

RESEARCH PAPER

Targeting the ABCG2-overexpressing multidrug resistant (MDR) cancer cells by PPAR γ agonists

Kenneth K W To^{1*} and Brian Tomlinson²

¹School of Pharmacy, The Chinese University of Hong Kong, Hong Kong, and ²Department of Medicine and Therapeutics, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong

*Correspondence

Kenneth K.W. To, Lo Kwee Seong Integrated Biomedical Sciences Building, Area 39, Shatin, New Territories, Hong Kong. E-mail: kennethto@cuhk.edu.hk

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BACKGROUND AND PURPOSE

Multidrug resistance (MDR), usually mediated by overexpression of efflux transporters such as P-gp, ABCG2 and/or MRP1, remains a major obstacle hindering successful cancer chemotherapy. There has been great interest in the development of inhibitors towards these transporters to circumvent resistance. However, since the inhibition of transporter is not specific to cancer cells, a decrease in the cytotoxic drug dosing may be needed to prevent excess toxicity, thus undermining the potential benefit brought about by a drug efflux inhibitor. The design of potent MDR modulators specific towards resistant cancer cells and devoid of drug-drug interactions will be needed to effect MDR reversal.

EXPERIMENTAL APPROACH

Recent evidence suggests that the PTEN/PI3K/Akt pathway may be exploited to alter ABCG2 subcellular localization, thereby circumventing MDR. Three PPAR γ agonists (telmisartan, pioglitazone and rosiglitazone) that have been used in the clinics were tested for their effect on the PTEN/PI3K/Akt pathway and possible reversal of ABCG2-mediated drug resistance.

KEY RESULTS

The PPAR γ agonists were found to be weak ABCG2 inhibitors by drug efflux assay. They were also shown to elevate the reduced PTEN expression in a resistant and ABCG2-overexpressing cell model, which inhibit the PI3K-Akt pathway and lead to the relocalization of ABCG2 from the plasma membrane to the cytoplasm, thus apparently circumventing the ABCG2-mediated MDR.

CONCLUSIONS AND IMPLICATIONS

Since this PPAR γ /PTEN/PI3K/Akt pathway regulating ABCG2 is only functional in drug-resistant cancer cells with PTEN loss, the PPAR γ agonists identified may represent promising agents targeting resistant cells for MDR reversal.

Abbreviations

ARBs, angiotensin II receptor blockers; MDR, multidrug resistance; PTEN, phosphatase and tensin homologue deleted on chromosome 10; TZDs, thiazolidinediones

Introduction

Despite the advances in the development of novel chemotherapeutic drugs, the occurrence of multidrug resistance (MDR) remains a major unresolved clinical problem hindering successful cancer chemotherapy. The most common mechanism of resistance is the active efflux of a variety of structurally unrelated anticancer drugs by ATP-binding cassette (ABC) transporters including P-glycoprotein (P-gp), ABCG2 and MRP1 (Gottesman *et al.*, 2002). An obvious strategy to restore drug sensitivity in MDR cancer cells is therefore to block drug efflux mediated by the ABC transporters. Over the past decade, tremendous efforts have been made to discover and develop such inhibitors or modulators in the clinic against P-gp, the most extensively studied MDR transporter (Sandor *et al.*, 1998; Tamaki *et al.*, 2011; Amiri-Kordestani *et al.*, 2012). However, the lack of specific and potent inhibitors against the MDR transporters and unpredictable pharmacokinetic drug-drug interactions has significantly hindered the development of transporter inhibitors in the clinic. Moreover, since the inhibition of transporters is not specific to cancer cells, it could also lead to impairment of the natural protective mechanism in the gastrointestinal tract and the drug excretion process at the hepatobiliary canaliculi. As a result, a decrease in the cytotoxic drug dosing may be needed to prevent excess toxicity, thus undermining the potential benefit obtained from having a drug efflux inhibitor. New transporter modulators with novel mechanisms targeting the resistance cancer cells are needed to realize the MDR reversal hypothesis.

ABCG2 is a relatively newer ABC transporter known to confer MDR, which has attracted wide attention recently because of its role in stem cell biology (Zhou *et al.*, 2001; Bunting, 2002) and the association of its genetic polymorphism with gout (Matsuo *et al.*, 2011). Growing evidence suggests that ABCG2 underlies the MDR of clinical samples from different cancer types (Ross *et al.*, 2000). Besides mediating MDR, ABCG2 is also known to play critical roles in absorption, drug elimination and tissue protection against toxins and xenobiotics in various organs (Kerr *et al.*, 2011). Due to the complex role played by ABCG2 in the body, it will be especially challenging to devise an ABCG2 inhibitor for MDR reversal.

The PI3K-Akt signalling pathway is a key regulator of cell cycle proliferation, growth, survival, protein synthesis and glucose metabolism, which therefore is implicated in many human diseases including cancer (Vivanco and Sawyers, 2002). PTEN (phosphatase and tensin homologue deleted on chromosome 10) is an important tumour suppressor gene that negatively regulates the PI3K/Akt pathway by dephosphorylating the lipid signalling intermediate PIP3 (Maehama and Dixon, 1998). Importantly, somatic inactivation of PTEN is prevalent in various cancer types and is associated with cancer susceptibility and tumour progression (Eng, 2003). Overactivation of the PI3K/Akt pathway as a result of PTEN loss is known to facilitate cancer growth and may predict resistance to anticancer drugs (Hafsi *et al.*, 2012). To this end, novel strategies to elevate PTEN expression and/or inhibit the PI3K/Akt pathway have been proposed for cancer prevention and chemotherapy (Patel *et al.*, 2001; Yap *et al.*, 2008; Cheng *et al.*, 2012; Garcia-Cao *et al.*, 2012).

Recently, inhibition of the PTEN/PI3K/Akt signalling pathway has been shown to modulate ABCG2-mediated drug transport via the translocation of ABCG2 from the plasma membrane to intracellular compartments in different cell model systems including side population cells in the bone marrow (Mogi *et al.*, 2003), gallbladder epithelial cells (Aust *et al.*, 2004), polarized ABCG2-overexpressing porcine kidney LLCPK1 cells (Takada *et al.*, 2005), hepatocellular carcinoma-derived cells (Hu *et al.*, 2008), glioma-derived stem-like cells (Bleau *et al.*, 2009) and ABCG2-overexpressing extracellular vesicles derived from MCF-7/MR breast cancer cells (Goler-Baron *et al.*, 2012). Importantly, Patel *et al.* have provided compelling data demonstrating that a synthetic PPAR γ agonist can up-regulate PTEN in human colorectal carcinoma Caco-2 cells and breast cancer MCF-7 cells, which correlated well with the suppression of proliferation in the treated cells. This activation of PTEN by a PPAR γ agonist was later shown to cause inhibition of the PI3K/Akt pathway, as evidenced by Akt dephosphorylation, thereby inhibiting cell proliferation (Teresi *et al.*, 2006). We therefore proposed to exploit PPAR γ agonist activity of existing drugs in clinical use to interact with the PTEN/PI3K/Akt pathway and probably also interfere with ABCG2 cellular localization in an attempt to circumvent MDR.

Angiotensin II receptor blockers (ARBs) represent a well-tolerated and increasingly prescribed class of anti-hypertensive agents used widely in combination drug regimens (Benndorf and Boger, 2008). Among the clinically available ARBs, telmisartan is well-known for its unique PPAR γ activating activities at therapeutically relevant concentrations (Benson *et al.*, 2004; Schupp *et al.*, 2004). Moreover, telmisartan and some other ARBs have been found to interact with ABC transporters including P-gp and ABCG2 (Kamiyama *et al.*, 2010; Weiss *et al.*, 2010), although the potential use of ARBs to reverse MDR has not been explored. It is also noteworthy that ARBs are generally considered to have low potential for drug-drug interaction because of their lack of significant interactions with cytochrome P450 monooxygenase (Schmidt and Schieffer, 2003).

Thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are synthetic PPAR γ ligands and were used as insulin-sensitizing antidiabetic drugs (Staels and Fruchart, 2005). They work by enhancing the insulin sensitivity in liver, muscle and adipose cells and by modulating lipid metabolism. Apart from their antidiabetic activity, some TZDs have in recent years caught attention for their potential anti-cancer activity. In particular, triglitazone and rosiglitazone were found to induce growth arrest and apoptosis in a broad spectrum of cancer cells, at least partially through the PPAR γ pathway (Eltner *et al.*, 1998; Koeffler, 2003).

The aim of this study was to evaluate the potential circumvention of ABCG2-mediated MDR by these commonly prescribed PPAR γ agonists. Intriguingly, remarkable down-regulation of PTEN was observed in our resistant and ABCG2-overexpressing cancer cell line model. Our data indicates that PPAR γ agonists up-regulate PTEN in the resistant cells to apparently circumvent drug resistance, probably via a dual mechanism by (i) inhibition of the PI3K/Akt pathway and (ii) driving the internalization of ABCG2 to the cytoplasm.

Methods

Chemicals

Mitoxantrone was purchased from Sigma Chemical (St Louis, MO, USA). Pheophorbide A (PhA) and fumitremorgin C (FTC) were kind gifts obtained from Dr Susan Bates (National Cancer Institute, NIH, Bethesda, MD, USA). Telmisartan was purchased from Changzhou Yabang Pharmaceutical Co., Ltd (Jiangsu Province, China). Rosiglitazone was obtained from GlaxoSmithKline. Pioglitazone was purchased from Amylin Pharmaceuticals, Inc (San Diego, CA, USA, USA). Irbesartan was from Zhejiang Apeloia Jiayuan Pharmaceutical Co. Ltd. (Zhejiang Province, China). Losartan and valsartan were purchased from Cayman Chemical (Ann Arbor, MI, USA). GW9662 was obtained from Sigma-Aldrich (St Louis, MO, USA). LY294002 was purchased from Selleck Chemicals (Houston, TX, USA).

