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Modulation of breast cancer resistance protein (BCRP/ABCG2) by non-basic chalcone analogues

*Yi Han, Meliana Riwanto, Mei-Lin Go, Pui Lai Rachel Ee**

Department of Pharmacy, Faculty of Science, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore

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ABSTRACT

Chalcones are biosynthetic precursors of flavonoids found to possess cytotoxic and chemopreventive activities. In this study, 17 non-basic chalcone analogues were synthesized and evaluated for their ability to modulate the function of either the human wild-type (482R) or mutant (482T) breast cancer resistance protein (BCRP/ABCG2) stably expressed in breast cancer MDA-MB-231 cells. At 5 μM, chalcones with 2,4-dimethoxy groups or 2,4-dihydroxyl groups on ring A were found to increase mitoxantrone accumulation to a greater extent than an established BCRP inhibitor, fumitremorgin C. At the same time, these chalcones had negligible effect on calcein accumulation in P-glycoprotein overexpressing MDCKII cells, indicating their potential as selective BCRP inhibitors. Functionally, these compounds were able to increase the sensitivity of BCRP-overexpressing cancer cells to mitoxantrone by 2–5-fold. The effect of chalcone compounds on both wild-type and mutant BCRP ATPase activity was also examined and variable effects were observed. A stimulatory effect was mostly observed with chalcones with 2,4-dimethoxy substitution on ring A which were earmarked as good BCRP inhibitors in the MX accumulation and cytotoxicity assays. These findings underscore the potential of methoxylated and hydroxylated chalcones as selective and potent inhibitors of BCRP whose mode of action may not involve the inhibition of ATPase activity.

1. Introduction

Multidrug resistance (MDR) remains a major obstacle to successful chemotherapy of many malignant diseases. Tumor cells often acquire resistance to a variety of anticancer agents with distinct chemical structures or mechanisms of action via the overexpression of ATP-binding cassette (ABC) transporters such as P-glycoprotein (Pgp) (MDR1/ABCB1) (Juliano and Ling, 1976), multidrug resistance-related protein 1 (ABCC1/MRP1) (Cole et al., 1992) and/or breast cancer resistance protein (BCRP/ABCG2) (Doyle et al., 1998). The latter is among the latest discovered to be involved in the MDR phenotype and is a half-transporter possessing a unique structure of only one nuclear binding domain and six transmembrane domains. Like Pgp and MRP1, BCRP displays broad substrate specificity and transports compounds that include chemotherapeutic agents such as mitoxantrone, topotecan, irinotecan, SN-38 and doxorubicin (Litman et al., 2000; Yang et al., 2000); fluorescent substances such as BODIPY-prazosin (Robey et al., 2001) and Hoechst 33342 (Kim et al., 2002); as well as chemical toxicants (Pavek et al., 2005). Substrate specificity has been found to be crucially dependent on mutations that alter the amino acid at position 482 of the BCRP protein. *In vitro* studies revealed that methotrexate is only transported by wild-type

* *Corresponding author*. Tel.: +65 6516 2653; fax: +65 6779 1554.
E-mail address: phaeplr@nus.edu.sg (P.L. Rachel Ee).
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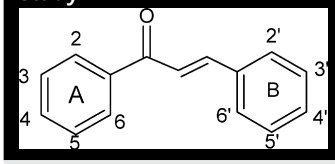
Table 1 – Structures of chalcones investigated in this study



Chalcone	Ring A	Ring B
1	2-OCH_3	H
2	2-OCH_3	2′-Cl
3	2-OCH_3	4′-Cl
4	2,4-OCH_3	H
5	2,4-OCH_3	2′-Cl
14	2,4-OCH_3	3′-Cl
6	2,4-OCH_3	4′-Cl
7	2,4-OCH_3	3′,4′-Cl
8	2,3,4-OCH_3	H
9	2,3,4-OCH_3	2′-Cl
10	2,3,4-OCH_3	4′-Cl
11	2,3,4-OCH_3	3′,4′-Cl
12	2-OH	H
13	2-OH	2′-Cl
15	2,4-OH	2′-Cl
16	2,4-OH	3′-Cl
17	2,4-OH	4′-Cl

(482R) BCRP and anthracyclines and rhodamine 123 only by mutant forms of BCRP (482T or 482G), whereas Hoechst33342 and mitoxantrone are transported by all variants (Yang et al., 2000; Honjo et al., 2001; Sarkadi et al., 2004).

