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Acridone derivatives: Design, synthesis, and inhibition of breast cancer resistance protein ABCG2

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Abstract—The breast cancer resistance protein (BCRP, ABCG2) is among the latest discovered ABC proteins to be involved in MDR phenotype and for which only few inhibitors are known. In continuing our program aimed at discovering efficient multidrug resistance modulators, we conceived and synthesized new acridones as ABCG2 inhibitors. The design of target molecules was based on earlier results dealing with ABCG2 inhibition with flavone and chromone derivatives. The human wild-type (R482) *ABCG2*-transfected cells were used for rational screening of inhibitory acridones. The synthesis of target compounds, the inhibitory activity against ABCG2, and structure–activity relationships are described. One of the acridones was even more potent than the reference inhibitor, GF120918, as shown by its ability to inhibit mitoxantrone efflux.

1. Introduction

The decreased accumulation of anticancer drugs in cancer cells may be mediated by several membrane proteins including the ABC (ATP-Binding Cassette) family of transporters.[1] The best studied are P-glycoprotein (P-gp/ABCB1) encoded by the *mdr*1 gene[2] and multidrug resistance protein (MRP1/ABCC1) encoded by the *mrp*1 gene.[3] More recently, the breast cancer resistance protein ABCG2 has been described as a new drug transporter.[4–6]

The structure of ABCG2 consists of 655 aminoacids with a molecular mass of 72.1 kDa. In contrast to P-gp/ABCB1 and MRP1/ABCC1, ABCG2 is a half-transporter containing only one ATP-binding site and six transmembrane domains.[7] However, ABCG2 is also able to induce cross resistance against various molecules, such as doxorubicin, topotecan, SN38, mitoxantrone, methotrexate, flavopiridol, zidovudine, and lamivudine.[8–11] It is interesting to highlight that ABCG2 is able to transport P-gp substrates but is not inhibited by most P-gp inhibitors, cardiovascular drugs, and immunosuppressors.[12,13] Since the discovery of BCRP in 1998, considerable efforts have been made to conceive inhibitors of this protein for acting in vivo as MDR modulators.[14,15] The ultimate therapeutical goal of such agents is a co-administration with the anticancer drugs to make the treatment more effective, with minimal drug side-effects.

GF120918 (Fig. 1) is an acridone-derived compound which inhibits both P-gp and ABCG2.[16–19] The antifungal agent, fumetrimorgin C,[20,21] and derivatives have been reported as specific inhibitors of ABCG2.[22] In recent reports, we have shown that 6-prenylchrysin and piperazine chromones act as powerful and specific inhibitors of ABCG2.[23,24]

Acridones are naturally occurring alkaloids which can be considered as aza-analogs of xanthones.[25] Acridones have been studied as P-gp, and to a lesser extent as MRP, inhibitors. In our continuing efforts to discover new MDR modulators, we targeted new acridone derivatives as inhibitors of the BCRP efflux pump, with the aim to determine structure–activity relationships. The

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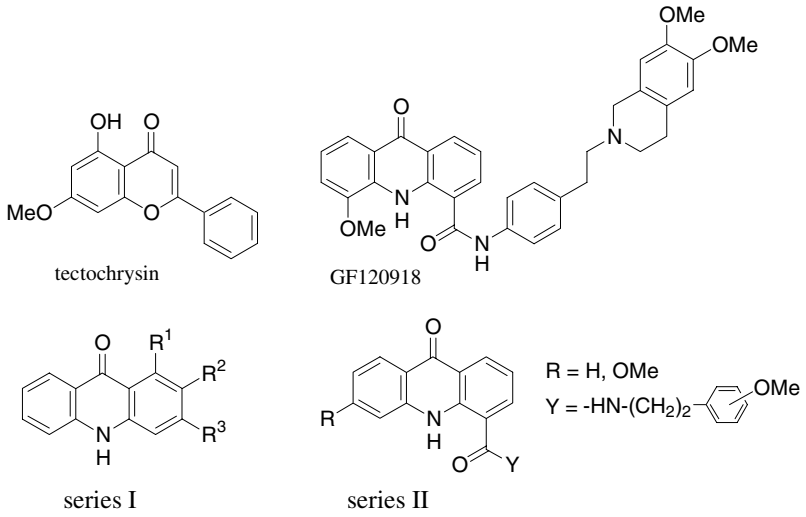


Figure 1. Structures of tectochrysin, GF120918, and the targeted acridones.

target compounds were designed from the following observations: (a) acridones, as represented by GF120918, have proved to be interesting inhibitors of the ABCG2 pump; (b) in our previous studies related to the investigation of flavonoids as potent MDR modulators, we have reported that tectochrysin (5-hydroxy-7-methoxyflavone) was an efficient inhibitor of ABCG2.[23] Having these elements in hand we conceived two series of acridones (Fig. 1): series I to check the substitution pattern found in tectochrysin, and series II related to the structure of GF120918 with targeted acridones having simple and easily accessible carboxamides at the 4-position.