Cell culture

MCF-7 and its drug-selected resistant subline MCF-7 FLV1000, and pcDNA3- or ABCG2-stably transfected HEK293 cells were obtained from Dr Susan Bates (National Cancer Institute). MCF-7 FLV1000 is overexpressing ABCG2, contributing to drug resistance, and was maintained in 1000 nM flavopiridol. No P-glycoprotein or MRP1 was detected in this resistant cell line. MCF-7 and MCF-7 FLV1000 cells, and the transfected HEK293 cells were maintained in DMEM and MEM medium, respectively, supplemented with 10% FBS, 100 units mL⁻¹ streptomycin sulfate and 100 units mL⁻¹ penicillin G sulfate, and incubated at 37°C in 5% CO₂. The resistant cells were grown in drug-free culture medium for more than 2 weeks before analysis. The ABCG2-transfected HEK293 cells expressed the wild type ABCG2 (i.e. R482) and were maintained at 1 mg mL⁻¹ G418.

Growth inhibition assay

Growth inhibitory effect of mitoxantrone with or without the concomitant treatment of the tested PPAR γ agonists was evaluated by the sulforhodamine B assay (Skehan *et al.*, 1990). Cells were seeded into 96-well microtitre plates in 100 μ L at a plating density of 5000 cells per well and allowed to incubate overnight. The cells were then treated with mitoxantrone at a range of concentrations in the presence or absence of a fixed concentration of tested PPAR γ agonist and allowed to incubate at 37°C in 5% CO₂ for 72 h. Each drug concentration was tested in quadruplicate and controls were tested in replicates of eight. The fold reversal of resistance was calculated by dividing the IC₅₀ for mitoxantrone or cisplatin (non-ABCG2 substrate as negative control) in the absence of PPAR γ agonist by that obtained in the presence of PPAR γ agonist. Each experiment was carried out independently at least three times. To determine whether differences between IC₅₀ values were significant, a Student's *t*-test was performed with *P* < 0.05 being considered significant.

Reverse transcription and real-time PCR

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA (1 μ g) was reverse transcribed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN, USA). Quantitative real-

time PCR was performed to measure ABCG2 transcript level using the KAPA SYBR FAST qPCR Kit (KapaBiosystems, Woburn, MA, USA) in a LightCycler 480 Instrument I (Roche Applied Science). The human GAPDH RNA was amplified in parallel as the internal control for normalization purpose. The specific primers used are as follows: ABCG2 (forward) 5'-TTTCCAAGCGTTCATTCAAAA-3' (reverse) 5'-TACGAC TGTGACAATGATCTGAGC-3' (To *et al.*, 2008); GAPDH (forward) 5'-AGCCACATCGCTCAGACAC-3' (reverse) 5'-GT TCAAACCTCTGCTCCTGA-3'; MDR1 (forward) 5'-CCCAT CATTGCAATAGCAGG-3' (reverse) 5'-GTTCAAACCTCTGCT CCTGA-3'. PCRs were performed at 95°C for 5 min, followed by 50 cycles of 95°C for 10 s and 60°C for 10 s. Fluorescence signal was acquired at the end of the elongation step of every PCR cycle (72°C for 10 s) to monitor the increasing amount of amplified DNA. Δ Ct was calculated by subtracting the Ct of GAPDH from that of ABCG2. $\Delta\Delta$ Ct was then calculated by subtracting the Δ Ct of the untreated cells from the Δ Ct of the treated cells. Fold change of gene expression was calculated by the equation $2^{-\Delta\Delta Ct}$.

Western blot analysis

Whole cell lysates prepared from MCF-7 or MCF-7 FLV1000 were separated by SDS-PAGE and subjected to immunoblot analysis with the respective antibodies (ABCG2, Kamiya Biomedical, Seattle, WA, USA; PPAR γ , Akt and pAkt, Cell Signaling Technology, Danvers, MA, USA; PTEN and GAPDH, Abcam, Cambridge, MA, USA respectively). The blots were developed with ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ, USA) and analysed with the FluroChem Q imaging system (Alpha Innotech, San Jose, CA, USA).

RNA interference

To construct a small interference hairpin-loop (sh) silencing vector against the human PPAR γ , two complementary oligonucleotides were synthesized, annealed and ligated into the Bgl II and EcoR I site of the pU6 vector (a kind gift from Dr Chen Yang Chao, School of Biomedical Sciences, CUHK, Hong Kong). The sequences were 5'-TGAATTATCTGATTGA GGCTTAtcaagagaTAAGCTTCAATCGGATGGTTCTTTTTTC-3' (forward) and 5'-TCGAGAAAAAAGAACCATCCGATTGAA GCTTAtctcttgaaTAAGCCTCAATCAGATAAATTCA-3' (reverse), which contained the oligonucleotide encoding a 20-mer hairpin sequence specific to the human PPAR γ mRNA [designed by using the siRNA Selection Program at the Whitehead Institute for Biomedical Research (<http://sirna.wi.mit.edu>)] (Yuan *et al.*, 2004), a TTCAAGAGA loop sequence separating the two complements, and a TTTTT terminator at the 3' end. A negative control shRNA vector targeting firefly luciferase (pU6-Luc) was constructed similarly with a target sequence of 5'-TGCGCTGCTGGTGCCAAC CCTATTCT-3' (To *et al.*, 2012). MCF-7 and MCF-7 FLV1000 cells were transfected with pU6-Luc or pU6-PPAR γ using Lipofectamine 2000 (Invitrogen). Silencing efficiency was assessed by reverse transcription (RT)-PCR and immunoblot analysis.

Flow cytometry

The cell surface ABCG2 expression and ABCG2-mediated efflux activity (i.e. transport activity) in MCF-7 FLV1000 cells

with or without the PPAR γ agonist pretreatment (24 h) were determined by flow cytometric assays as described previously (Robey *et al.*, 2004). To measure the cell surface ABCG2 expression, trypsinized cells were incubated in 2% BSA/Dulbecco's PBS with either phycoerythrin-conjugated anti-ABCG2 antibody 5D3 (eBioscience, San Diego, CA, USA) or phycoerythrin-conjugated mouse IgG2b negative control antibody (eBioscience) according to manufacturer's instructions for 30 min at room temperature. The anti-ABCG2 5D3 antibody recognizes an extracellular epitope of the protein (Ozvegy-Laczka *et al.*, 2005; Polgar *et al.*, 2010), therefore its signal represents cell surface ABCG2 expression. The cells were then washed with Dulbecco's PBS and subsequently analysed. Surface expression of ABCG2 was calculated as the difference in mean channel numbers between the 5D3 antibody and the negative control antibody histograms. ABCG2 transport activity was also examined in the PPAR γ agonists-pretreated cells. MCF-7 and MCF-7 FLV1000 after 24-h pretreatment of PPAR γ agonists (telmisartan 10 μ M, pioglitazone 5 μ M, rosiglitazone 25 μ M) were trypsinized and were incubated in 1 μ M PhA with or without 10 μ M ABCG2-specific inhibitor FTC in complete medium (phenol red-free RPMI 1640 with 10% FBS) at 37°C in 5% CO $_2$ for 30 min. Subsequently, the cells were washed with cold complete medium and then incubated for 1 h at 37°C in PhA-free medium continuing with FTC to generate the FTC/efflux histogram, or without FTC to generate the efflux histogram. The FTC-inhibitable PhA efflux was determined as the difference in mean fluorescence intensity (Δ MFI) between the FTC/efflux and efflux histograms, which indicates the ABCG2-mediated transport activity. The evaluation of ABCG2 transport activity was also repeated by replacing PhA with a fluorescent anticancer drug (mitoxantrone 10 μ M). Cells were finally washed with cold Dulbecco's PBS and placed on ice in the dark until analysis by flow cytometry.

Telmisartan, pioglitazone and rosiglitazone were also evaluated for direct competition with PhA for ABCG2-mediated efflux. In these experiments, an ABCG2-stable transfected HEK293 and an ABCG2-overexpressing drug resistant MCF-7 FLV1000 cell line were used. The cells, without drug pretreatment, were incubated directly with the three individual PPAR γ agonists in complete medium for 30 min at 37°C. Subsequently, the cells were washed with cold complete medium and then incubated for 1 h at 37°C in PhA-free medium continuing with the same PPAR γ agonist to obtain the efflux histogram. The ABCG2-specific inhibitor FTC was used as a control for comparison. Inhibitors of the transporter will shift the inhibitor/efflux histogram to the right, indicating retention of the fluorescent substrate in the cells.

Samples were analysed on an LSRFortessa Cell Analyzer (BD Biosciences, San Jose, CA, USA). Phycoerythrin fluorescence was detected with a 488-nm argon laser and a 585-nm bandpass-filter. PhA fluorescence was detected with a 488-nm argon laser and a 670-nm bandpass filter. Mitoxantrone fluorescence was detected with a 635-red diode laser and a 670-nm bandpass filter. At least 10 000 events were collected for all flow cytometry studies. Cell debris was eliminated by gating on forward versus side scatter and dead cells were excluded based on propidium iodide staining. All assays were performed in three independent experiments.