One strategy to overcome MDR is to co-administer inhibitors of ABC transporters and anticancer drugs in order to increase intracellular substrate accumulation by preventing ATP-dependent drug efflux. In fact, several generations of compounds have been examined for their potential as Pgp and MRP1 inhibitors in preclinical and clinical studies, while relatively few are reported for BCRP inhibitors (Ahmed-Belkacem et al., 2006). Fumitremorgin C (FTC) is the first compound identified but its intensive neurotoxicity has precluded further *in vivo* applications (Nishiyama and Kuga, 1986; Allen et al., 2002). Some other drugs used clinically such as calcium channel blockers (Zhang et al., 2005) and corticosteroids (Cooray et al., 2006) were also found to be effective as BCRP inhibitors. However, drug–drug interactions may be a potential problem in patients taking these drugs concomitantly with BCRP substrate drugs. Apart from existing drugs, some natural products have been evaluated for their effect on BCRP activity in recent years. Flavonoids are among the most studied and characterized BCRP modulators (Cooray et al., 2004; Imai et al., 2004; Zhang et al., 2004; Ahmed-Belkacem et al., 2005; Katayama et al., 2007), while some curcuminoids (Chearwae et al., 2006) and ginsenosides (Jin et al., 2006) have also been shown recently to interfere with BCRP activity. In addition, chemical libraries of acridones and benzyopyranones have been tested for their effectiveness as BCRP inhibitors (Boumendjel et al., 2005, 2007).

Chalcones are bioprecursors of flavonoids that bear the structure of 1,3-diphenylprop-2-en-1-one (Table 1). This class of compounds has been reported to possess many biological activities such as anticancer proliferation (Liu and Go, 2006), antimalarial (Liu et al., 2001) and Pgp inhibitory activities (Bois et al., 1998, 1999). In an earlier study, we found that methoxylated chalcones with basic substituents on either ring A or B were good inhibitors of Pgp but negligible inhibitors of BCRP (Liu et al., 2008). For example, the basic chalcone 1-[5-(1-ethylpiperidin-4-yl)-2,4-dimethoxyphenyl]-3-phenylprop-2-en-1-one (Fig. 1A) increased calcein accumulation in Pgp-overexpressing MDCKII cells and enhanced the accumulation of the anticancer drug doxorubicin in MCF-7 cells selected for the overexpression of Pgp. However, it did not increase the uptake of mitoxantrone (MX, a substrate of BCRP) in MCF-7 cells that overexpressed BCRP. In contrast, a non-basic chalcone (**6**, Fig. 1B) showed a reverse profile—**6** did not increase calcein accumulation in Pgp-overexpressing cells but increased MX accumulation significantly in BCRP-overexpressing MCF-7 cells. We thus concluded that by introducing a basic group, Pgp inhibition was enhanced at the expense of BCRP inhibition. Conversely, it follows that omitting the basic functionality resulted in chalcones with more BCRP than Pgp inhibition. As chalcones have not been recognized as selective BCRP inhibitors or modulators, it was of interest to explore their potential in this area. Hence, we have synthesized several analogues of **6** and evaluated their effectiveness as specific BCRP inhibitors. The compounds were investigated for their abilities to increase MX accumulation in human breast cancer cells (MDA-MB-231) that were transfected with the human wild-type (482R) and mutant (482T) BCRP cDNA. These BCRP variants were used to shed light on the inhibitory specificities of the synthesized compounds. The same compounds were also tested for their influence on MX cytotoxicity profiles, as well as their effects on BCRP ATPase activity.

2. Materials and methods

2.1. *Chemistry*

Melting points were determined in open capillary tubes on a Gallenkamp melting point apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Spectrospin 300 Ultrashield spectrometer operating at 300 MHz. Spectra were analyzed using the Bruker 1D Win NMR software and chemical shifts (δ, ppm) were reported with tetramethylsilane as an internal standard. Mass spectra were collected on LcQ Finnigan MAT mass spectrometer fitted with an atmospheric chemical ionization (APCI) probe. Analytical thin layer chromatography was carried out on precoated silica gel 60 F254 (230–400 mesh) supported on aluminium sheets. Silica gel 60 (0.040–0.063 mm) was used for preparative column chromatography. Combustion analyses (C, H) were determined on a PerkinElmer PE 2400 CHN/CHNS Elemental Analyzer by the Department of Chemistry, National University of Singapore. Reagents used in the synthetic procedures were of synthetic or analytical grade. The syntheses and characterization of chalcones **1–11** have been described in earlier reports (Liu et al., 2001; Liu and Go, 2006) and chalcones **14–17** were synthesized in a similar manner as reported (Liu et al., 2001). The syntheses of the remaining chalcones **12–13** are reported in the following paragraph. The yields of the synthesized compounds, melting points and spectroscopic data are given in Table 1 (supporting material).

