Journal of Medicinal Chemistry

Brief Article

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Substituted Chromones as Highly Potent Nontoxic Inhibitors, Specific for the Breast Cancer Resistance Protein

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Supporting Information

ABSTRACT: A series of 13 disubstituted chromones was synthesized. Two types of substituents, on each side of the scaffold, contributed to both the potency of ABCG2 inhibition and the cytotoxicity. The best compound, 5-(4-bromobenzyloxy)-2-(2-(5-methoxyindolyl)ethyl-1-carbonyl)-4*H*-chromen-4-one (**6g**), displayed high-affinity inhibition and low cytotoxicity, giving a markedly high therapeutic index. The chromone derivative specifically inhibited ABCG2 versus other multidrug ABC transporters and was not transported. It constitutes a highly promising candidate for *in vivo* chemosensitization of ABCG2-expressing tumors.

INTRODUCTION

Cancer cells display a strong ability to acquire resistance to anticancer drugs, termed multidrug resistance (MDR) phenotype, which constitutes a critical hurdle to cancer therapy. While several mechanisms can mediate cellular resistance to chemotherapeutic agents, the energy-dependent drug efflux mediated by ATP-binding cassette (ABC) transporters is well recognized. Overexpression of multidrug ABC transporters in cancer cells alters anticancer drugs efficacy by significantly reducing their accumulation into the intracellular compartment.

The first ABC transporter identified to be responsible for MDR was P-glycoprotein, encoded by the *MDR1/ABCB1* gene. It was shown to confer resistance to a wide panel of chemotherapeutic drugs, including anthracyclines, taxanes, and *vinca*-alkaloids.[1,2] A second multidrug ABC transporter was called "multidrug-resistance associated protein" (MRP1)[3]/ABCC1. MRP1 confers resistance to a smaller range of drugs, including *vinca*-alkaloids, anthracyclines, and epipodophyllotoxins. ABCG2 was recently discovered, simultaneously in three independent groups, and then received different denominations: ABCP for its overexpression in placenta,[4] BCRP for its isolation from resistant breast cancer cells,[5] and MXR for its ability to confer resistance to mitoxantrone.[6] ABCG2 has been demonstrated to confer resistance to a wide variety of anticancer agents.[6]

Overcoming multidrug resistance against anticancer agents is of critical importance for future clinical treatments. This strategy can be achieved through effective inhibitors of multidrug ABC transporters involved in the MDR phenotype. ABCG2 inhibitors have then been investigated in many studies, leading to identification of potent inhibitors.[7,8] Fumitremorgin C (FTC) was the first discovered selective ABCG2 inhibitor;[9] however, its serious neurotoxicity precluded any clinical application. In order to provide less toxic and more potent inhibitors, synthetic FTC analogues were designed as indolyldiketopiperazines, among which Ko143 was found to be the most promising *in vitro*, despite some residual cytotoxicity.[10,11] GF120918/elacridar and XR9576/tariquidar, initially developed as third-generation compounds able to strongly inhibit ABCB1, were also found to inhibit ABCG2.[12,13] XR9576/tariquidar was used as a scaffold for designing ABCG2-specific inhibitors by introducing targeted structural modifications.[14,15] We also used GF120918/elacridar as a template for designing acridones as ABCG2-selective inhibitors[16] (Figure 1A). One of them was recently shown to retain *in vivo* activity through potentiation of irinotecan antitumor activity,[8,17] despite some cytotoxicity.