Analysis of transporter inhibition kinetics

The inhibition kinetic of the ABCG2-mediated efflux of PhA (an ABCG2 specific substrate) by the tested PPAR γ agonists was followed as described previously with minor modification (Zhang *et al.*, 2010). Briefly, ABCG2-stably transfected HEK293/ABCG2 cells were incubated with various concentration of PhA (1, 2, 5 and 10 μ M) in the presence of four designated concentrations of the PPAR γ agonists for 3 h at 37°C. The cells were then collected, centrifuged and washed once with cold PBS, and resuspended in the medium without PhA in the absence or presence of the PPAR γ agonists. Subsequently, cells were incubated for 10 min at 37°C to allow for efflux, centrifuged and washed three times with cold PBS. In the control samples, the incubations were kept at 0°C. Finally, the intracellular concentration of PhA was determined by flow cytometric analysis as above. The quantity of PhA efflux by ABCG2 was calculated by subtracting values obtained at 37°C from that at 0°C. The inhibitory effect of the three PPAR γ agonists on ABCG2 was then analysed by Dixon plot. In the Dixon plot, the K_i was estimated from the linear regression of reciprocal plot of PhA efflux rate versus the PPAR γ agonist concentration.

Immunofluorescence

Immunofluorescence studies were performed as previously described (Polgar *et al.*, 2004). Briefly, cells were seeded on sterile glass coverslips in 6-well culture dishes and grown overnight. Drug treatments with the various PPAR γ agonists (24 h) were carried out in these cells growing on glass coverslips. Afterwards, the cells were fixed with 4% formaldehyde and permeabilized with ice-cold methanol, and followed by blocking in a buffer containing 2 mg mL $^{-1}$ BSA, 0.1% Triton-X100 and 5% goat serum. Immunostaining was performed by incubation with a 1:100 dilution of the mouse monoclonal anti-ABCG2 antibody, BXP-21 (Kamiya Biomedical) and a 1:1000 dilution of the rabbit polyclonal anti-calnexin antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 2 h at room temperature. Non-specific antibody binding was then removed by washing twice in PBS. This was followed by incubation with secondary Rhodamine-conjugated donkey anti-mouse antibody and FITC-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Nuclei were counterstained with the DNA dye DAPI at 0.5 μ g mL $^{-1}$. Confocal images were generated on an Olympus FV1000-ZCD laser scanning confocal system fitted with an IX81 inverted microscope.

Results

PPAR γ agonists sensitized ABCG2-overexpressing resistant cancer cells

The cytotoxicity of mitoxantrone (ABCG2 substrate) or cisplatin (non-ABCG2 substrate) was evaluated in the absence or presence of telmisartan, pioglitazone or rosiglitazone in MCF-7 and its ABCG2-overexpressing resistant MCF-7 FLV1000 cells. This MCF-7 FLV1000 cell line has been routinely used as a reliable model for studying ABCG2-mediated multidrug resistance because only ABCG2 is up-regulated among all other major transporters (Robey *et al.*, 2001). The

Table 1

Cell proliferation was determined by SRB assay as described in Materials and Methods. Data are presented as means \pm SDs from at least three independent experiments performed in quadruplicate. The fold reversal of resistance was calculated by dividing the IC₅₀ for mitoxantrone (Top Panel) or cisplatin (Bottom Panel) in the absence of PPAR γ agonist by that obtained in the presence of PPAR γ agonist. The specific ABCG2 inhibitor, FTC, was used as the positive control. The three PPAR γ agonists when tested alone, telmisartan, pioglitazone and rosiglitazone, did not affect cell proliferation at concentrations below 20 μ M, 5 μ M and 60 μ M respectively. * $P < 0.05$, versus that obtained in the absence of inhibitor

	IC ₅₀ \pm SD (fold reversal)			
	MCF-7	MCF-7 FLV1000 (ABCG2-overexpression)	HEK293/pcDNA3 (stably transfected)	HEK293/ABCG2 (stably transfected)
Mitoxantrone alone	0.0097 \pm 0.0035 μ M	2.116 \pm 0.261 μ M	0.83 \pm 0.42 nM	46.2 \pm 3.5 nM
+FTC 5 μ M	0.0100 \pm 0.0021 μ M (1.0)	0.023 \pm 0.010 μ M (92)	0.79 \pm 0.37 nM (1.1)	1.08 \pm 0.36 nM (42.8)
+Telmisartan 1 μ M	0.0152 \pm 0.0251 μ M (0.6)	0.378 \pm 0.101* μ M (5.6)	1.42 \pm 0.69 nM (0.6)	28.8 \pm 6.9 nM (1.6)
+Telmisartan 2 μ M	0.0087 \pm 0.0022 μ M (1.1)	0.168 \pm 0.038* μ M (12.6)	1.80 \pm 0.72 nM (0.5)	9.6 \pm 2.1* nM (4.8)
+Telmisartan 10 μ M	0.0110 \pm 0.0260 μ M (0.9)	0.048 \pm 0.035* μ M (44.1)	1.08 \pm 0.63 nM (0.8)	1.6 \pm 0.5* nM (28.9)
+Pioglitazone 1 μ M	0.0109 \pm 0.0312 μ M (0.9)	1.519 \pm 0.253 μ M (1.4)	0.94 \pm 0.52 nM (0.9)	35.2 \pm 5.4 nM (1.3)
+Pioglitazone 2 μ M	0.0081 \pm 0.0045 μ M (1.2)	0.369 \pm 0.062* μ M (5.7)	0.88 \pm 0.49 nM (0.9)	24.1 \pm 3.6 nM (1.9)
+Pioglitazone 5 μ M	0.0042 \pm 0.0044 μ M (2.3)	0.162 \pm 0.031* μ M (13.1)	1.12 \pm 0.46 nM (0.7)	10.5 \pm 2.8* nM (4.4)
+Rosiglitazone 1 μ M	0.0126 \pm 0.0035 μ M (0.8)	0.175 \pm 0.032* μ M (12.1)	0.86 \pm 0.26 nM (1.0)	29.5 \pm 3.2 nM (1.6)
+Rosiglitazone 10 μ M	0.0082 \pm 0.0041 μ M (1.2)	0.119 \pm 0.031* μ M (17.8)	1.19 \pm 0.35 nM (0.7)	9.2 \pm 2.4* nM (5.0)
+Rosiglitazone 50 μ M	0.0039 \pm 0.0042 μ M (2.5)	0.095 \pm 0.021* μ M (22.3)	1.18 \pm 0.48 nM (0.7)	4.8 \pm 1.0* nM (9.6)
Cisplatin alone	11.23 \pm 2.11 μ M	12.45 \pm 1.43 μ M	2.02 \pm 0.34 μ M	2.15 \pm 0.86 μ M
+FTC 5 μ M	12.56 \pm 1.87 μ M (1.0)	11.98 \pm 1.37 μ M (1.0)	1.99 \pm 0.36 μ M (1.0)	2.35 \pm 0.76 μ M (0.9)
+Telmisartan 2 μ M	10.98 \pm 1.87 μ M (1.0)	13.15 \pm 2.11 μ M (0.9)	2.26 \pm 0.65 μ M (0.9)	1.98 \pm 0.74 μ M (1.1)
+Telmisartan 10 μ M	11.25 \pm 2.09 μ M (1.0)	12.78 \pm 1.88 μ M (1.0)	2.19 \pm 0.49 μ M (0.9)	2.05 \pm 0.68 μ M (1.0)
+Pioglitazone 2 μ M	10.76 \pm 2.02 μ M (1.0)	11.67 \pm 1.54 μ M (1.1)	1.86 \pm 0.59 μ M (1.1)	2.11 \pm 0.48 μ M (1.0)
+Pioglitazone 5 μ M	12.98 \pm 1.72 μ M (0.9)	12.19 \pm 1.76 μ M (1.0)	2.01 \pm 0.43 μ M (1.0)	1.98 \pm 0.56 μ M (1.1)
+Rosiglitazone 10 μ M	11.76 \pm 2.14 μ M (1.0)	12.64 \pm 1.76 μ M (1.0)	1.59 \pm 0.86 μ M (1.3)	2.08 \pm 0.59 μ M (1.0)
+Rosiglitazone 50 μ M	10.88 \pm 1.65 μ M (1.0)	11.54 \pm 1.28 μ M (1.1)	1.89 \pm 0.46 μ M (1.1)	2.16 \pm 0.47 μ M (1.0)

concentration of the PPAR γ agonists tested alone in the assays did not affect cell growth. Of note, pioglitazone was only used at concentrations up to 5 μ M, which is limited by its aqueous solubility. As summarized in Table 1 (upper panel), all three PPAR γ agonists tested were able to sensitize MCF-7 FLV1000 cells to mitoxantrone but not a non-ABCG2 substrate cisplatin. Similar potentiation of mitoxantrone cytotoxicity was also obtained in an ABCG2-stably transfected HEK293 human kidney embryonic cell line (Table 1, lower panel), albeit a lower degree of resistance reversal was attained.

PPAR γ agonists inhibited ABCG2-mediated drug efflux

To understand the underlying mechanism(s) of resistance reversal, the competition between the PPAR γ agonists and a fluorescent ABCG2 substrate (PhA) for efflux was studied in the ABCG2-overexpressing MCF-7 FLV1000 and HEK293/ABCG2 cells by flow cytometric analysis. The read-out of the assay is the accumulation of the fluorescent ABCG2 substrate (PhA) after a 1-h drug-free efflux. Inhibition of ABCG2-mediated efflux is indicated by a shift to higher intracellular fluorescence signal. As illustrated in Figure 1, all three PPAR γ

agonists were found to inhibit the efflux of the fluorescent ABCG2 substrate in a concentration-dependent manner, with telmisartan being the most potent one followed by rosiglitazone and pioglitazone. Using the specific and potent ABCG2 inhibitor (FTC) as a reference for comparison, the inhibition of ABCG2 by the three PPAR γ agonists was found to be only mild to moderate.

