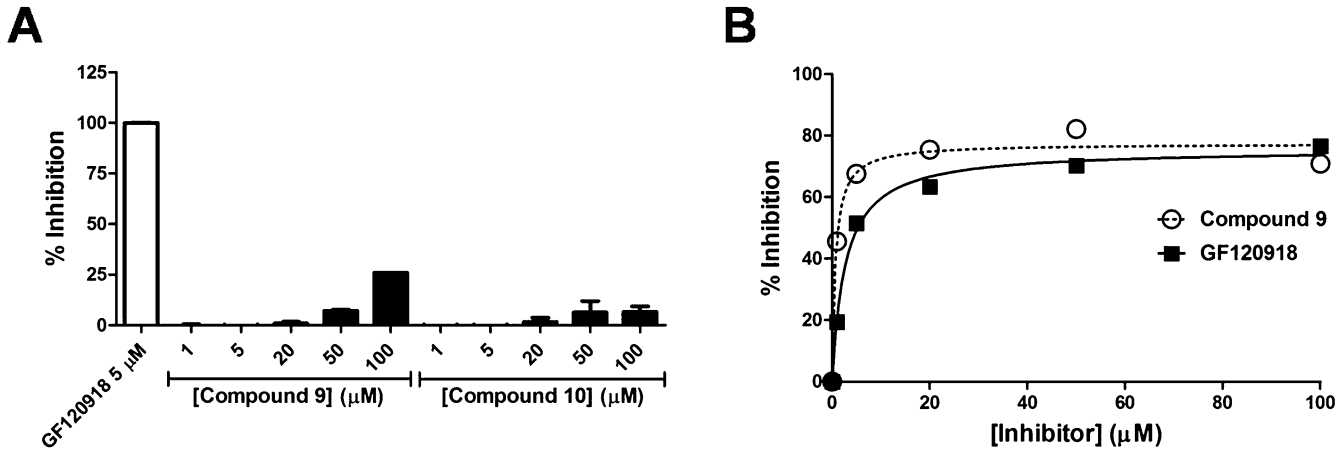

Figure 1. Inhibition of rhodamine 123 efflux in HEK293 cells transfected by either wild-type ABCB1 or R482T ABCG2 constructs. (A) Effects of compounds **9** and **10** on ABCB1-mediated efflux. The percent inhibition was determined by flow cytometry, using GF120918 as a control (100% inhibition). The data are the mean ± SD of two independent experiments. (B) Effects of compound **9** (○) and GF120918 (■) on R482T-ABCG2-mediated efflux. The percent inhibition was determined by flow cytometry and normalized using cells transfected with empty pcDNA3.1 as a control. Data are the mean ± SD of two independent experiments.