To continue our investigation of ABCG2-selective inhibitors, the acridone moiety was replaced by a chromone core (Figure 1B), with substituents aimed at further increasing inhibitory potency and lowering cytotoxicity. The substituents grafted to the chromone scaffold were chosen among those reported to induce favorable effects in other ABCG2 inhibitors. The results

Received: October 18, 2011
Published: December 13, 2011

dx.doi.org/10.1021/jm201404w | *J. Med. Chem.* 2012, 55, 966−970

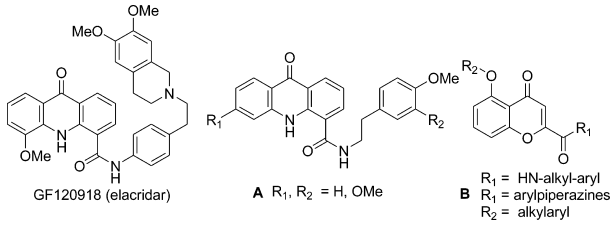


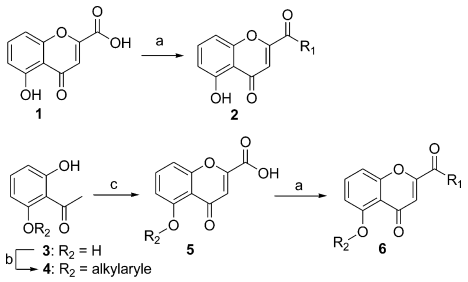
Figure 1. Acridones including a mono- or a dimethoxyphenylethylamine substituent (A) were previously designed from GF120919/elacridar. The acridone moiety is here replaced by a chromone core derivatized by two main substituents: R_1 and R_2 (B derivatives).

show that two different types of substituents contributed both to increase ABCG2 inhibition and to lower cytotoxicity. The best compound, among a series of 13 derivatives, could be considered as an excellent ABCG2 inhibitor and a quite interesting candidate for future *in vivo* experiments.

CHEMICAL SYNTHESIS

The access to the target compounds required the synthesis of key chromone-2-carboxylic acids **1** and **5** (Scheme 1). The synthesis

Scheme 1. Synthesis of 5[a]



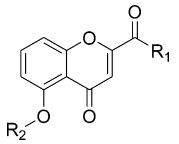
[a]Conditions: (a) R_1-NH-R_2, BOP-Cl, DMF. (b) Ar-$(CH_2)n$-Br, K_2CO_3, acetone. (c) (i) Diethyloxalate, EtO^-Na^+/EtOH; (ii) KOH, EtOH/H_2O. For R_1 and R_2, see Table 1.

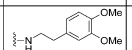
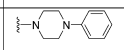
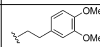
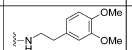
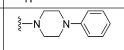
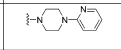
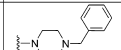
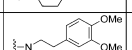
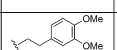
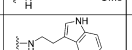
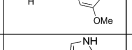
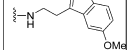
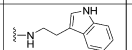
of **1** was performed according to our previous report.[18,19] The synthesis of **5** was achieved as shown in Scheme 1. 2,6-Dihydroxyacetophenone reacted with a suitable aryl alkyl halide (1 equiv) in the presence of K_2CO_3 in acetone to provide the mono *O*-alkylated derivative **4**. The latter was condensed with ethyloxalate using sodium ethoxide as a base to yield the desired ester, which was then hydrolyzed to give the corresponding carboxylic acid **5**. Finally, the latter compound was condensed with either aryl alkyl amines or *N*-arylpiperazines to afford the final compounds **6**.

BIOLOGICAL EVALUATION AND DISCUSSION

Inhibition of ABCG2 Drug-Efflux Activity. Thirteen substituted chromones were synthesized and analyzed, by flow cytometry, for their ability to inhibit ABCG2-mediated mitoxantrone efflux from transfected HEK293 cells. The R_1 and R_2 substituents were chosen from those known in other compounds to favor ABCG2 inhibition: arylethylamines and arylpiperazines for R1, and benzyl derivatives for R_2.[16,18,19] They indeed greatly contributed to chromone inhibition as illustrated in Table 1. The unsubstituted chromone **1** was totally ineffective, whereas some inhibition was produced by either R_1 (in **2a** and **2b**) or R_2 (in **5a**) as a substituent. The inhibition produced by **5a** suggests that the carboxyl group does not fully prevent membrane permeation; therefore, it