Inhibition kinetics of ABCG2-mediated PhA efflux by PPAR γ agonists

To further elucidate the mode of interaction between the PPAR γ agonists with ABCG2, the efflux of the fluorescent ABCG2 substrate (PhA) at four different concentrations was studied in the absence and presence of the PPAR γ agonists. The resulting inhibition curves were transformed to a Dixon plot and analysed by linear regression. As demonstrated in the Dixon plots (Figure 2), all curves intersect at the x -axis, suggesting non-competitive inhibition. The K_i values for telmisartan, pioglitazone and rosiglitazone were found to be 14.3, 28.5 and 25.9 μ M, respectively, indicating a relatively weak interaction with ABCG2. For comparison, we have identified axitinib (a molecular targeting tyrosine kinase inhibitor) as a potent ABCG2 inhibitor from an

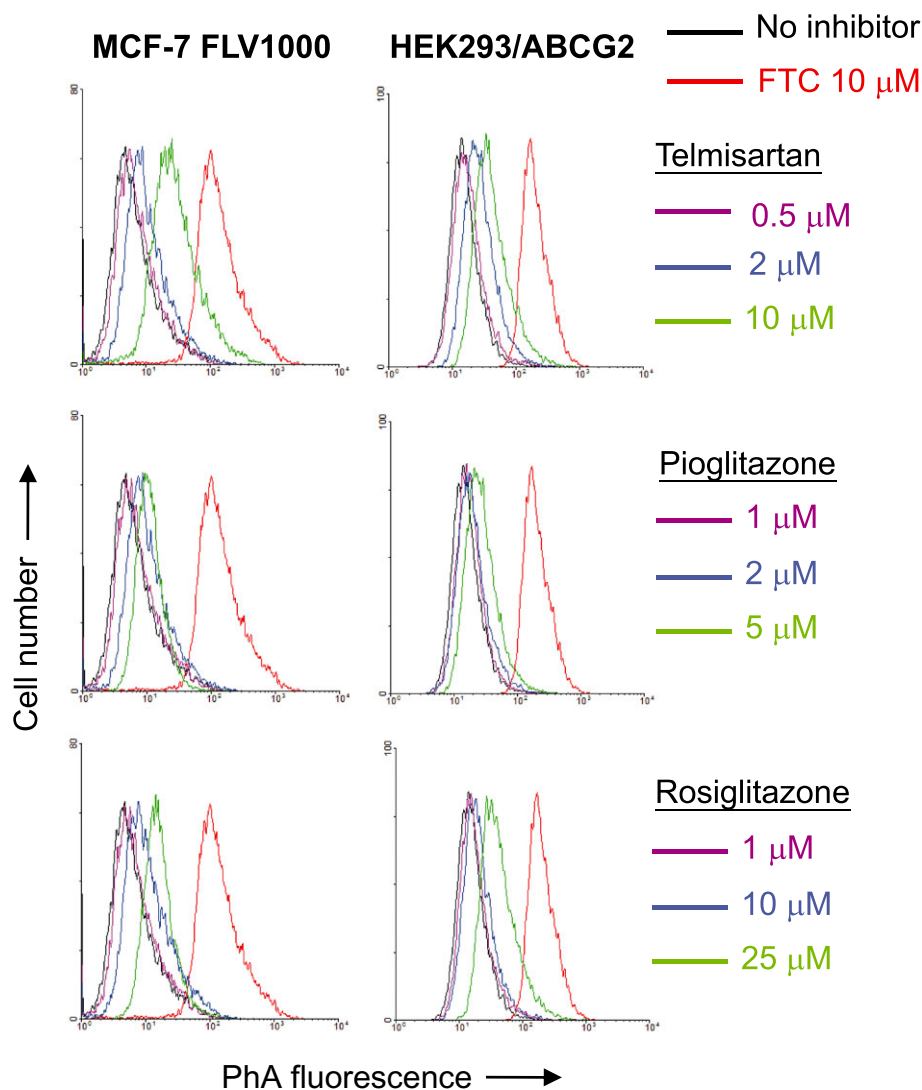


Figure 1

Direct inhibition of ABCG2-mediated pheophorbide A (PhA) efflux in ABCG2-overexpressing MCF-7 FLV1000 and ABCG2-stably transfected HEK293/ABCG2 cells. Cells (no pretreatment) were incubated with 1 μM PhA alone (black), 1 μM PhA with the various PPAR γ agonists at the indicated concentrations (various colours) or 1 μM PhA with 10 μM FTC (red) at 37°C for 30 min. PhA fluorescence retention in the cells after a 1-h PhA-free efflux was measured by flow cytometry. Representative histograms from three independent experiments are shown.

independent project with a K_i of 0.85 μM (detailed graph is not shown).

ABCG2 mRNA and total cellular protein levels were not affected by PPAR γ agonists

Besides the direct inhibition of ABCG2 transport function, the reversal of ABCG2-mediated MDR could also be mediated by decreased transporter expression. The effect of the three PPAR γ agonists on ABCG2 mRNA and protein expression was examined in MCF-7 and MCF-7 FL1000 cells by RT-real time PCR and immunoblot analysis. Our results indicated that 24-h treatment with the three PPAR γ agonists (at concentrations up to 10, 5, and 25 μM for telmisartan, pioglitazone and rosiglitazone, respectively) did not affect ABCG2 expression at both mRNA and protein levels (Figure 3).

PPAR γ agonists reduce surface expression of ABCG2 protein and transport activity

Since the direct inhibition of ABCG2 transport activity by the three PPAR γ agonists was only fairly moderate (Figure 1), it is unlikely to contribute to the remarkable enhancement of mitoxantrone cytotoxic effect especially in the ABCG2-overexpressing MCF-7 FLV1000 cells (Table 1). As shown in Figure 3, total cellular ABCG2 expression was not affected; therefore, we sought to investigate the possible change in localization of the transporter upon PPAR γ agonists treatment.

By flow cytometric analysis, ABCG2 surface expression was found to be substantially reduced in the PPAR γ agonists 24-h pretreated MCF-7 FLV1000 cells (Figure 4). The effect on the parental MCF-7 cells was very minimal because of the low

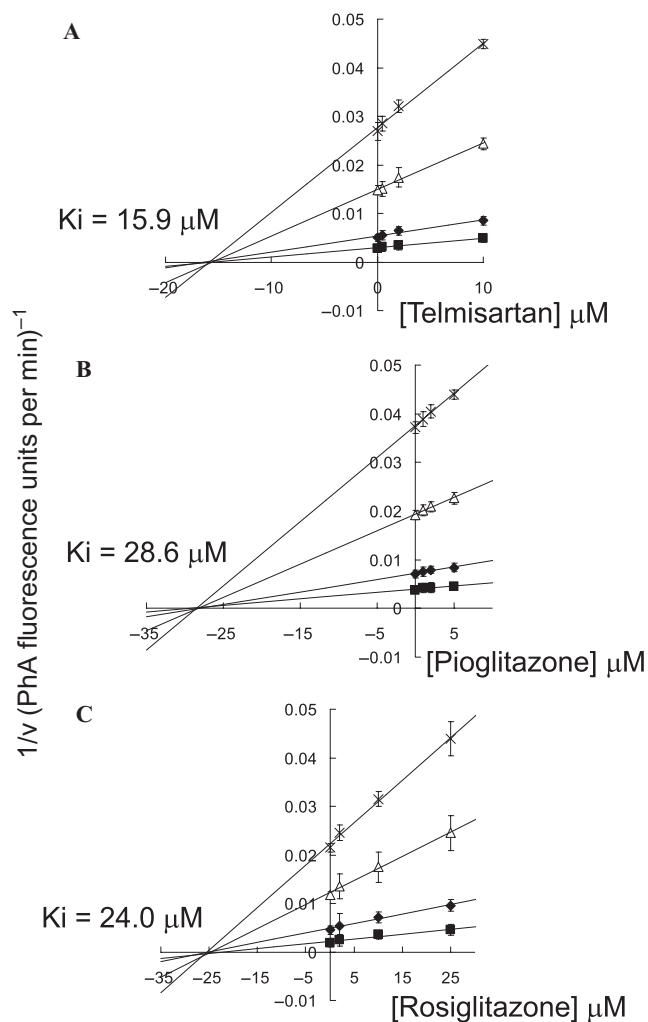


Figure 2

Inhibition kinetic of ABCG2-mediated efflux of PhA by PPAR γ agonists. HEK293/ABCG2 cells were incubated with different concentrations of PhA (1, 2, 5, and 10 μ M) in the presence of four concentrations of telmisartan (0, 0.5, 2, or 10 μ M), pioglitazone (0, 1, 2, or 5 μ M) or rosiglitazone (0, 2, 10, or 25 μ M) for 3 h. After a brief wash, the cells were allowed to incubate in PhA-free medium continuing with or without the PPAR γ agonist incubations to allow for efflux. The quantity of PhA efflux was measured for 10 min by flow cytometry, which was calculated by subtracting the fluorescence signal obtained at 37°C from that at 0°C. The reciprocal of PhA efflux rate is plotted against the concentration of the PPAR γ agonist using the Dixon plot. The intersection point represents the K_i value. Since the lines converge on the x axis, the PPAR γ agonists tested are likely non-competitive inhibitors of ABCG2. Each data point is presented as the mean \pm SEM from three independent experiments.

ABCG2 expression level in these cells. Consistent with the reduction in ABCG2 surface expression, FTC-inhibitable efflux of two ABCG2 fluorescent substrates [PhA and mitoxantrone (mito)] in MCF-7 FLV1000 (i.e. Δ MFI), representing the ABCG2-mediated transport activity, was also significantly decreased in the PPAR γ agonists 24-h treated MCF-7 FLV1000 cells (Figure 5; left panel – PhA and right panel – mito).