2. Chemistry

Acridones of series I were prepared in two steps (Scheme 1): condensation of 2-chlorobenzoic acid with substituted aniline in the presence of copper as a catalyst, and then cyclization under acidic conditions according to Tercio et al.[26] The selective demethylation of methoxyl group adjacent (*ortho*-position) to the carbonyl group was conducted in the presence of $AlCl_3$ in acetonitrile. When the methoxyl group was not at the *ortho*-position, then the demethylation required the use of BBr_3 in CH_2Cl_2.

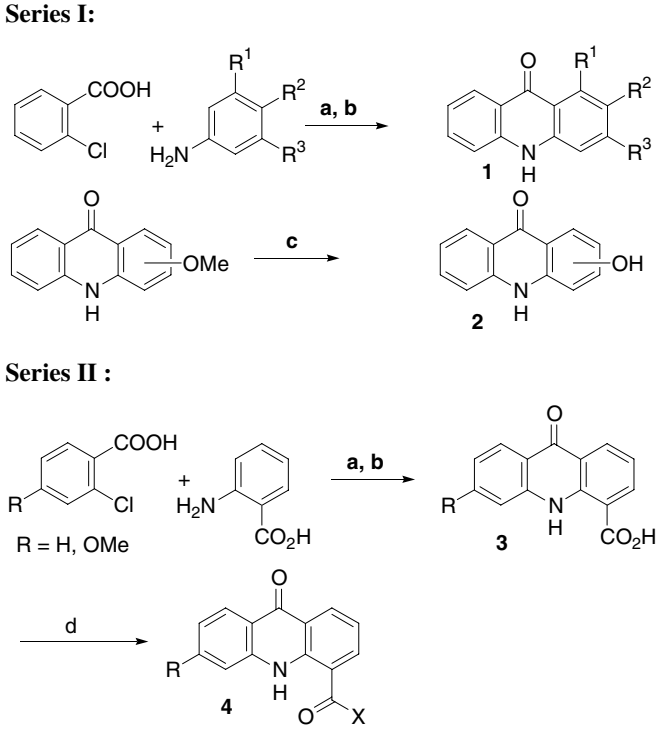
The preparation of acridones of series II required the synthesis of carboxylic acid **3** (Scheme 1). The latter was obtained by reacting 2-chlorobenzoic or 2-chloro-4-methoxybenzoic acid with 2-aminobenzoic acid according to the same procedure mentioned above. Finally, target acridones (**4**) were obtained by coupling acids **3** with conveniently substituted amines in the presence of DCC as a coupling agent.

3. Results and discussion

Due to its recent discovery, we know only few inhibitors of ABCG2.[13] Our extensive studies on MDR-reversal agents[27,28] led us to investigate a number of structurally related acridones as potential inhibitors, with the aim to identify MDR modulators and to establish structure–activity relationships for the inhibition of mitoxantrone efflux by *ABCG2*-transfected cells. The GF120918-sensitive drug efflux activity of human wild-type (R482) *ABCG2*-transfected cells was used for rational screening of inhibitory acridones and establishment of structure–activity relationships.

The inhibitory activity against ABCG2 is reported in Table 1, expressed as IC_{50} values. One of the first targeted acridone compounds was the 1-hydroxy-3-methoxyacridone (**2c**), which possesses a comparable substitution pattern as tectochrysin (Fig. 1), but this compound turned out to be inactive. However, methylation of the 5-OH group (**1c**) was highly beneficial since the IC_{50} dropped down to 7.9 ± 2.8 μM. The 1,3-position of the two methoxy groups (present in acridone **1c**) seemed to be important, since the 1,2-methoxylation (acridone **1d**) dramatically decreased the activity. It is worthwhile mentioning that the presence of a single methoxy group at C-2 (**1b**) was even more beneficial than the two methoxy groups at C-1 and C-3 (**1c**). As shown in Table 1, the perturbation of the substitution pattern of **1c** (compounds **2a**–**2d**) was harmful for the inhibition activity.