Table 1. Inhibition of Mitoxantrone Efflux in *ABCG2*-Transfected Cells[a]



Compound	R_1	R_2	% inhibition		IC_{50} (μM)
			at 1 μM	at 5 μM	
1	OH	H	-1.2 ± 2.3	-2.0 ± 2.8	
2a		H	34.4 ± 5.2	62.5 ± 2.4	
2b		H	5.5 ± 2.1	9.8 ± 2.5	
5a	OH		6.1 ± 1.7	26.7 ± 4.6	
6a					0.17
6b			21.3 ± 6.7	66.1 ± 12.4	
6c			18.1 ± 2.1	46.2 ± 3.1	
6d			16.5 ± 2.4	48.3 ± 3.2	
6e					0.47
6f					0.18
6g					0.11
6h					0.20
6i					0.16

[a]Percent inhibition was determined by flow cytometry, relative to 5 μM GF120918 as a control producing 100% inhibition. Data are the mean ± SD of at least three independent experiments. For the most active compounds where a full inhibition was observed, the IC_{50} value was determined using a range of concentrations.

cannot be fully excluded that **1** may cross the membrane, despite its low logP, and then not bind to ABCG2. A maximal inhibition, with high affinity (IC_{50} = 0.17 μM), required both types of substituents, as in **6a**, with dimethoxyphenethylamine as R_1 and benzyl as R_2. We then examined the effects produced by various arylethylamines and arylpiperazines as R_1, and substituted benzyls as R_2. The nature of R_1 was critical, since a low inhibition was observed with either a phenylpiperazine in **6b**, a pyridylpiperazine in **6c**, or a benzylpiperazine in **6d**. In contrast, the methoxytryptamine in **6f** appeared as efficient as the dimethoxyphenethylamine in **6a**. The nature of the substituent at the aryl moiety of R_2 was also found to be important. As shown in Table 1, the introduction of a bromine was highly beneficial (**6g** vs **6f** and **6h**), probably due to the size of the halogen and/or the hydrophobic group at this position of the molecule. The impact of both the size and hydrophobicity of R_2 can be clearly seen in compounds **6f**–**6i**: they should be hydrophobic (**6g** vs **6e** and **6h**) but not too big (**6f** vs **6h**). The five chromones **6a**, **6f**, **6g**, **6h**, and **6i** which displayed a similarly high potency for inhibition were at least 2-to-3-fold more efficient than previously reported flavones,[20] acridones,[16] boeravinones,[21] and other types of flavonoids, including natural

dx.doi.org/10.1021/jm201404w | J. Med. Chem. 2012, 55, 966–970

compounds.[22−24] Tariquidar/XR9576 derivatives, modified to be ABCG2 specific, were also less potent inhibitors,[15] and some of them produced only a partial inhibition of mitoxantrone efflux activity, limited to 40−60%.[14]

Specificity for ABCG2 and Intrinsic Cytotoxicity. The six most potent inhibitors of ABCG2-mediated mitoxantrone efflux, for which an IC_{50} value was determined in Table 1, as well as **2a**, were assayed against P-glycoprotein/ABCB1 and MRP1/ABCC1 (Table SI-1 Supporting Information). They all appeared fully specific for ABCG2, since they did not produce any inhibition of either mitoxantrone efflux by ABCB1 or calcein efflux by ABCC1, even at high concentrations up to 5 μM, corresponding to 45-fold the IC_{50} reported for **6g** in Table 1. Such a selectivity was not observed for well-known dual compounds such as GF120918/elacridar[12] and XR9576/tariquidar,[13] which inhibit P-glycoprotein even better than ABCG2. The most potent ABCG2 inhibitor presently known, Ko143, is not fully selective, since it was also found to inhibit P-glycoprotein at a 40-fold higher concentration than that for ABCG2 (IC_{50} = 1 μM); similarly, an IC_{50} of 2 μM was obtained for MRP1.[11] It is worth mentioning that some chromone derivatives at high concentrations, especially **2a** and **6a**, slightly stimulated P-glycoprotein-mediated drug efflux activity, indicating that they indeed bound to P-glycoprotein but interacted differently than with ABCG2. The P-glycoprotein-binding site appears not to be inhibitory or not to promote a conformational change required for inhibition.