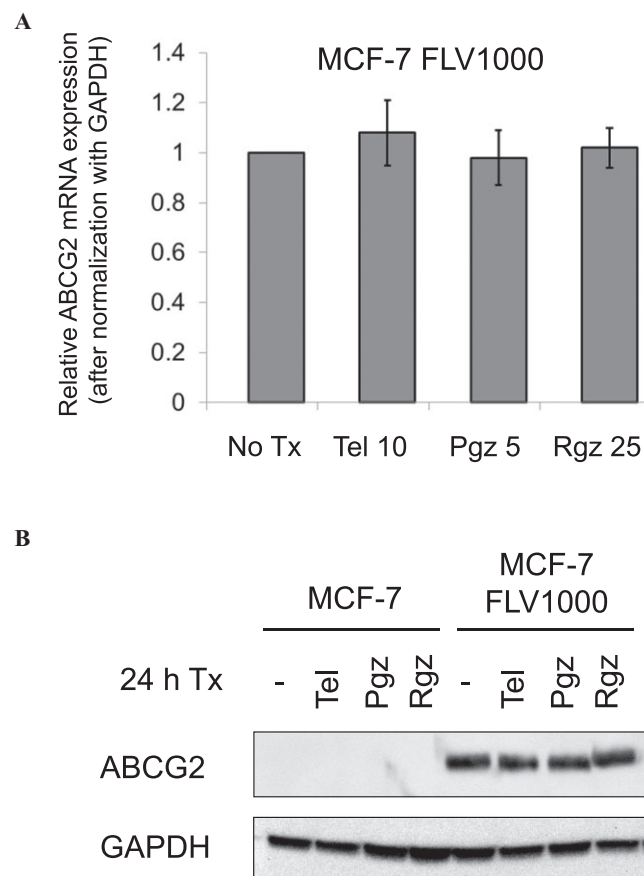


Figure 3

Quantitative PCR and immunoblot analysis showing that treatment with PPAR γ agonists did not affect ABCG2 expression at both mRNA and total cellular protein levels. (A) PCR analysis in MCF-7 FLV1000 cells treated with the three PPAR γ agonists for 24 h [at concentrations found to inhibit ABCG2-mediated transport; telmisartan (Tel): 10 μ M; pioglitazone (Pgz): 5 μ M; rosiglitazone (Rgz): 25 μ M]. mRNA expression were normalized with GAPDH. Relative ABCG2 mRNA levels were expressed relative to that in the untreated resistant MCF-7 FLV1000 cells. ABCG2 expression in the parental MCF-7 cells is only about 1/1500 times that in the resistant MCF-7 FLV1000 cells, which was also not affected by the PPAR γ agonists treatment (not shown in the figure). Mean \pm SD from three independent experiments is shown. (B) Immunoblot analysis under the same experimental conditions as in (A) above.

PPAR γ agonists led to the internalization of ABCG2 into cell cytoplasm

The decrease in the cell surface expression of ABCG2 (Figure 4) without a significant change in the total protein level (Figure 3) suggest a possible alteration in the cellular localization of the transporter after treatment with the three PPAR γ agonists. To further investigate the subcellular localization of ABCG2 after PPAR γ agonist treatment, cells were examined by immunofluorescence confocal microscopy. The untreated MCF-7 FLV1000 cells showed primarily plasma membrane staining with little intracellular signal (Figure 6). However, both intracellular and cell surface staining could be observed in the PPAR γ agonists-treated cells (Figure 6). The

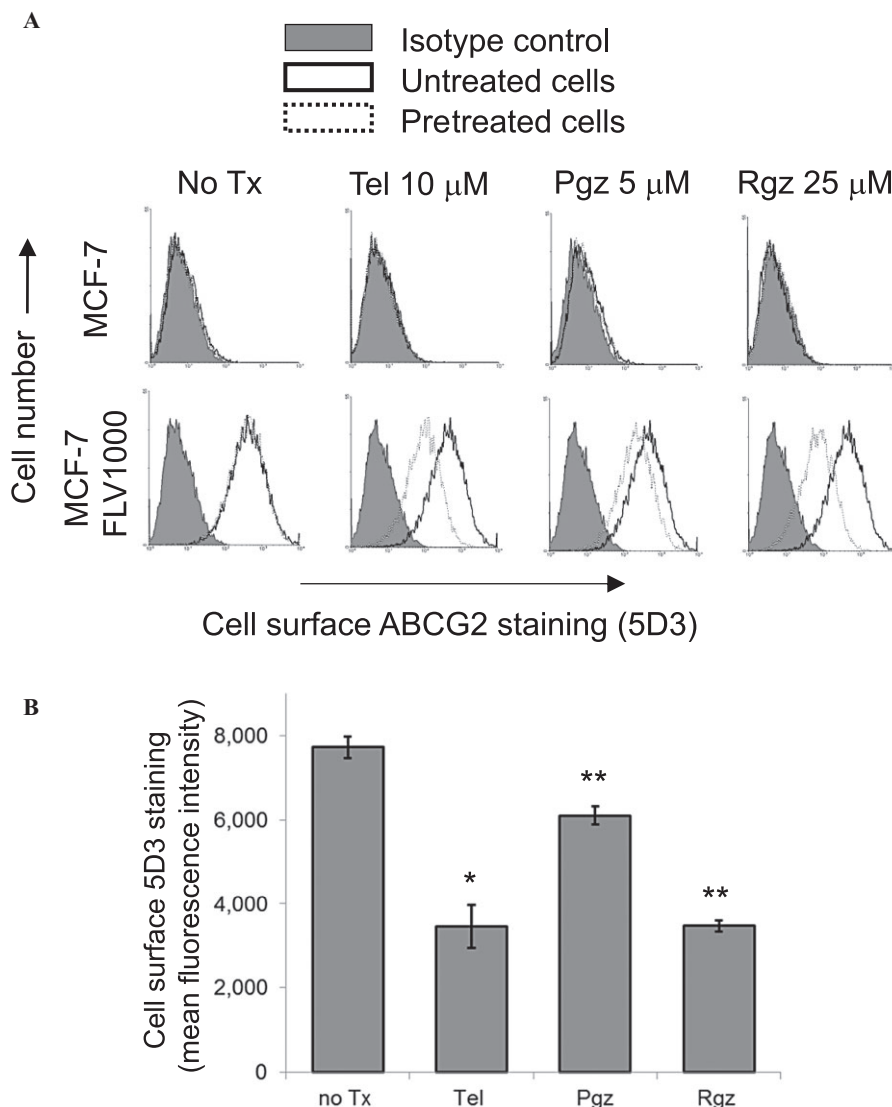


Figure 4

ABCG2 surface expression of PPAR γ agonists-treated MCF-7 and its ABCG2-overexpressing MCF-7 FLV1000 cells (24-h treatment). (A) Representative histograms showing the cell surface staining of ABCG2 by the 5D3 monoclonal antibody, which recognizes an extracellular epitope of ABCG2, of the untreated and PPAR γ agonists-treated cells. Cells were trypsinized and incubated for 30 min in phycoerythrin (PE)-labelled negative control antibody (shaded histogram) or 5D3 antibody (solid line: untreated cells; dashed line: PPAR γ agonists-treated cells) and analysed in a FACSort flow cytometry. The distance between the 5D3 histogram (solid or dashed lines representing untreated and pretreated cells, respectively) and the shaded negative control antibody histogram provide an indication of the amount of ABCG2 protein expressed on the cell surface. The assays were repeated in three independent experiments. (B) Cell surface 5D3 staining of the resistant MCF-7 FLV1000 cells before and after a 24-h treatment with the indicated PPAR γ agonists (telmisartan 10 μ M, pioglitazone 5 μ M, or rosiglitazone 25 μ M) was quantified by subtracting the fluorescence signal from 5D3 labelling by that from the control IgG isotype labelling. Mean \pm SD from three independent experiments is shown. *, $P < 0.05$; **, $P < 0.01$; compared with the untreated MCF-7 FLV1000 cells.

overlapping staining of ABCG2 (red) with the ER marker (green) in MCF-7 FLV1000 cells treated with the three PPAR γ agonists provides strong evidence for the subcellular location of ABCG2. The indispensable role of PPAR γ activation in the internalization of ABCG2 was further demonstrated by the recovery of cell surface expression of ABCG2 via genetic knockdown (by siRNA; Figure 6) or chemical antagonism of PPAR γ (by GW9662 200 nM; Supporting information Figure S1).