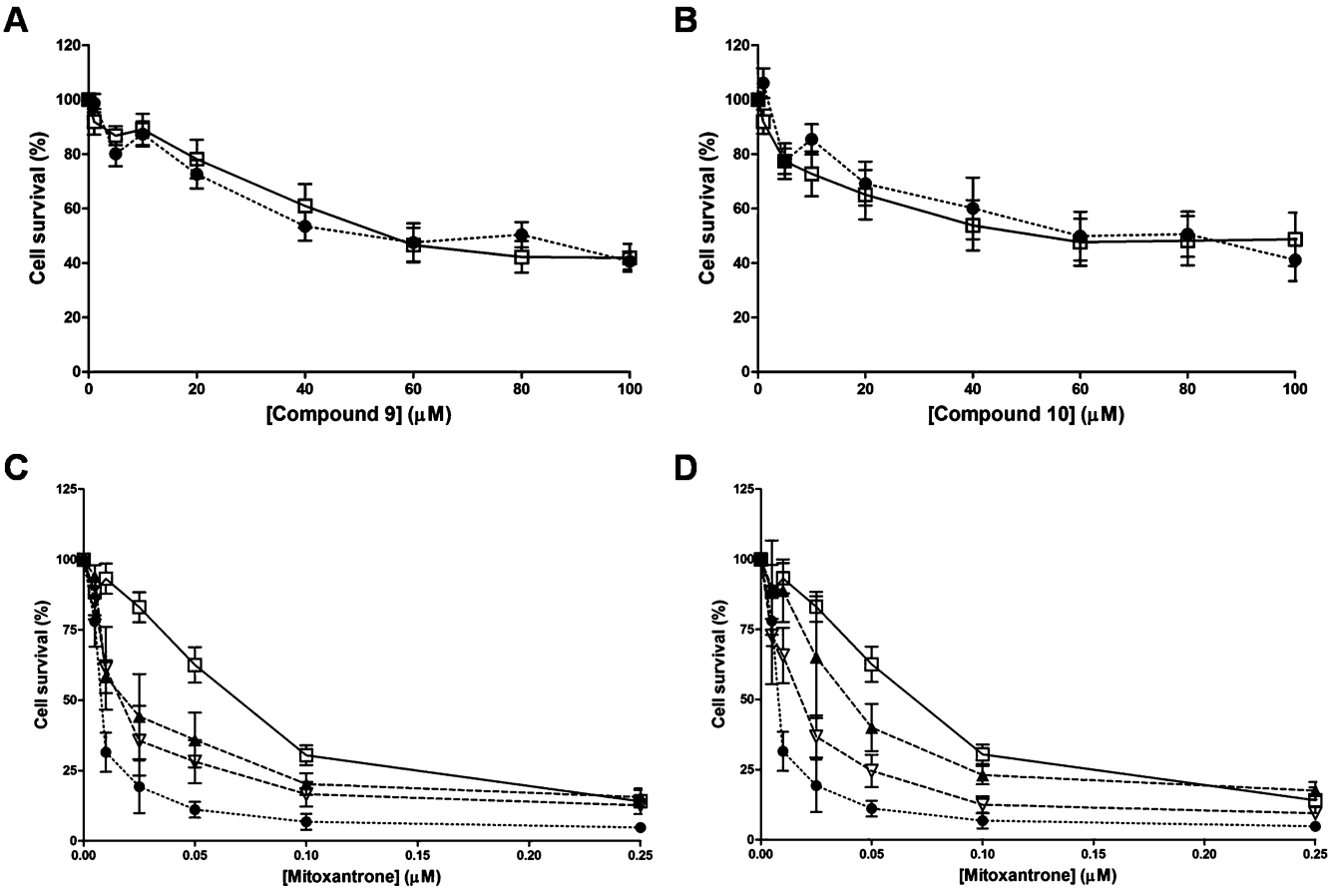

Figure 2. Cytotoxicity of compounds **9** and **10** and sensitization to mitoxantrone. Cell survival was determined by MTT assays as described in Methods. (A, B) Cell viability of HEK293-*ABCG2 (R482 wild-type)* (□) and HEK293-*pcDNA3.1* control cells (●) upon 72-h treatment with either compound **9** or compound **10** at increasing concentrations, as indicated. (C, D) Cell viability of HEK293-*ABCG2* and HEK293-*pcDNA3.1* cells upon co-treatment with compound **9** (C) or compound **10** (D) at either 0.2 μM (▲) or 1 μM (▽) and mitoxantrone (0−0.25 μM) for 72 h. Parallel experiments with only mitoxantrone were performed with *ABCG2*-transfected (□) and control (●) 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.

0.16 μM, a value comparable to that obtained with the reference inhibitor GF120918. Methoxy substitution at position 4′ was important since a 3-fold decrease in affinity was observed when the methoxy group was shifted to position 5′ in compound **8** (IC_{50} = 0.47 μM); this may also explain the lower efficiency of compound **10** (IC_{50} = 0.29 μM), despite the