The highest activity of this series was reached with 1-methoxy-3-trifluoromethyl acridone (**1e**) (IC_{50} = 0.77 ± 0.07 μM). However, all these acridones were less efficient than GF120918 both in terms of affinity ($IC_{50} = 0.41 \pm 0.21$ μM) and maximal inhibition (one-fifth to one-third lower). Here again, attempts to demethylate the methoxy group (**2d**) led to a complete loss of activity and hence confirmed the importance of a methoxy group at position C-1. In order to check the N–H role, we N-methylated the most active compounds in this series and found that N-methylation led to inactive compounds. These results indicated that the N–H function was probably acting as a hydrogen-bond donor. This assumption was supported by the fact that the



Scheme 1. Reagents and conditions: (a) Cu, K_2CO_3, isoamylic alcohol, 160 °C, 10 h; (b) H_3PO_4/P_2O_5, 110 °C, 3 h; (c) i—$AlCl_3$, CH_3CN, 110 °C, 14 h (for demethylation of OMe at 1-position); ii—BBr_3, CH_2Cl_2, 24 h (demethylation of OMe at other positions); (d) H_2NR_2, DCC, Et_3N.

replacement of the N–H by an oxygen to obtain xanthones led to completely inactive compounds (results not shown).

In series II, the coupling of a methoxylated phenethyl group led to acridone analogs with IC_{50} values in the submicromolar range, comparable to GF120918 (0.41 ± 0.21 μM). Table 1 and Figure 2 show that (**4c**) even induced a higher maximal inhibition (112.3 ± 8.9%), with respect to (**4a**) (71.3 ± 12.0%), indicating a positive effect of the methoxy group at position 6. This might be compared to the increased inhibition observed for Ko143 versus Ko134.[29] In contrast, a second methoxy group at position 3 of the lateral phenyl group (**4d**) was detrimental for affinity (IC_{50} = 1.38 ± 0.22 μM) and also for maximal inhibition (94.9 ± 6.1%). The role played by a monomethoxyphenethyl and a dimethoxyphenethyl group was already reported in an earlier study with benzopyranone derivatives, showing the requirement for at least one methoxy group.[24] Methoxylation at the left-aromatic ring seemed not to be important. When the monomethoxy or dimethoxyphenethyl group was linked to the carbonyl group via a piperazine unit, fluorescent compounds were obtained.

On the basis of the structure–activity elements drawn from the present study, it can be concluded that it is possible to conceive simple and potent ABCG2 inhibitors. The easy and short synthesis of such compounds makes them attractive for further development.

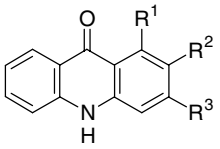
4. Experimental

4.1. Materials and methods

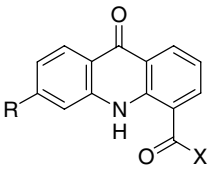
^1H and ^{13}C NMR spectra were recorded on a Bruker AC-200 (200 MHz for ^1H, 50 MHz for ^{13}C) and Bruker AC-400 instrument (400 MHz for ^1H, 100 MHz for ^{13}C). Chemical shifts are reported as δ values (ppm) relative to Me_4Si as an internal standard. EI and DCI mass spectra were recorded on a Fisons Trio 1000 instrument. Elemental analyses were performed by the Analytical Department of CNRS, Vernaison, France. Thin-layer chromatography (TLC) was carried out using Merck silica gel F-254 plates (thickness 0.25 mm). Flash chromatography was carried out using Merck silica gel 60, 200–400 mesh. All solvents were distilled prior to use. Chemicals and reagents were obtained either from ALDRICH or ACROS companies and used as obtained.