PPAR γ agonists reverse the PTEN loss in MCF-7 FLV1000 cells and inhibit Akt activation, presumably leading to internalization of ABCG2

Since the PTEN/PI3K/Akt signalling pathway has been proposed to regulate plasma membrane localization of ABCG2 (Bleau *et al.*, 2009), PTEN status and inhibition of Akt were examined in the PPAR γ agonists treated MCF-7 and MCF-7

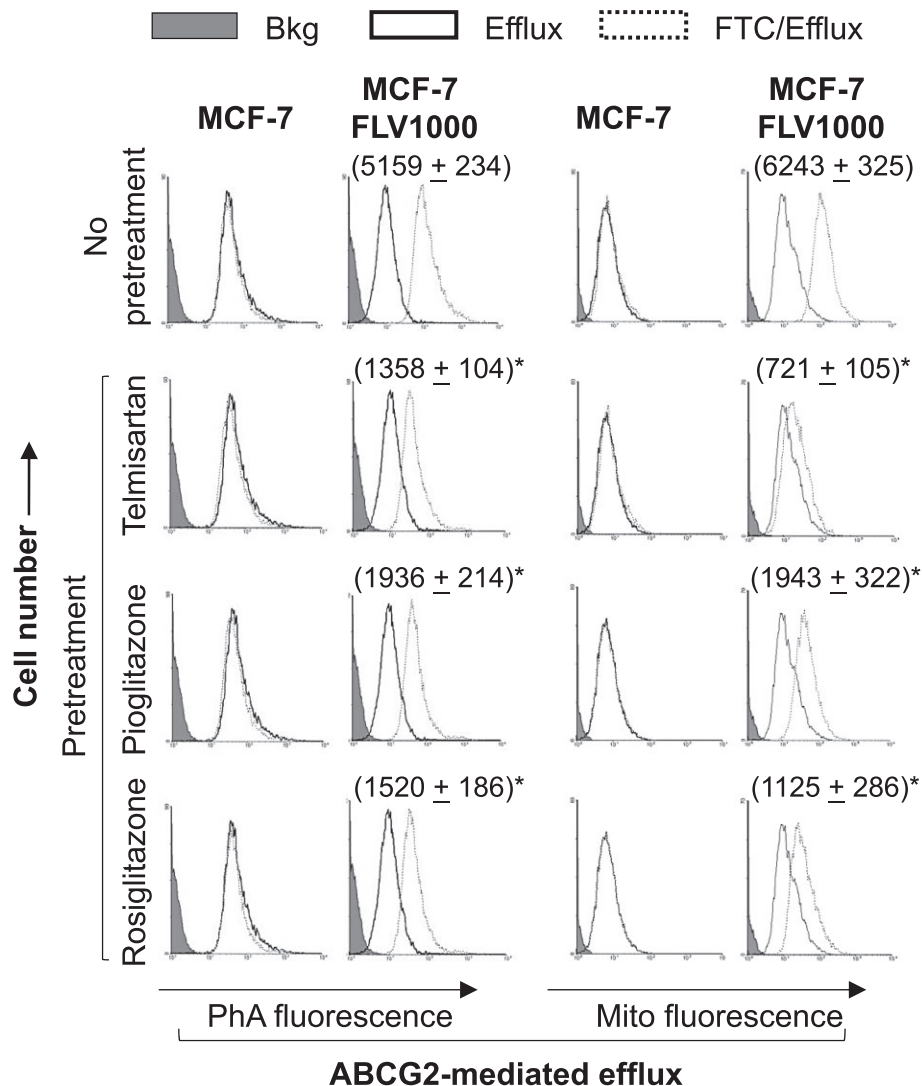


Figure 5

Flow cytometric analysis of ABCG2 transport activity in cells pretreated with PPAR γ agonists (telmisartan 10 μ M; pioglitazone 5 μ M; rosiglitazone 25 μ M; 24-h treatment). Left panel: Cells were incubated for 30 min in complete media containing 1 μ M pheophorbide A (PhA) with or without the ABCG2-specific inhibitor FTC (solid line – efflux/without FTC; dashed line – efflux/with FTC). The fluorescence signal between the efflux/with and efflux/without FTC histograms (i.e. Δ MFI) represents the ABCG2-mediated transport activity in the cells. Right panel: The same analysis as the left panel but PhA was replaced with a fluorescent anticancer drug mitoxantrone 10 μ M (mito). Negligible Δ MFI was obtained for the parental MCF-7 cells. Δ MFI values for the resistant MCF-7 FLV1000 under the different PPAR γ agonist treatments are reported in parenthesis (* $P < 0.05$, compared with untreated MCF-7 FLV1000 cells).

FLV1000 cells. A remarkably lower PTEN protein expression was noted in the resistant MCF-7 FLV1000 than in the parental MCF-7 cells (Figure 7). Interestingly, the down-regulated PTEN level in the MCF-7 FLV1000 cells was found to be elevated by treatment with all three PPAR γ agonists tested. This up-regulation of PTEN was dependent on PPAR γ because it was abolished by the chemical PPAR γ antagonist (GW9662, 200 nM) or genetic knockdown by PPAR γ siRNA. Active PTEN is known to inhibit the activation of its downstream effector Akt (i.e. phosphorylation) via its lipid phosphatase activity to inhibit cell proliferation. According to our immunoblot analysis (Figure 7), phosphorylated Akt (p-Akt) was only detected at a basal level in MCF-7 cells because of normal

cellular proliferation whereas elevated p-Akt was observed in the resistant MCF-7 FLV1000 cells probably because of PTEN down-regulation in the latter cells. Intriguingly, treatment of MCF-7 FLV1000 cells with all three PPAR γ agonists resulted in a significant decrease in p-Akt, concomitant with the increase in PTEN expression described above. The decrease in p-Akt by the PPAR γ agonists is PPAR γ -dependent because it was not observed in cells concomitantly exposing to the PPAR γ antagonist GW9662 or PPAR γ specific siRNA. The involvement of PTEN/PI3K/Akt pathway in the internalization of ABCG2 by PPAR γ agonists was further examined by the use of PI3K inhibitor (LY294002). As illustrated in Figure 8, treatment of MCF-7 FLV1000 by LY294002 for 16 h was found to

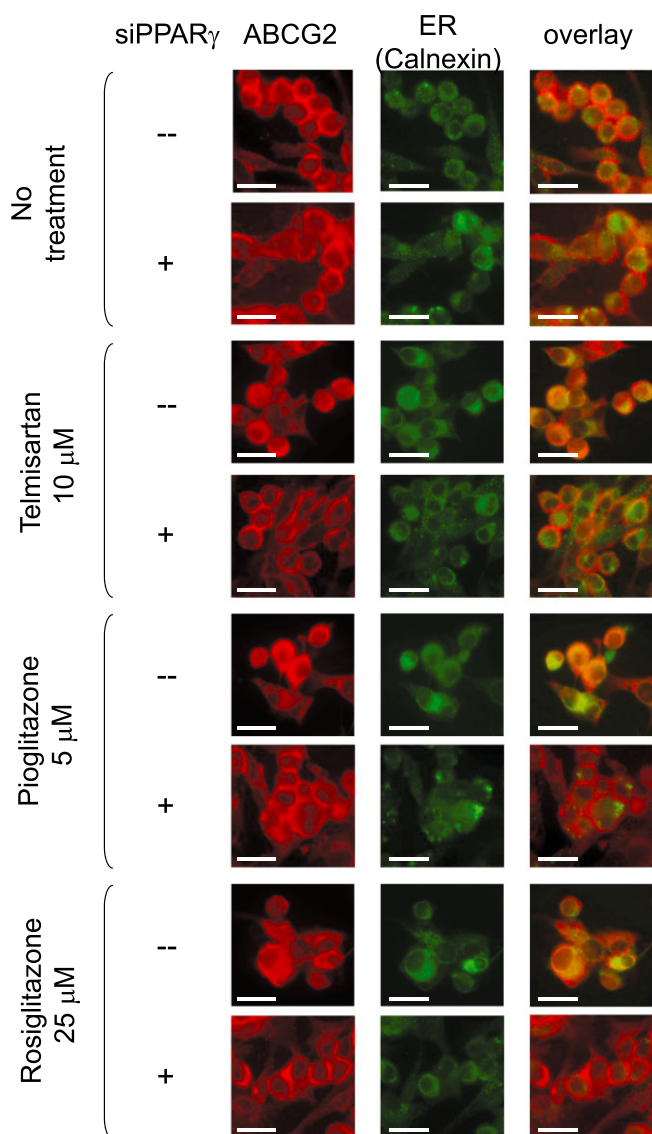


Figure 6

Immunofluorescence analyses of the translocation of ABCG2 in MCF-7 FLV1000 cells before and after treatment with PPAR γ agonists. Confocal microscopy of untreated MCF-7 FLV1000 cells or cells after a 24-h treatment with telmisartan (10 μ M), pioglitazone (5 μ M), or rosiglitazone (25 μ M) was performed following fixation with paraformaldehyde and permeabilization with ice-cold methanol. Immunostaining was conducted for 2 h at room temperature with the anti-ABCG2 BXP-21 antibody and anti-calnexin (ER marker) antibody. Nuclei were counter-stained with DAPI (blue), which is not shown for clarity. Control untreated MCF-7 FLV1000 cells shows predominantly cell surface expression of ABCG2 (red). In contrast, MCF-7 FLV1000 cells treated with the various PPAR γ agonists exhibited both cell surface and intracellular compartment localization of ABCG2. This is illustrated by the colocalization of ABCG2 with the ER marker calnexin (green) after drug treatment. When PPAR γ was silenced by siRNA, the predominant cell surface expression of ABCG2 was restored in PPAR γ agonists-treated MCF-7 FLV1000 cells. Scale bar, 50 μ m.