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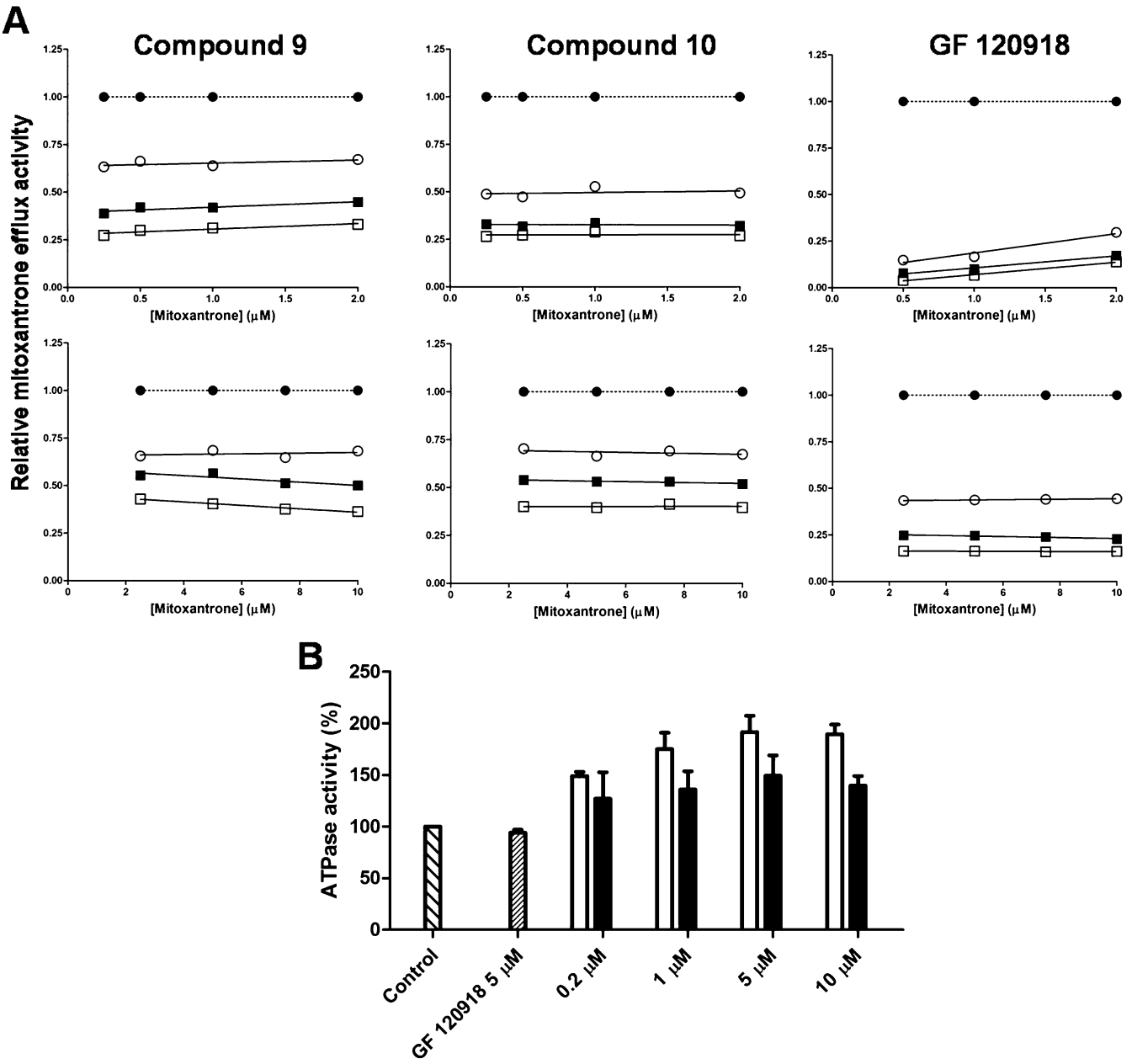

Figure 3. Different mechanism of compounds **9** and **10** in comparison with GF120918. (A) Different types of inhibition produced by compound **9**, compound **10**, and **GF120918** at (●) 0 μM, (○) 0.1 μM, (■) 0.5 μM, and (□) 2 μM on ABCG2-mediated mitoxantrone efflux, as measured at low concentrations (upper panels) and high concentrations (lower panels) of the substrate mitoxantrone. (B) Stimulation by compound **9** (white bars) and compound **10** (black bars) of vanadate-sensitive ABCG2 ATPase activity, by contrast to the lack of GF120918 effects. The data represent three independent experiments performed in triplicate.

presence of an additional methoxy group at position 4 with an improved inhibition. By contrast, substitution at position 2 in compound **11** (and compound **6**) was clearly disadvantageous, compared to substitution at position 4 in compound **10** (and compound 7). The negative effect of C-2 methoxy substitution was also confirmed by the poor inhibition of compound **3** (in comparison with compounds **4** and **5**) and of compound **11** (in comparison with compound **8**). In addition to position 4′, the position 3′ of the right aromatic ring needed to be substituted since, at 5 μM each, compound **4** was much less active than compound **9**, and compound **7** was less active than compound **10**. The advantageous effect of 4- (*versus* 5-) substitution was also found in compound **5** by comparison to compound **4**, at 5 μM each.

The high inhibition potency of compound **9** against mitoxantrone efflux has a 2-fold higher affinity than 6-prenylchrysin (IC_{50} of 0.29 μM), which was previously characterized by our group as a good inhibitor lead compound.[26] The fact that the hydroxylated transport substrate resveratrol was converted into the potent inhibitor compound **9** by complete substitution with methoxy groups was consistent with our previous observations that methoxylation increases both inhibition efficiency and binding affinity of flavones, when comparing tectochrysin to chrysin.[26]