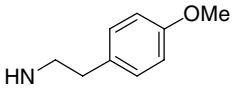
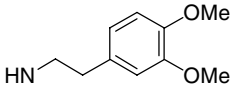
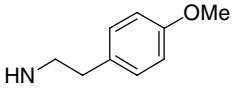
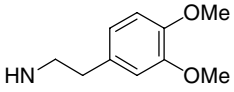
4.1.1. 1,3-Dimethoxy-10*H*-acridin-9-one (1c). A mixture of 2-chlorobenzoic acid (1.56 g, 10 mmol), 3,5-dimethoxyaniline (1.53 g, 10 mmol), powdered copper (25 mg), and K_2CO_3 (1.5 g) in isoamylic alcohol (10 mL) was heated at 160 °C for 10 h. After cooling, the alcohol was evaporated under vacuum, and the residue was dissolved in hot water (120 mL) and acidified with concentrated HCl. The precipitate was washed with hot water, dissolved in ethyl acetate, and the solution was dried over Na_2SO_4 and then evaporated. The crude product was purified over silica gel chromatography

Table 1. Inhibition of mitoxantrone efflux by wild type ABCG2-transfected cells



Compound	R^1	R^2	R^3	IC_{50}[a] (μM)	% maximal inhibition versus GF120918[a]
1a	OMe	H	H	6.3 ± 2.1	74 ± 7.2
1b	H	OMe	H	4.8 ± 2.3	66 ± 6.6
1c	OMe	H	OMe	7.9 ± 2.8	65 ± 7.0
1d	OMe	OMe	H	>20	
1e	OMe	H	CF_3	0.77 ± 0.07	81 ± 8.2
2a	OH	H	H	>20	
2b	H	OH	H	>20	
2c	OH	H	OMe	>20	
2d	OH	H	CF_3	>20	



Compound	X	R	IC_{50}[a] (μM)	% maximal inhibition versus GF120918[a]
4a		H	0.35 ± 0.16	71.3 ± 12.
4b		H	0.45 ± 0.07	90.2 ± 4.9
4c		OMe	0.56 ± 0.16	112.3 ± 8.9
4d		OMe	1.38 ± 0.22	94.9 ± 6.1
GF120918	—		0.41 ± 0.21	(100)

[a] The values are averages from three-independent experiments.

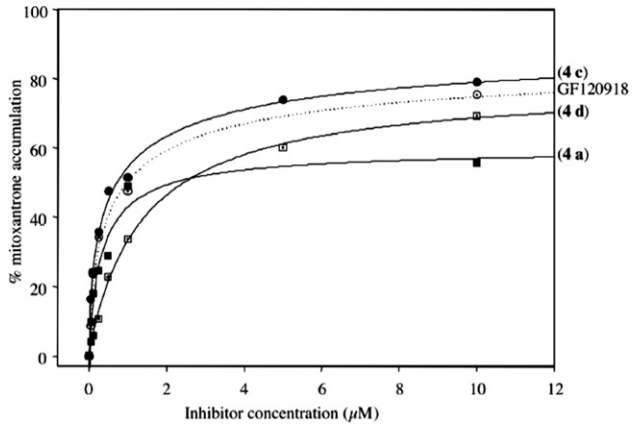


Figure 2. Concentration-dependent mitoxantrone intracellular accumulation as a result of ABCG2-mediated efflux inhibition. Effects of (**4a**), (**4c**), and (**4d**) as compared to GF120918.

column eluted with ethylacetate/hexane (1:1) to obtain acridone **1c** as a yellow powder.

The cyclization was performed as following:[26] a solution of P_2O_5 (3.75 g) in H_3PO_4 (1.5 mL) was heated under N_2 steam at 110 °C during 1 h. To the latter solution was added the compound to be cyclized, and the solution was heated at 110 °C for 3 h. After cooling, ice was added and the mixture was alkalinized by adding NaOH (3 N). A beige precipitate was formed; it was washed with H_2O and crystallized in H_2O–CH_3OH. (53% yield); mp (H_2O/CH_3OH) 259–261 °C; ^1H NMR (DMSO-d_6) : δ 11.36 (s, 1H); 8.08 (d, 1H, $J = 8$ Hz); 7.60 (t, 1H, $J = 8.4$ Hz); 7.37 (d, 1H, $J = 8$ Hz); 7.16 (t, 1H, $J = 7.2$ Hz); 6.45 (d, 1H, $J = 2$ Hz); 6.24 (d, 1H, $J = 2$ Hz); 3.86 (s, 3H); 3,82 (s, 3H). MS (EI): *m/z* 255 $[M]^{+\cdot}$. Anal. Calcd for $C_{15}H_{13}NO_3$: C, 70.58; H, 5.13; N, 5.49. Found: C, 70.49; H, 5.01; 5.39.