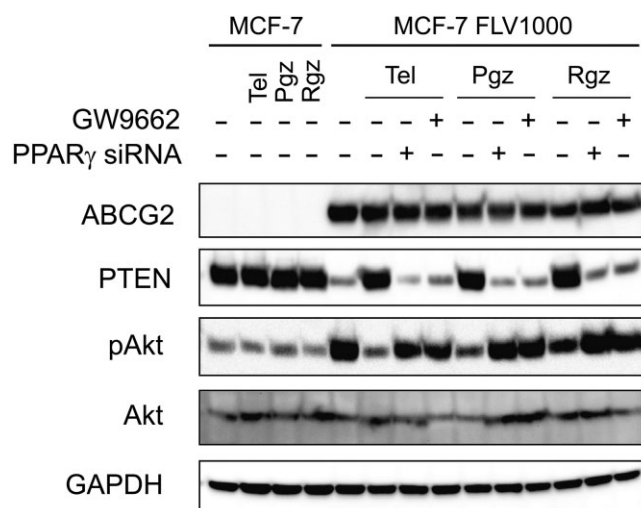


Figure 7

Immunoblot analysis showing that ABCG2 overexpression in the resistant MCF-7 FLV1000 cells is associated with PTEN loss and activation of Akt. The three PPAR γ agonists tested (Tel: telmisartan 10 μ M; PgZ: pioglitazone 5 μ M; Rgz: rosiglitazone 25 μ M; 24-h treatment) were found to correct this PTEN loss and decrease Akt phosphorylation. The specific effect of the PPAR γ agonists on PTEN and Akt was confirmed by PPAR γ knockdown with the chemical inhibitor GW9662 (200 nM) and a PPAR γ specific siRNA. Representative results from three independent and reproducible experiments were shown.

cause concentration-dependent translocation of ABCG2 from plasma membrane to the subcellular compartment (Figure 8A and B), mirroring the effect of the three PPAR γ agonists (Figure 6).

Discussion and conclusions

Various ABC transporters, including ABCG2, are often overexpressed in multidrug resistance cancer cells. There has been great interest in the development of novel inhibitors towards these ABC transporters as a strategy to circumvent multidrug resistance. Post-translational regulation of ABCG2 has been extensively studied in relation to the PI3K/PTEN/Akt signalling pathway in a variety of cancer cells (Mogi *et al.*, 2003; Aust *et al.*, 2004; Takada *et al.*, 2005; Hu *et al.*, 2008; Bleau *et al.*, 2009; Goler-Baron *et al.*, 2012). Inhibition of this pathway appears to facilitate the translocation of ABCG2 from plasma membrane to intracellular compartment, thus leading to apparent circumvention of the transporter-mediated drug resistance. Since the PI3K/Akt pathway is also regulating a number of other oncogenic effectors driving tumour growth and survival (Kitamura *et al.*, 2001; Chang *et al.*, 2003), inhibition of this pathway may be especially beneficial and may represent a druggable target for sensitization of cancer cells to chemotherapy.

PTEN is an important tumour suppressor gene that inhibits the PI3K/Akt pathway to mediate cell cycle arrest and apoptosis (Stambolic *et al.*, 1998; Salmena *et al.*, 2008). While PTEN down-regulation is frequently observed in

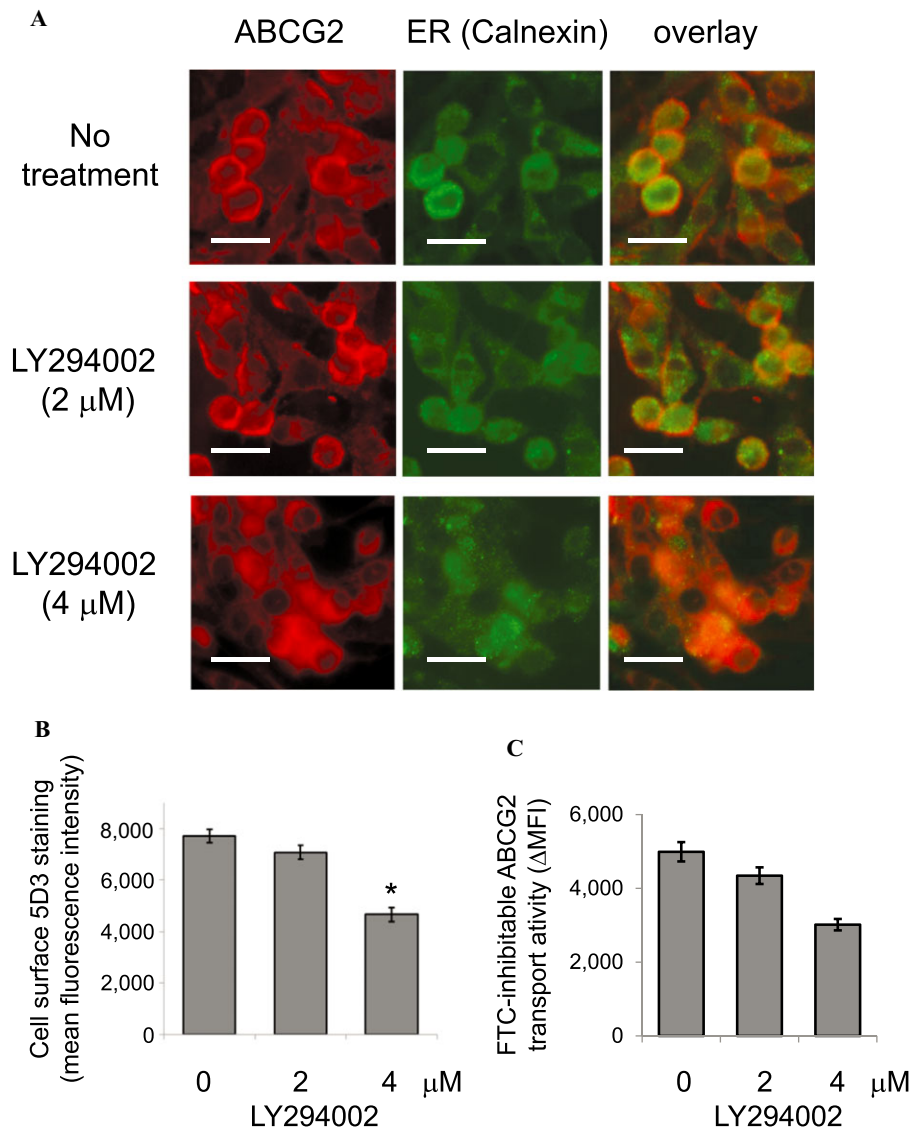


Figure 8

Immunofluorescence and flow cytometric analyses of the translocation of ABCG2 in MCF-7 FLV1000 cells before and after treatment with the PI3K inhibitor LY294002. (A) Confocal microscopy of MCF-7 FLV1000 cells after a 16-h treatment with 2 μ M or 4 μ M of LY294002 was performed as described in Figure 6. Representative images taken from three independent experiments are shown. Scale bar, 50 μ m. (B) Cell surface 5D3 staining of MCF-7 FLV1000 cells after treatment with LY294002 was performed as described in Figure 4. (C) ABCG2 transport activity (FTC-inhibitable PhA efflux) in MCF-7 FLV1000 cells treated with LY294002 (16 h; 2 or 4 μ M) was examined as in Figure 5. * $P < 0.05$, compared with untreated MCF-7 FLV1000 cells.

cancer, we have observed a remarkable decrease in PTEN expression in our resistant and ABCG2-overexpressing cancer cell line model relative to the respective parental cells (Figure 7). It follows that strategies to up-regulate PTEN level may be used to specifically target these ABCG2-overexpressing resistant cancer cells. Since PPAR γ is known to up-regulate PTEN transcriptionally (Patel *et al.*, 2001), three PPAR γ agonists (telmisartan, rosiglitazone and pioglitazone) which have been in clinical use were investigated for their modulation on the PTEN/PI3K/Akt pathway and possible circumvention of ABCG2-mediated drug resistance. Telmisartan is the only member of the ARB class of antihyp-

ertensive agents exhibiting PPAR γ activity whereas the TZD-class antidiabetic drugs (i.e. rosiglitazone and pioglitazone) represent the first few synthetic compounds investigated as PPAR γ ligands (Zhang *et al.*, 1996). Our goal is to generalize some insights for further clinical development. To this end, the MDR reversing concentration of the three PPAR γ agonists revealed from the present study (Table 1) is in fact achievable *in vivo*. According to a few recent pharmacokinetic studies, C_{max} of telmisartan 80 mg, pioglitazone 40 mg and rosiglitazone 8 mg was found to be around 1.3 (Smith *et al.*, 2000), 2.4 (Jaakkola *et al.*, 2005) and 1.7 μ M (Cox *et al.*, 2000) respectively.

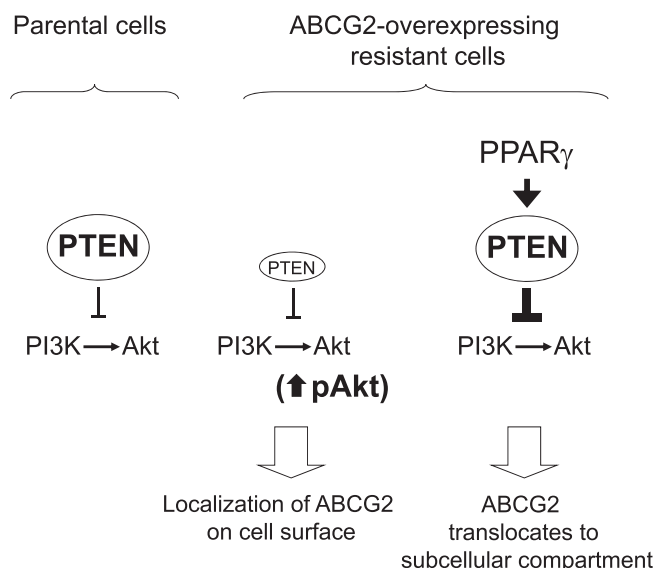


Figure 9

A working model illustrating the involvement of PTEN loss and the activation of Akt in the localization of ABCG2 in the resistant MCF-FLV1000 cells. While the aetiology for the dramatic decrease in PTEN expression in the resistant cells is still not clear, PPAR γ agonist treatments were found to reverse the PTEN loss in the resistant cells, subsequently inhibiting Akt, and leading to the re-localization of ABCG2 from the cell surface to the cytoplasm.