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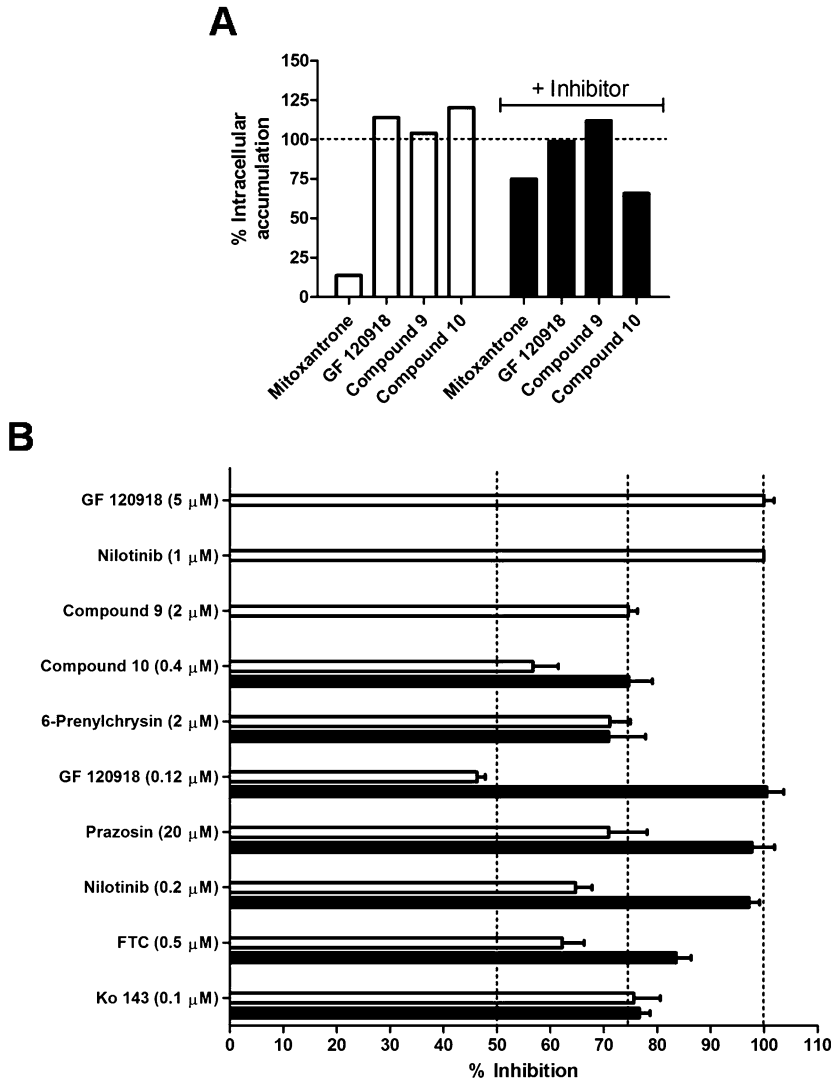

Figure 4. Absence of transport of different inhibitors, in contrast to substrates, and bimodulation of mitoxantrone efflux. (A) All compounds were tested at 2 μM, and their percent intracellular accumulation was determined by HPLC−MS and normalized using *pcDNA3.1*-transfected cells as a control (100%). GF120918 (5 μM) was used to inhibit the transport of mitoxantrone, prazosine, and compounds **9** and **10**, whereas Ko143 (0.3 μM) was used to inhibit the transport of GF120918. The initial amounts of compounds found in control cells were the following: compound **9** (1.94 ng/mL), compound **10** (2.2 ng/mL), mitoxantrone (107 ng/mL), and GF120918 (101 ng/mL). (B) Bimodulation was assayed by flow cytometry as above. The compounds were used either alone at the indicated concentration (white bars) or in combination with 2 μM compound **9** (black bars). Data are the mean ± SD of four independent experiments.

The drug-efflux inhibition by these methoxy derivatives appears to be quite specific for ABCG2, since no inhibition of either ABCB1-mediated mitoxantrone efflux or ABCC1-mediated calcein efflux was observed with any compound, at least up to a 5 μM concentration. By contrast, the reference inhibitor GF120918 was even more potent toward ABCB1 (IC_{50} = 0.034 μM) than toward ABCG2 (IC_{50} = 0.120 μM). The specificity of the compounds was also studied with respect to the efflux of another usual substrate, namely rhodamine 123. Figure 1A shows that the GF120918-sensitive efflux of rhodamine 123 by ABCB1 required very high concentrations, up to 100 μM of compounds **9** and **10**, to be partly inhibited (25% and 10%, respectively), in agreement with other data published for compound **9**.[31] Rhodamine 123 efflux could also be studied using the R482T ABCG2 mutant: a maximum of 75% inhibition was then obtained at 10−20 μM, with an IC_{50} value of 0.45 μM (Figure 1B). Interestingly, the maximal inhibition produced by GF120918 was not complete and GF120918 showed an affinity lower than that of compound **9** (IC_{50} of 2.7 μM). This contrasts with the inhibition of mitoxantrone efflux by wild-type ABCG2 reported in Table 2, suggesting that GF120918 and methoxy stilbenes may use different mechanisms to inhibit ABCG2-mediated drug efflux.