The intrinsic cytotoxicity of substituted chromones was monitored at increasing concentrations on cell survival upon a 72-h growth period (Figure 2). Among the most potent

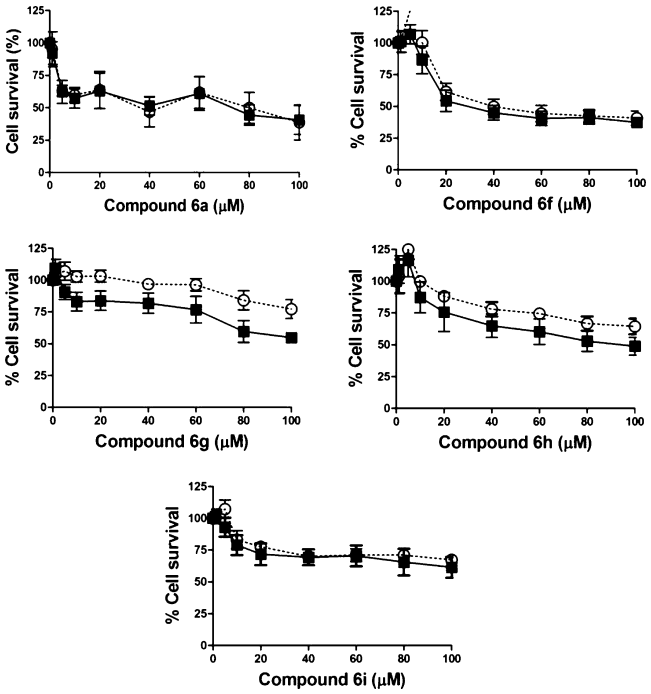


Figure 2. Cytotoxicities of compounds. Cell survival of HEK293-*ABCG2* cells (■) and HEK293-pcDNA3.1 cells (○), upon a 72-h treatment with the compounds at increasing concentrations, was determined by MTT assays as described in the Experimental Section.

inhibitory compounds, **6g** displayed a very low cytotoxicity on control HEK293 cells, with less than 25% cell death at 100 μM, the highest concentration tested. It then appeared much less cytotoxic than previously reported inhibitors, such as acridone 4d/MBLI-87 (30% cytotoxicity at 5−10 μM)[8] and hydrophobic flavones displaying IG_{50} values in the range of 9−16 μM.[20]

Bromine substitution of the R_2 phenyl ring was actually important, since its absence in **6f** considerably increased cytotoxicity (IG_{50} = 35 μM). An intermediate effect was observed with methylbenzyl in **6h** and with a phenylethyl in **6i**. By contrast, the R_1 dimethoxyphenylethylamine group in **6a** (also present in the above acridones) was much more cytotoxic, especially at low concentrations when compared to **6f** (35−40% cytotoxicity versus no cytotoxicity at 10 μM); an IG_{50} value around 60 μM could be estimated. Therefore, both R_1 and R_2 substituents greatly contributed to chromone cytotoxicity, with methoxytryptamine and bromobenzyl, respectively, producing minimal effects. It should be noticed that no cross-resistance was ever observed, since the IG_{50} value was never higher for transfected versus control cells, suggesting that the substituted chromones were not transported by ABCG2.