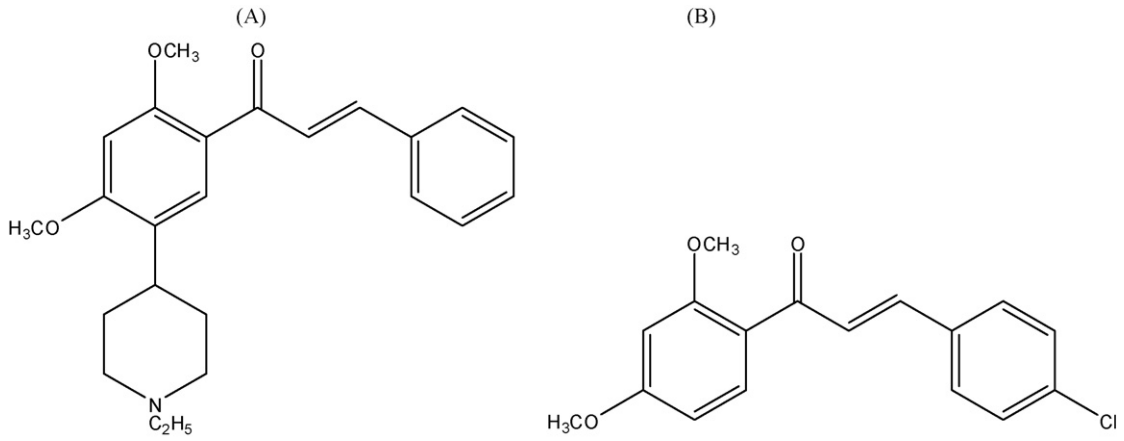


Fig. 1 – Structures of (A) the basic chalcone 1-[5-(1-ethylpiperidin-4-yl)-2,4-dimethoxyphenyl]-3-phenylprop-2-en-1-one, and (B) chalcone 6.

2.1.1. *General method for the synthesis of chalcones* 12 *and* 13

A mixture of 2-hydroxyacetophenone (1.36 g, 10 mmol) and the benzaldehyde (10 mmol) in ethanolic KOH (20%, w/v, 10 ml) was heated in an oil bath at 130 °C for 3–5 min. On cooling, the reaction mixture was poured into crushed ice, acidified with HCl, and kept at low temperatures (2–3 °C) overnight for the separated residue to solidify. The solid was then removed by filtration, washed several times with water and recrystallized with methanol.

2.2. *Materials*

All cell culture reagents were purchased from Invitrogen Singapore Pte. Ltd. (Singapore) unless otherwise stated. Calcein acetoxymethyl ester (calcein-AM), cyclosporine A, MX and FTC were purchased from Sigma–Aldrich Chemical Co. (Singapore). 3-(4,5-Dimethylthiazol-2-il)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from MP Biomedicals Inc. (Solon, OH, US). Mouse monoclonal antibodies for Western blot analyses of BCRP, Pgp, MRP1 and MRP2 were from Signet Laboratories Inc. (Dedham, MA, US) while the anti-mouse secondary antibody was from Amersham Biosciences Inc. (Piscataway, NJ, US).

2.3. *Cell culture*

All the cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. The breast cancer cell line, MDA-MB-231, stably transfected with expression vectors for wild-type 482R BCRP (MDA-MB-231/R cells), mutant 482T BCRP (MDA-MB-231/T cells) and pcDNA3.1 (MDA-MB-231/V cells) were kindly provided by Dr. Douglas D. Ross (University of Maryland Greenebaum Cancer Center, Baltimore, US). They were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 1% penicillin/streptomycin and 1 mg/ml G418. Mardin–Darby canine kidney cells transfected with expression vectors for human MDR1, MRP1 and MRP2 (MDCKII/MDR1, MDCKII/MRP1 and MDCKII/MRP2) were generous gifts from Dr. Anton Berns (Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, Amsterdam, Netherlands). They were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were subcultured when they reached 80–90% confluency and used within 10 passages for all assays.

2.4. *Western blotting*

20 μg of cell lysates from MDA-MB-231/V, MDA-MB-231/R and MDA-MB-231/T cells were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Bio-Rad Laboratories Pte. Ltd., Singapore). Membranes were blocked overnight and probed with primary antibodies followed by peroxidase conjugated anti-mouse secondary antibody. The following primary antibodies were used: anti-BCRP (BXP-21), anti-Pgp (C219), anti-MRP1 (MRPr-1) and anti-MRP2 (M2III-6). Proteins were visualized using the Supersignal West Pico chemiluminescence reagent (Pierce Chemicals, Rockford, IL).