Figure 2A,B shows the effects of the two most potent methoxy stilbenes on cell growth, as determined by a cell-survival MTT assay. Compounds **9** and **10** appeared to have very low intrinsic cytotoxicity (around 10% and 20%, respectively, at 10 μM). In both cases, their IG_{50} (concentration producing a 50% inhibition of cell growth) value was ≥60 μM, giving a high therapeutic ratio IG_{50}/IC_{50} of 200−400 for cytotoxicity *versus* inhibition of drug efflux. In addition, the same curves obtained with either *ABCG2*-transfected cells or control cells indicated no apparent cross-resistance, suggesting that the compounds were not transported by ABCG2. By contrast, GF120918 was more cytotoxic on control cells (IG_{50} around 20 μM, not shown here) and produced cross-resistance

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at concentrations higher than 5 μM, suggesting that GF120918 might be transported at high concentrations. Both methoxy stilbenes sensitized the growth of *ABCG2*-transfected cells to mitoxantrone (Figure 2C,D), with a stronger effect produced by compound **9**, in comparison to compound **10** at submicromolar concentration, in agreement with its higher affinity to inhibit mitoxantrone efflux (*cf.* Table 2).

Different Molecular Mechanisms of ABCG2-Specific Methoxy Stilbenes and the Dual Inhibitor GF120918. The difference in maximal inhibition of both types of inhibitors on mitoxantrone efflux (around 75% *versus* 100% at 10 μM mitoxantrone, *cf.* Table 2) justified the further investigation of the inhibition mechanism. When the mitoxantrone concentration varied from 0.5 to 10 μM, Lineweaver–Burk double-reciprocal plots showed a downward curvature, indicating a negative cooperativity. A Hill number of 0.7 was calculated, suggesting the involvement of at least two interacting mitoxantrone-transporting sites within a functional ABCG2 oligomer (not shown here). The type of inhibition was therefore studied at both low and high mitoxantrone concentrations (Figure 3A). For compounds **9** and **10**, the inhibition always appeared to be noncompetitive, since the degree of inhibition only depended on the inhibitor concentration and not on the substrate concentration (at either low or high concentrations). By contrast, the GF120918 inhibition appeared to be partly competitive at low mitoxantrone concentrations, for each inhibitor concentration, since the extent of inhibition was stronger at low *versus* high substrate concentrations (between 0.5 and 2 μM); however, at higher substrate concentrations (2.5–10 μM) the inhibition appeared to be noncompetitive. The partially competitive inhibition of GF120918 at low mitoxantrone concentrations was confirmed by the fact that the IC_{50} for GF120918 was clearly lower at low substrate concentrations. This was not the case for either compounds **9** or **10**. This suggests that the slight concentration-dependence observed for compound **9** at low concentrations is not significant and that both methoxy *trans*-stilbenes were noncompetitive inhibitors.

Figure 3B shows that the effects produced by the different compounds on ABCG2 ATPase activity were also quite distinct. Indeed, while GF120918 at 5 μM did not display any effect, both methoxy stilbenes produced a strong stimulation of vanadate-sensitive ATP hydrolysis, up to 200% for compound **9**, and 150% for compound **10**, at the same, low, concentrations that inhibit mitoxantrone efflux.

The specificity of compounds **9** and **10** for ABCG2 is consistent with their noncompetitive inhibition indicating a binding site distinct from the transport site. This transport site is expected to have many common features with ABCB1 since both transporters efflux a number of common substrates including mitoxantrone, Hoechst 33342, and prazosine. The interaction at this transport site was more sensitive to the R482T mutation than the interaction at the ABCG2-specific site, as monitored here by the relative loss in efficiency against rhodamine 123 and mitoxantrone efflux.[26] Methoxy stilbenes probably bind to the same site as 6-prenylchrysin, since both types of ABCG2-specific inhibitors display the same partial inhibition (around 75%), possibly due to their rather small size. Also the combined action of the two compounds does not increase this maximal inhibition. Such a specific site, which is able to bind other natural products such as rotenoids [27] and acridones,[24] was characterized in detail and allowed the elaboration of a molecular model for typical inhibitors.[28,29]