4.1.2. 1-Hydroxy-3-methoxy-10*H*-acridin-9-one (2c).[30] To a solution of **1c** (0.64 g, 2.5 mmol) in acetonitrile (90 mL) was added $AlCl_3$ (9 g), and the mixture was heated at 110 °C for 14 h. After cooling to room temperature, the solution was poured into HCl (2%, 180 mL) and heated at 80 °C for 30 min. The solution was evaporated to around 200 mL. The precipitate formed was washed with H_2O and crystallized in EtOH (95%). (50% yield); mp 233–235 °C (EtOH); ^{1}H NMR (DMSO-d_6): δ 11.97 (s, 1H); 8.17 (dd, 1H, J_1 = 8 Hz, J_2 = 1.2 Hz); 7.74 (dt, 1H, J_1 = 1.6 Hz, J_2 = 7.2 Hz); 7.49 (d, 1H, J = 8.4 Hz); 7.29 (dt, 1H, J_1 = 0.8 Hz, J_2 = 7.2 Hz); 6.38 (d, 1H, J = 2.4 Hz); 6.16 (d, 1H, J = 2.4 Hz); 3.85 (s, 3H). MS (EI): *m*/*z* 241 $[M]^{+\cdot}$. Anal. Calcd for $C_{14}H_{11}NO_3$: C, 69.70; H, 4.60; N, 5.81. Found: C, 69.66; H, 4.52; N, 5.77.

4.1.3. 2-Methoxy-10*H*-acridin-9-one (1b). It was prepared by following the same procedure as for **1c**. (27% yield); mp > 270 °C (H_2O–CH_3OH); ^{1}H NMR (DMSO-d_6): δ 11.78 (s, 1H); 8.23 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.6 Hz); 7.69 (dt, 1H, J_1 = 1.6 Hz, J_2 = 6.8 Hz); 7.63 (d, 1H, J = 2.8 Hz); 7.54–7.50 (m, 2H); 7.41 (dd, 1H, J_1 = 2.8 Hz, J_2 = 8.8 Hz); 7.22 (dt, 1H, J_1 = 1.3 Hz, J_2 = 6.8 Hz); 3.88 (s, 3H). MS (EI): *m*/*z* 225 $[M]^{+\cdot}$. Anal. Calcd for $C_{14}H_{11}NO_2$: C, 74.65; H, 4.92; N, 6.22. Found: C, 74.53; H, 4.85; N, 6.13.

4.1.4. 2-Hydroxy-10*H*-acridin-9-one (2b). It was prepared starting from **1b** as follows. To an ice cooled solution of **1b** (0.45 g, 2 mmol) in CH_2Cl_2(10 mL) was slowly added BBr_3 (10 mL from a 1 M solution in CH_2Cl_2). The mixture was stirred at room temperature for 24 h and then added to ice water which led to the formation of green precipitate. The latter was filtered off, washed with water, and the dried under vacuum to yield **2b** as a yellow powder. (54% yield); mp > 270 °C; ^{1}H NMR (DMSO-d_6): δ 11.64 (s, 1H); 8.19 (d, 1H, J = 7.6 Hz); 7.67 (t, 1H, J = 7.2 Hz); 7.55 (d, 1H, J = 2.8 Hz); 7.50 (d, 1H, J = 8.4 Hz); 7.46 (d, 1H, J = 8.8 Hz); 7.28 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.8 Hz); 7.19 (t, 1H, J = 8 Hz). MS (EI): *m*/*z* 211 $[M]^{+\cdot}$. Anal. Calcd for $C_{13}H_9NO_2$: C, 73.92; H, 4.29; N, 6.63. Found: C, 73.89; H, 4.22; N, 6.57.

4.1.5. 1-Methoxy-10*H*-acridin-9-one (1a). It was prepared following the same procedure as for **1c**. The final compound was purified by silica gel column chromatography eluted with ethyl acetate:hexane 1:1 to yield **1a** as a yellow powder. Yield 41%; mp 267–269 °C; ^{1}H NMR (DMSO-d_6): δ 11.62 (s, 1H); 8.19 (d, 1H, J = 8 Hz); 8.13 (d, 1 H, J = 8.8 Hz); 7.69 (t, 1H, J = 8.4 Hz); 7.48 (d, 1H, J = 8 Hz); 7.23 (t, 1H, J = 8 Hz); 6.89 (d, 1H, J = 2.4 Hz); 6.86 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.8 Hz); 3.95 (s, 3H). MS (EI): *m*/*z* 225 $[M]^{+\cdot}$. Anal. Calcd for $C_{14}H_{11}NO_2$: C, 74.65; H, 4.92; N, 6.22. Found : C, 74.56; H, 4.88; N, 6.09.