The therapeutic index was extremely high for **6g**, around 2000 as defined by the ratio between IG_{50} for cytotoxicity and IC_{50} for inhibition. This value is higher than that of 300−400 estimated for Ko143, which, although being more potent for ABCG2 inhibition (IC_{50} = 0.05−0.1 μM), is however much more cytotoxic (IG_{50} = 18−34 μM).[11] For other inhibitors, the therapeutic index is much lower: around 40 for 6-prenylchrysin[20] and even lower for acridones.[8,16]

Chemosensitization of Cell Growth due to Permanent ABCG2 Inhibition. The efficiency of **6g** to inhibit ABCG2-mediated mitoxantrone efflux was confirmed by its ability to chemosensitize cell growth to the drug (Figure 3A). The nearly full chemosensitization obtained at 0.2 μM was consistent with the IC_{50} of 0.11 μM determined in Table 1, and it suggests that **6g** was not metabolized during the 72-h duration of chemosensitization. Very similar results were obtained (Figure 3C) when mitoxantrone was replaced by SN-38, the actively transported metabolite of irinotecan/CPT-11. A similar behavior of **6a** was also observed toward either mitoxantrone (Figure 3B) or SN-38 (Figure 3D).

The two potent chromones **6g** and **6a** did not significantly modify the expression of ABCG2 even at high concentrations, over a 72-h incubation, as monitored by Western blot with BXP-21 monoclonal antibody (Figure SI-1A Supporting Information). This effect excluded the possibility that inhibition could be related to a decreased expression of the transporter. The lack of compound transport was directly confirmed by HPLC fractionation and mass spectrometry quantification (Figure SI-1B Supporting Information). No significant decrease in the amount of either **6g** or **6a** was observed in ABCG2-transfected cells versus control cells, as well as no increased amount upon inhibition of ABCG2 by GF120918. This is consistent with the lack of transport observed with other polyphenolic inhibitors such as 6-prenylchrysin,[20] acridones,[16] and methoxystilbenes.[25] Similarly to the other ABCG2-specific inhibitors, acridones[8] and methoxystilbenes,[25] **6a** was found to produce a noncompetitive inhibition toward mitoxantrone (not shown here). The present chromone derivatives including compound **6g** are not transported and inhibit mitoxantrone efflux in a noncompetitive way. Therefore, they may be considered as "true" inhibitors, by difference with other inhibitory compounds, such as tyrosine kinase inhibitors, which are themselves transported.[8,26]

In conclusion, the chromone derivative **6g** may be considered as an excellent *in vitro* ABCG2 inhibitor when taking into account its high-affinity and complete inhibition, selectivity

dx.doi.org/10.1021/jm201404w | J. Med. Chem. 2012, 55, 966−970

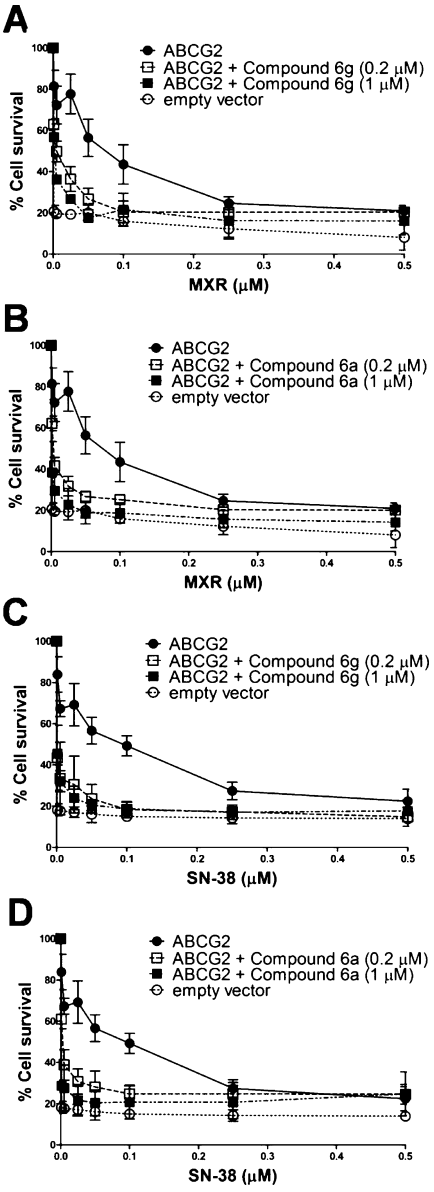


Figure 3. Sensitization of cell growth to mitoxantrone and SN-38. Cell viability of HEK293-*ABCG2* cells was determined upon cotreatment for 72 h with mitoxantrone or SN-38 (at 0−0.5 μM) and either compound **6a** or **6g**. Parallel experiments with only mitoxantrone or SN-38 were performed with HEK293-*pcDNA3.1* and *ABCG2*-transfected cells. The values represent the mean ± SD of percent cell viability with respect to the untreated control. Data were collected from at least three independent experiments performed in triplicate.