Absence of Transport of ABCG2 Inhibitors, and Combination toward a Complete Modulation of Mitoxantrone Efflux. The two methoxy stilbenes, compounds **9** and **10**, were not transported in *ABCG2*-transfected cells at 2 μM, a saturating inhibition concentration, when assayed by HPLC separation and mass spectrometry titration (Figure 4A). By contrast, the positive control with mitoxantrone showed an 80% efflux, which was largely blocked in the presence of GF120918 acting as an ABCG2 inhibitor. As expected, addition of GF120918 did not modify accumulation of compound **9**, although it produced some decrease in that of compound **10**. Transport was also observed with the other substrate prazosin (not shown here). GF120918 itself was not transported when assayed at 2 μM by the same procedure, and no transport was observed with 6-prenylchrysin (not shown here), another ABCG2-specific inhibitor that we previously characterized.[26] The lack of transport of methoxy stilbenes agrees with their noncompetitive inhibition. In contrast, the dual inhibitor GF120918, which fully inhibits mitoxantrone efflux with high affinity, appears able to be transported at high (but not low) concentrations, in agreement with its partially competitive inhibition. This suggests that its binding site partly overlaps with the mitoxantrone binding/transport site.

Since the methoxy stilbenes were not transported, in common with GF120918 at low concentration, we checked the possibility of using these compounds together since they apparently act with different mechanisms and probably bind to distinct sites, in order to reach a complete inhibition at low inhibitor concentrations. Figure 4B shows the effects of different compounds in combination with compound **9**. In agreement with the data of Table 2, compound **9** alone at 2 μM produced a maximal inhibition of 75%, significantly lower than the 100% inhibition produced by 5 μM GF120918. Interestingly, the combination of compound **9** with either 0.4 μM of compound **10** or 2 μM 6-prenylchrysin (a saturating inhibition concentration) did not further increase the maximal inhibition of compound **9** alone, suggesting that the three compounds might bind to the same site. In contrast, combination with non-saturating concentrations of either GF120918 (0.12 μM) (a partly competitive inhibitor that appeared to be transported at high concentrations) or one of the two transported substrates prazosin and nilotinib[32] led to complete inhibition, consistent with the fact that both GF120918 and nilotinib alone at saturation (5 μM and 1 μM, respectively) produced a complete inhibition. An intermediate situation was observed with another ABCG2-specific inhibitor, fumitremorgin C (FTC), which partly increased the inhibition.

Methoxy stilbenes thus appear to be useful for modulating ABCG2 drug-efflux activity in two alternative ways: either alone, if 75% inhibition is sufficient to increase anticancer drug bioavailability and toxicity (with the advantage of not fully inhibiting the physiological transport activity in normal cells), or in combination with other inhibitors, such as GF120918 or a transport substrate such as prazosin or nilotinib[32] (as shown here, but probably as well with other substrates such as curcumin[33]) with the advantage of yielding complete inhibition at low concentrations and thus limiting side effects.

These new inhibitors are characterized by a very low cytotoxicity, in contrast with 6-prenylchrysin,[26] and provide a 10-fold higher therapeutic ratio (200–400 *versus* 20–40). In addition, at low concentrations these compounds chemosensitize cell growth to mitoxantrone, indicating that no major intracellular metabolization appears to occur. This is not the case with curcumin, which is

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easily metabolized.[34] Methoxy stilbenes therefore appear to be good candidates for *in vivo* experiments and are expected to be more efficient than previously used compounds.[24,25,35,36]

METHODS

Materials. Mitoxantrone, rhodamine 123, 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.

The ^{1}H spectra were run in $CDCl_3$ using a Varian Unity Inova spectrometer operating at 500 MHz. Mass spectra were recorded in ESI positive mode on a Micromass ZQ2000 spectrometer (Waters). All reactions were monitored by TLC on commercially available precoated plates (silica gel 60 F_{254}), and the products were visualized with cerium sulfate solution; silica gel 60 (25−40 μm) was employed as stationary phase for preparative column flash chromatography.