4.1.6. 1-Hydroxy-10*H*-acridin-9-one (2a). This compound was prepared starting from **1a** and following the same procedure as for **2b**. After treatment with ice, the product was extracted with ethyl acetate and the residue was chromatographed over silica gel column eluted with ethyl acetate/hexane 1:1. Yield 71%; mp 219–221 °C; ^{1}H NMR (DMSO-d_6): δ 11.48 (s, 1H); 10.47 (1H, s); 8.16 (d, 1H, J = 8.4 Hz); 8.05 (d, 1H, J = 8.8 Hz); 7.65 (t, 1H, J = 8 Hz); 7.44 (d, 1H, J = 8 Hz); 7.19 (t, 1H, J = 8 Hz); 6.78 (s, 1H); 6.71 (d, 1H, J = 8.8 Hz); 3.37 (s, 3H). MS (EI): *m*/*z* 211 $[M]^{+\cdot}$. Anal. Calcd for $C_{13}H_9NO_2$: C, 73.92; H, 4.29; N, 6.63. Found: C, 73.84; H, 4.19; N, 6.54.

4.1.7. 1-Methoxy-3-trifluoromethyl-10*H*-acridin-9-one (1e). Yield 31%; mp 259–261 °C; ^{1}H NMR (DMSO-d_6): δ 11.97 (s, 1H); 8.17 (d, 1H, J = 8 Hz); 7.71 (t, 1H, J = 8 Hz); 7.49 (d, 1H, J = 8.4 Hz); 7.28 (t, 1H, J = 8 Hz); 7.22 (d, 1H, J = 2.4 Hz); 7.18 (d, 1H, J = 2.4 Hz); 3.95 (s, 3H). MS (EI): *m*/*z* 293 $[M]^{+\cdot}$. Anal. Calcd for $C_{15}H_{10}F_3NO_2$: C, 61.44; H, 3.44; N, 4.78. Found: C, 61.37; H, 3.30; N, 4.70.

4.1.8. 1-Hydroxy-3-trifluoromethyl-10*H*-acridin-9-one (2d). A solution of **1e** (0.23 g, 0.7 mmol) in HBr (48%, 2.5 mL) was refluxed for 24 h under a N_2 steam, then added to ice water and alkalinized with $NaHCO_3$. The precipitate formed was chromatographed over silica gel column eluted with ethyl acetate/hexane 1:1. (20% yield); mp 259–261 °C; ^{1}H NMR (DMSO-d_6): δ 12.47 (s, 1H); 8.22 (d, 1H, J = 8 Hz); 7.84 (t, 1H, J = 7.2 Hz); 7.58 (d, 1H, J = 8.4 Hz); 7.37 (t, 1H, J = 7.2 Hz); 7.22 (s, 1H); 6.74 (s, 1H). MS (EI): *m*/*z* 279 $[M]^{+\cdot}$. Anal. Calcd for $C_{14}H_8F_3NO_2$: C, 60.22; H, 2.89; N, 5.02. Found: C, 60.11; H, 2.80; N, 4.97.

4.1.9. 1,2-Dimethoxy-10*H*-acridin-9-one (1d). Yield 40%; mp > 270 °C; ^{1}H NMR (DMSO-d_6): δ 8.20 (d, 1H, J = 7.6 Hz); 7.66 (t, 1H, J = 8.4 Hz); 7.57 (s, 1H); 7.49 (d, 1H, J = 8.4 Hz); 7.21 (t, 1H, J = 7.6 Hz); 6.97 (s, 1H); 3.92 (s, 3H); 3.85 (s, 3H). MS (EI): *m*/*z* 255 $[M]^{+\cdot}$. Anal. Calcd for $C_{15}H_{13}NO_3$: C, 70.58; H, 5.13; N, 5.49. Found: C, 70.56; H, 5.07; N, 5.42.