Our working model is presented in Figure 9. Briefly, the PTEN/PI3K/Akt signalling pathway is believed to regulate the subcellular localization of ABCG2 in cancer cells. In our resistant cell line model, significant PTEN down-regulation was noted, which is believed to allow activation of Akt for predominant ABCG2 expression on cell surface. PPAR γ agonists, by transcriptionally up-regulating PTEN, inhibit Akt phosphorylation and lead to translocation of ABCG2 from the plasma membrane to the intracellular compartment.

However, there have been some controversies about the involvement of the PI3K/Akt axis in regulating plasma membrane localization of ABCG2 (Hegedus *et al.*, 2012; Imai *et al.*, 2012). In these studies, ABCG2 translocation was not observed upon treatment with inhibitors of the downstream effectors of the PI3K/Akt/mTOR pathway (i.e. PI3K inhibitors: LY294002 and wortmannin; mTOR inhibitor: rapamycin). In our ABCG2-overexpressing resistant MCF-7 FLV1000 cell line model, translocation of ABCG2 from cell surface to subcellular compartment (Figure 8A and B) and decreased ABCG2 transport activity (Figure 8C) was observed when cells were treated with LY294002 (a PI3K inhibitor) for 16 h, which mirrors the effect of the three PPAR γ agonists tested (Figure 5 and 6). The underlying reason for the discrepancy is not clear. Nonetheless, our observed circumvention of ABCG2-mediated MDR is likely associated with this PPAR γ /PTEN/PI3K/Akt pathway because the concomitant administration of the chemical PPAR γ antagonist GW9662 or genetic knock-down of PPAR γ were found to abolish the observed elevation of PTEN (Figure 7), inhibition of Akt (Figure 7), internalization of ABCG2 (Figures 6 and Supporting information Figure S1) and apparent reversal of ABCG2-mediated

resistance (Supporting information Table S1) by the PPAR γ agonists.

PPAR γ is a ligand-mediated nuclear transcription factor that regulates gene expression by binding to PPAR γ response elements (PPRE) within the promoter of its target gene. To this end, three functional PPRE have been identified within a 150-bp long enhancer region located at about 4 kb upstream of the human ABCG2 promoter and were shown to mediate the upregulation of ABCG2 in human myeloid dendritic cells (Szatmari *et al.*, 2006). In our study, ABCG2 expression was not changed in the breast cancer cell lines used (Figure 3) and a few other colon cancer cell lines (data not shown). Various splice variants of the ABCG2 5'-untranslated leader exons have been reported and were shown to be related to alternative promoter utilization (Zong *et al.*, 2006; Campbell *et al.*, 2011; Natarajan *et al.*, 2011). An alternative leader exon E1U located at approximately 73 kb upstream from the reported ABCG2 transcriptional start site, has been identified and studied in bone marrow samples (Campbell *et al.*, 2011). To our knowledge, E1U is the only alternative leader exon identified that spans the PPRE. Coincidentally, PPAR γ activation has only been shown to increase the expression of the functional ABCG2 transporter in dendritic cells for protection against endogenous substances and xenobiotics (Szatmari *et al.*, 2006). This tissue-specific alternative promoter usage may explain why ABCG2 is not changed in the breast cancer cell line tested in our study.

Telmisartan tested in our study was known to be unique among other ARB class of antihypertensive agents in that it is the only one exhibiting PPAR γ agonist activity. Indeed, this PPAR γ -mediated pleiotropic effect of telmisartan was shown to cause induction of insulin sensitivity and a reduction of low-density lipoprotein cholesterol (Benndorf and Boger, 2008), which will be beneficial in patients in need for diabetes and/or lipid control. For comparison, three other ARBs including the classical losartan and two newer ones valsartan and irbesartan, which have no or very minimal PPAR γ agonist effect (Benson *et al.*, 2004; Schupp *et al.*, 2004), were also investigated for possible circumvention of ABCG2-mediated MDR. As presented in Supporting information Figure S2, all of these ARBs did not affect PTEN expression and consequently no inhibition of Akt was observed in MCF-7 FLV1000. They were also not able to alter the predominant cell surface localization of ABCG2 in the resistant MCF-7 FLV1000 (Supporting information Figure S3). Interestingly, these ARBs were also not found to be direct inhibitors for ABCG2 as demonstrated by flow cytometry-based drug efflux assay (Supporting information Figure S4). The possible pleiotropic effects of the tested PPAR γ agonists on other ABC transporters associated with multidrug resistance (MDR-1/P-gp and MRP-1) was also evaluated by quantitative real-time PCR. While basal expression levels of MDR-1/P-gp and MRP-1 in our MCF-7 FLV1000 cell model were both lower than those in the parental MCF-7 cells (by about 50%), they were not altered by the treatment with the PPAR γ agonists tested (Supporting information Figure S5). It is noteworthy that pharmacokinetic interaction between telmisartan and other ABCG2 substrate drugs is expected to be fairly minimal because of its weak inhibition on ABCG2-mediated transport activity (Figure 1). The final proof will await clinical pharmacokinetic study in patients concomitantly on telmisartan and

other ABCG2 substrate drugs. To this end, although telmisartan has been shown to inhibit significantly P-gp transport (Weiss *et al.*, 2010), it was not found to adversely affect the safety profile of digoxin (a well-known P-gp substrate drug) (Stangier *et al.*, 2000).

In summary, the three PPAR γ agonists tested were shown to reverse ABCG2-mediated MDR by (i) exerting moderate inhibitory effect on ABCG2 transport activity; and (ii) facilitating the translocation of ABCG2 from cell surface to intracellular compartments via a PTEN/PI3K/Akt pathway. Since PPAR γ agonists are also known to exert tumour suppressor and apoptotic effects by mechanisms other than the PI3K/Akt signalling axis (Bonofiglio *et al.*, 2011), the potential dual mechanism of resistance reversal advocates more in-depth investigation for their optimal clinical use for MDR circumvention.

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Conflict of interest

There are no potential conflicts of interest to be disclosed.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Table S1 Cell proliferation data showing that GW9662 alone did not affect cell viability but it abolished the circumvention of ABCG2-mediated resistance in MCF-7 FLV1000 by the PPAR γ agonists tested. Data are presented as means \pm SDs from at least three independent experiments performed in quadruplicate. The fold reversal of resistance was calculated by dividing the IC₅₀ for mitoxantrone in the absence of PPAR γ agonist by that obtained in the presence of PPAR γ agonist. * $P < 0.05$, versus that obtained in mitoxantrone alone in the same cell line.

Figure S1 Immunofluorescence analyses of the translocation of ABCG2 in MCF-7 FLV1000 cells before and after treatment with PPAR γ agonists. Confocal microscopy was performed as described in Figure 8. In the presence of the chemical PPAR γ antagonist (GW9662, 200 nM), the predominant cell surface expression of ABCG2 was restored in PPAR γ agonists-treated MCF-7 FLV1000 cells. Scale bar, 50 μ m.

Figure S2 Immunoblot analysis showing that other angiotensin II receptor blockers [ARBs; including losartan (Lo), valsartan (Val) and irbesartan (Irbe)], which have minimal PPAR γ agonist effect, did not correct PTEN loss and affect Akt phosphorylation in MCF-7 FLV1000 cells. MCF-7 FLV1000 cells were treated for 24 h with these three ARBs at 50 μ M before harvested for immunoblot analysis. Representative

results from three independent and reproducible experiments are shown.

Figure S3 Immunofluorescence analyses of the plasma membrane localization of ABCG2 in MCF-7 FLV1000 cells before and after treatment with a few ARBs (losartan, valsartan, and irbesartan; at 50 μ M for 24 h) with minimal PPAR γ agonist effect. Confocal microscopy was performed as described in Figure 6. (A) Representative images taken from three independent experiments are shown. Predominant cell surface expression of ABCG2 was still observed after treatment with these ARBs. Scale bar, 50 μ m. (B) Cell surface 5D3 staining of MCF-7 FLV1000 cells after the indicated treatments was performed as in Figure 4. Mean \pm SD from three independent experiments is shown. There is no remarkable change in ABCG2 cell surface expression.

Figure S4 Flow cytometric PhA efflux analysis showing that losartan, valsartan and irbesartan did not directly compete for ABCG2-mediated PhA efflux. The assay was performed as described in Figure 1 in MCF-7 FLV1000 or HEK293/ABCG2 cells incubated for 30 min with the indicated concentrations of the three ARBs tested. PhA fluorescence retention in the cells after a 1-h PhA-free efflux was measured by flow cytometry. Representative histograms from three independent experiments are shown.

Figure S5 Quantitative PCR analysis showing that the tested PPAR γ agonists did not affect expression levels of other ABC transporters [(A) MDR-1/P-gp and (B) MRP-1] commonly associated with multidrug resistance. Parental MCF-7 and resistant MCF-7 FLV1000 cells were treated with the three tested PPAR γ agonists for 24 h [at concentrations found to inhibit ABCG2-mediated transport; telmisartan (Tel): 10 μ M; pioglitazone (Pgz): 5 μ M; rosiglitazone (Rgz): 25 μ M]. Total RNA was then harvested for RT-PCR analysis. Relative MDR-1/P-gp and MRP-1 mRNA levels were expressed relative to that in the untreated parental MCF-7 cells, after normalization with GAPDH. It is noted that the basal expression of both MDR-1/P-gp and MRP-1 are lower in the resistant MCF-7 FLV1000 than in the parental MCF-7 cells. The PPAR γ agonists tested did not significantly affect MDR-1/P-gp and MRP-1 expression in MCF-7 FLV1000 cells. Mean \pm SD from three independent experiments is shown.