The previously reported *trans*-4-methoxystilbene (compound **1**),[37] *trans*-3,5-dimethoxystilbene (compound **2**),[38] *trans*-2,3,4′-trimethoxystilbene (compound **3**),[39] *trans*-3,5,4′-trimethoxystilbene (compound **4**),[40] *trans*-3,4,4′-trimethoxystilbene (compound **5**),[41] *trans*-3,4,5,4′-tetramethoxystilbene (compound **7**),[42] *trans*-3,5,3′,5′-tetramethoxystilbene (compound **8**),[40] *trans*-3,5,3′,4′-tetramethoxystilbene (compound **9**),[40] and *trans*-3,4,5,3′,5′-pentamethoxystilbene (compound **10**)[43] were synthesized according to a general protocol based on an Arbuzov rearrangement followed by the Horner−Emmons−Wadsworth reaction; the spectral data of the isolated products were in perfect agreement with the literature; the ^{1}H NMR data of compound **10**, which were not previously reported, are the following: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.84 (s, 6H, -OCH_3), 3.88 (s, 3H, -OCH_3), 3.92 (s, 6H, 3-OCH_3), 6.41 (t, *J* = 2.0, 1H, H-4′), 6.67 (d, *J* = 2.0, 2H, H-2′ and H-6′), 6.74 (s, 2H, H-2 and H-6), 6.95 (d, *J* = 16.5, 1H, H-7), 7.02 (d, *J* = 16.5, 1H, H-8).

The previously unreported compounds *trans*-2,3,5,4′-tetramethoxystilbene (compound **6**) and *trans*-2,3,5,3′,5′-pentamethoxystilbene (compound **11**) were synthesized in two steps: (i) a mild *m*-CPBA direct aromatic hydroxylation, according to a previous work of some of us, which afforded the intermediates 2-hydroxy-3,5,4′-trimethoxystilbene and 2-hydroxy-3,5,3′,5′-tetramethoxystilbene; (ii) subsequent methylation with dimethyl sulfate to obtain the final products. A solution of *m*-CPBA in CH_2Cl_2 (0.150 mmol/mL) was added to a stirred solution of the substrate in CH_2Cl_2 (0.105 mmol/mL) at RT. The reaction mixture was then washed with a $NaHSO_3$ solution and subsequently with saturated aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4), filtered, and concentrated *in vacuo*; the residue was submitted to flash chromatography on a 3 cm × 25 cm silica gel column, eluted with EtOAc in *n*-hexane (from 0% to 30%) to give the hydroxylated stilbenoids; these intermediates (0.107 mmol) were submitted to methylation using dimethyl sulfate (15 μL) in acetone (20 mL) in presence of anhydrous potassium carbonate (0.107 mmol).

***trans*-2,3,5,4′-Tetramethoxystilbene (6).** ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.78 (s, 3H, OCH_3), 3.84 (s, 6H, OCH_3), 3.86 (s, 3H, -OCH_3), 6.43 (d, *J* = 3.0, 1H, H-4) 6.69 (d, *J* = 3.0, 1H, H-6), 6.91 (d, *J* = 8.5, 2H, H-3′ and H-5′), 7.11 (d, *J* = 16.5, 1H, H-a), 7.32 (d, *J* = 16.5, 1H, H-8), 7.50 (d, *J* = 8.5, 2H, H-2′ and H-6′).

***trans*-2,3,5,3′,5′-Pentamethoxystilbene (11).** ^{1}H NMR ($CDCl_3$, 500 MHz) δ 3.79 (s, 3H, -OCH_3), 3.84 (s, 9H, -OCH_3), 3.86 (s, 3H, -OCH_3), 6.41 (t, *J* = 2.0, 1H, H-4′), 6.45 (d, *J* = 2.0, 1H, H-4), 6.69 (d, *J* = 2.0, 1H, H-6), 6.70 (d, *J* = 2.0, 2H, H-2′ and H-6′), 7.02 (d, *J* = 16.5, 1H, H-7), 7.41 (d, *J* = 16.5, 1H, H-8).

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. Human fibroblast HEK293 cell lines transfected with either wild-type or mutant *ABCG2* (HEK293-*R482* cells) or the empty vector (HEK293-*pcDNA3.1* cells) were obtained as previously.[25] HEK293 cell lines transfected with either *MDR1* or *MRP1* were kindly provided by Dr. S. E. Bates (NCI, NIH, Bethesda, MD). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM high glucose), supplemented with 10% fetal bovine serum (FBS), 1% penicilin/streptomycin, and drug supplemented in some cases with either 0.75 mg mL^{-1} G418 (HEK293-*pcDNA3.1*, HEK293-*R482* or *R482T*), 2 mg mL^{-1} G418 (HEK293-*MDR1*), or 5 μM etoposide (HEK293-*MRP1*).