for ABCG2, absence of transport, very low cytotoxicity, and extremely high therapeutic index. It constitutes a highly promising candidate for future *in vivo* experiments, such as in mice xenografted with human ABCG2-expressing tumors, for which a significant sensitization to irinotecan/CPT-11 was recently observed with the acridone 4d/MBLI-87[8,17] despite relatively low inhibition potency and therapeutic index.

EXPERIMENTAL SECTION

Chemistry. NMR spectra were recorded on a 400 MHz Brüker AC-400 instrument. Chemical shifts (δ) are reported in ppm relatively to Me_4Si (internal standard). Electrospray ionization ESI mass spectra were acquired by the Analytical Department of Grenoble University on an Esquire 300 Plus Brüker 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), and 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.

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

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 embryonic kidney HEK293 cell lines transfected with ABCG2 (HEK293-*ABCG2*) or the empty vector (HEK293-*pcDNA3.1*) were obtained as previously described.[22] The HEK293 cell lines transfected with *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% penicillin/streptomycin, and supplemented with either 0.75 mg/mL G418 (HEK293-*pcDNA3.1* and HEK293-*ABCG2*), 2 mg/mL G418 (HEK293-*MDR1*), or 5 μM etoposide (HEK293-*MRP1*).

ABCG2-Mediated Drug Transport. The efflux assays were determined according to ref 27 with minor modifications. HEK293-*ABCG2* and control cells 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 5 μM mitoxantrone for 30 min (HEK293-*ABCG2* cells) for 30 min at 37 °C, in the presence or absence of compounds at various concentrations. After cell washing with phosphate buffer saline (PBS), the cells were trypsinized, and 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 without inhibitor.

Cytotoxicity Assays. HEK293-*ABCG2* 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 increasing concentrations of compounds for 72 h at 37 °C under 5% CO_2. For the sensitization experiments, after overnight incubation, the cells were concomitantly treated with compounds and increasing concentrations of either mitoxantrone or SN-38 for 72 h at 37 °C under 5% CO_2. In both cases, cell viability was evaluated with an MTT colorimetric assay.[28] Control experiments were performed with DMEM high glucose containing 0.1% DMSO (v/v). The results were expressed as percentage of viable versus control cells, taken as 100%.

ASSOCIATED CONTENT

Supporting Information

Table SI-1, Figure SI-1, characterization of chemical compounds, and biological methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

#Both senior investigators equally contributed to the work

ACKNOWLEDGMENTS

Drs. R. W. Robey and S. E. Bates, NCI-NIH, Bethesda, MD, are acknowledged for providing HEK293 cells transfected by either *ABCB1* (MDR-19 clone) or *ABCC1*, and GlaxoSmithKline is acknowledged for GF120918. We thank Dr. Anthony Coleman

for rereading the manuscript and improving the English. G.V. was the recipient of a mobility fellowship from the Brazilian CAPES (Process number 2303/10-8). E.G.-B. and C.G. were recipients of doctoral fellowships from the Région Rhône-Alpes and the Ligue Nationale Contre le Cancer, respectively. Financial support was provided by the CNRS and Université Lyon 1 (UMR 5086), the Ligue Nationale Contre le Cancer (Equipe labellisée Ligue 2011), the Région Rhône-Alpes (CIBLE 2010), and an international grant from French ANR and Hungarian NIH (2010-INT-1101-01).

■ ABBREVIATIONS USED

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

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