4.1.10. 9-Oxo-9,10-dihydroacridine-4-carboxylic acid 4-methoxyphenethyl amide (4a). Yield 5%; mp 225–227 °C; ^{1}H NMR ($CDCl_3$): δ 8.63 (d, 1H, J = 8 Hz); 8.45 (d, 1H, J = 8 Hz); 7.75-7.67 (m, 2H); 7.43 (d, 1H, J = 8.4 Hz); 7.32–7.15 (m, 4H); 6.91–6.86 (m, 2H); 6.57 (br s, 1H); 3.85–3.76 (m, 2H); 3.81 (s, 3H); 2.96 (t, 2H, J = 6.8 Hz). MS (EI): *m*/*z* 372 $[M]^{+\cdot}$. Anal. Calcd for $C_{23}H_{20}N_2O_3$: C, 74.18; H, 5.41; N, 7.41. Found: C, 74.00; H, 5.33; N, 7.41.

4.1.11. 9-Oxo-9,10-dihydroacridine-4-carboxylic acid 3,4-dimethoxyphenethyl amide (4b). Yield 7%; mp 205–207 °C; ^{1}H NMR ($CDCl_3$): δ 8.60 (d, 1H, J = 8 Hz); 8.44 (d, 1H, J = 8 Hz); 7.78 (d, 1H, J = 7.2 Hz); 7.70 (t, 1H, J = 8 Hz); 7.43 (d, 1H, J = 8.4 Hz); 7.31 (d, 1H, J = 8 Hz); 7.14 (t, 1H, J = 7.6 Hz); 6.86–6.80 (m, 2H); 6.74 (br s, 1H); 3.88 (s, 3H); 3.87 (s, 3H); 3.82–3.77 (m, 2H); 2.97 (t, 2H, J = 6.8 Hz). MS (EI): *m*/*z* 402 $[M]^{+\cdot}$. Anal. Calcd for $C_{24}H_{22}N_2O_4$: C, 71.63; H, 5.51; N, 6.96. Found: C, 71.55; H, 5.39; N, 6.92.

4.1.12. 6-Methoxy-9-oxo-9,10-dihydroacridine-carboxylic acid-(4-methoxyphenethyl) amide (4c). Yield 30%; mp 219–221 °C; ^{1}H NMR ($CDCl_3$): δ 12.20 (s, 1H); 8.60 (d, 1H, J = 8 Hz); 3.39 (d, 1H, J = 8 Hz); 7.75 (br s,

1H); 7.30–6.70 (m, 7H); 3.95 (br s, 2H); 3.80 (s, 6H); 3.0 (br s, 2H). MS (EI): *m/z* 402 $[M]^{+\cdot}$. Anal. Calcd for $C_{24}H_{22}N_2O_4$: C, 71.63; H, 5.51; N, 6.96. Found: C, 71.49; H, 5.38; N, 6.89.

4.1.13. 6-Methoxy-9-oxo-9,10-dihydroacridine-carboxylic acid-(3,4-dimethoxyphenethyl) amide (4d). Yield 18%; mp 177–179 °C; ^{1}H NMR ($CDCl_3$): δ 12.24 (s, 1H); 8.60 (d, 1H, J = 8 Hz); 8.34 (d, 1H, J = 8 Hz); 7.73 (d, 1H, J = 7.6 Hz); 7.12 (t, 1H, J = 7.6 Hz); 6.89–6.75 (m, 5H); 3.98 (s, 3H); 3.87 (s, 6H); 3.69 (m, 2H); 2.97 (t, 1H, J = 6.4 Hz). MS (EI): *m/z* 432 $[M]^{+\cdot}$. Anal. Calcd for $C_{25}H_{24}N_2O_5$: C, 69.43; H, 5.59; N, 6.48. Found: C, 69.30; H, 5.47; N, 6.43.

4.1.14. Flow cytometry. HEK-293 cells, transfected by either *ABCG2* or the empty vector (control cells), were incubated with 5 μmol/L mitoxantrone for 30 min at 37 °C in the presence or absence of various concentrations of inhibitors added as DMSO solutions (0.5% final concentration), washed in PBS, and incubated in substrate-free medium with the same inhibitor concentration for 1 h. Intracellular drug fluorescence was monitored with a FACscan flow cytometer (Becton–Dickinson, Mountain View, CA). The maximal fluorescence (100%) was the difference between mean fluorescence of control cells and *ABCG2*-transfected cells, incubated with substrate but without inhibitor. The highest inhibitor concentration tested did not significantly modify the fluorescence of control cells. Cells without drug were included as an autofluorescence control.

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