ABCG2- and ABCB1-Mediated Drug Transport. HEK293 cells were seeded at a density of 1 × 10^5 cells/well into 24-well culture plates and, after 48 h, exposed to 10 μM mitoxantrone (HEK293-*R482* and HEK293-*MDR1* cells) or 10 μM rhodamine 123 (HEK293-*R482T* and HEK293-*MDR1* cells) for 30 min at 37 °C, in the absence or presence of compounds at various concentrations. After cell washing with phosphate buffered saline (PBS) and incubation for 1 h in substrate-free medium with compounds at the same concentrations, the intracellular drug fluorescence was monitored with a FACS Calibur cytometer (Becton Dickinson). At least 10,000 events were collected, for which the maximal fluorescence (taken as 100%) was the difference between the geometric mean fluorescence of cells incubated with 5 μM GF120918 and cells without inhibitor. The Hill number of mitoxantrone efflux in HEK293 cells transfected by either wild-type ABCG2 or R482T ABCG2 contructs was determined from the curves (sigmoidal -3 parameters) fitted in the SigmaPlot 11.0 software and calculated by the equation $f = ax^b/(c^b + x^b)$, where *b* is the Hill number.

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

Cytotoxicity Assays. HEK293-*R482* and HEK293-*pcDNA3.1* cells were seeded into 96-well culture plates at a 1 × 10^4 cells/well density. After overnight incubation, the cells were treated with various concentrations of compounds **9** and **10** for 72 h at 37 °C under 5% CO_2. For the sensitization experiments, after overnight incubation, the cells were concomitantly treated with compound **9** or compound **10** and increasing concentrations of mitoxantrone for 72 h at 37 °C under 5% CO_2. In both cases, cell viability was evaluated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay. Control experiments were performed with high glucose DMEM containing 0.1% of DMSO (v/v). The results were expressed as percentage of viable cells *versus* control cells taken as 100%.

ATPase Activity. ATPase activity was determined as previously[44] by quantifying the release of inorganic phosphate from ATP by a colorimetric assay. Membranes from HEK293-*R482* cells (10 μg proteins) were incubated for 30 min at 37 °C in 50 mM NaCl, 50 mM Tris pH 7.5 buffer supplemented with 5 mM ATP, 5 mM NaN_3, 7 mM $MgCl_2$, 0.1 mM EGTA, 2 mM oubain, the effectors (compound **9**, compound **10** and GF120918) with or without vanadate 0.6 mM, at a final volume of 60 μL. After incubation, the reaction was stopped by adding 30 μL of 10% SDS for 10 min at 4 °C and then 180 μL of the solution B: 5 mL of solution A (2.16 g ammonium molybdate dissolved in 35 mL of 15 mM zinc acetate) dissolved in 20 mL of freshly prepared 20 μM ascorbic acid pH 5.0. After 20-min incubation at 37 °C, the released phosphate was colorimetrically quantified at 620 nm.

Transport by HPLC and Mass Spectrometry. HEK293-*R482* and HEK293-*pcDNA3.1* cells were seeded at a density of 5 × 10^5 cells/well into 6-well culture plates. After 48-h incubation, cells were treated with the compounds for 30 min at 37 °C under 5% CO_2, then washed with PBS, and trypsinized. They were suspended in 1 mL of PBS and centrifuged at 1,000 × *g* for 5 min. The pellet was suspended in 1 mL of PBS, and 50 μL was removed for protein quantification by the BCA method. Cells were again centrifuged and suspended in 50 μL of methanol and stored at −80 °C until further analysis.

Intracellular quantification of the different compounds was performed using a triple quadrupole tandem mass spectrometer with an electrospray source coupled to a liquid chromatography (LC−ESI-

MS/MS) from ThermoFisher. The data were acquired using the positive ion mode. The SRM transitions were m/z 384.3 → 247.2 for prazosin, 445.5 → 88.0 for mitoxantrone, 323.0 → 267.0 for 6-phenylchrysin, 564.0 → 252.0 for GF120918, 331.0 → 177.2 for compound **10**, 301.1 →146.2 for compound **9**, and 349.0 → 305.0 for camptothecin used as internal standard. The chromatographic separation was achieved on HypersilGold 100 mm × 2.1 mm column (ThermoFisher, USA). A first method was used for HPLC analysis of compounds **9** and **10**, using a mobile phase constituted by ammonium acetate buffer (pH 6, 50 mM), propanol-2, and acetonitrile. A second method was performed for the other compounds with a mobile phase constituted by water and acetonitrile both with 0.1% formic acid. For both methods a gradient elution mode was used. The I.S. was added and the mixture was then submitted to vortex (for 30 s) and to centrifugation (for 5 min at 13,000 × g). Calibration curves and quality control of different compounds were prepared by spiking blank cells with appropriate standard solutions. The organic layer was removed and evaporated to dryness under a stream of nitrogen. The residues were suspended in 200 μL of mobile phase, and 10 μL was injected in the HPLC device.

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