2.5. *Flow cytometry*

The effects of the chalcones on the intracellular accumulation of a fluorescent BCRP substrate, MX, were determined by flow cytometry as previously described (Zhang et al., 2004). Briefly, cells cultured in 75 cm^2 flasks were trypsinized, washed with serum-free RPMI 1640 medium and PBS and resuspended in serum-free medium at a cell density of 10^6 cells/ml. The cells were pre-incubated with 5 μM of chalcones or vehicle (0.1% DMSO) for 15 min followed by incubation with MX (3 μM) for 40 min. FTC, a specific BCRP inhibitor, was used as a positive control. The cells were then washed thrice and resuspended in cold PBS prior to analysis in the CyAn™ Research Flow Cytometer (Dako, Glostrup, Denmark). Cells were excited at 488 nm with an argon laser and the emission recorded via a 670 nm long-pass filter for MX fluorescence. The raw data were recorded and analyzed with the Summit Version 4.3 software (Dako, Glostrup, Denmark). MX cellular levels were normalized to vehicle control levels, which was taken as 100%. Three or four independent experiments were performed.

2.6. Calcein-AM uptake assay

Calcein-AM accumulation into MDR1-overexpressing MDCKII/MDR1 cells was performed in the presence and absence of chalcones as previously described (Liu et al., 2008). Briefly, MDCKII/MDR1 and MDCKII/WT cells at 80–90% confluency were trypsinized, seeded in 96-well plates at a cell density of 5.0×10^4 cells/well, and incubated at 37 °C for 24 h in 5% CO_2 atmosphere. The cell monolayer in each well was carefully washed with PBS, followed by incubation with test compounds at a concentration of 10 μM, prepared in DMSO–Hank's buffered saline solution (HBSS) for 30 min. After 30 min incubation, an aliquot of calcein-AM in DMSO–HBSS was added to each well to give a final concentration of 2 μM. The maximum concentration of DMSO per well was 1% (v/v). After incubation (10 min), the fluorescence of each well was measured on a microplate reader with $\lambda_{excitation}$ of 485 nm and $\lambda_{emission}$ of 535 nm. Concurrent determinations were made with positive control, cyclosporine A, at 10 μM. The accumulation of calcein-AM in the presence of chalcones was calculated as the percentage ratio of the control value (in the absence of chalcones).

2.7. Cytotoxicity assays

The cytotoxicity profiles of MX in MDA-MB-231/V, MDA-MB-231/R and MDA-MB-231/T cells in the absence or presence of chalcones were evaluated using MTT assays. Cells were seeded into wells of 96-well plates in triplicates and incubated in a humidified atmosphere of 37 °C. After 24 h, culture medium in each well was replaced with fresh medium containing increasing concentrations (0–500 μM) of MX or vehicle (0.1% DMSO). Chalcones were added concomitantly to each well in a range of concentrations of 0.5, 1 and 2 μM. After 72 h of drug exposure, the drug-containing medium was aspirated, followed by the addition of MTT solution (1 mg/ml). The plates were incubated for 4 h at 37 °C prior to analysis. The purple formazan crystals formed were solubilized in DMSO and the absorbance measured at 595 nm. 100% cell survival was defined as the absorbance of cells exposed to control vehicle only (0.1% DMSO). The percentage survival of tested groups was calculated by dividing the absorbance values of tested groups by that of control vehicle. Growth inhibition by MX (IC_{50} value) either alone or with the chalcones was obtained by fitting a nonlinear, sigmoidal regression model to our data using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). The equation used by the software is a four-parameter logistic equation, the Hill equation and given as $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{(\text{LogIC}_{50}\text{-X})\text{HillSlope}})$, where X is the logarithm of concentration and Y is the response.

2.8. Crude membrane preparation and vanadate-sensitive ATPase assay

Crude membranes of MDA-MB-231/R and MDA-MB-231/T cells were prepared as described previously (Ambudkar, 1998). The protein concentrations were determined using the Bio-rad protein assay dye reagent and aliquots of the protein were kept at −80 °C until use. Protein concentrations of the crude membrane preparations were typically 5 mg/ml. The vanadate-sensitive ATPase assay was based on a colorimetric method as described previously (Xia et al., 2007). Crude membranes of MDA-MB-231/R and MDA-MB-231/T cells (10 μg protein) were first incubated for 90 min in assay medium (5 mM ATP, 50 mM KCl; 2 mM DTT; 50 mM MOPS–Tris; pH 7.0) in the presence of 1 mM EGTA, 2 mM ouabain and 5 mM NaN_3. Test compounds were then added in various concentrations in DMSO. DMSO was present at 1% in the final reaction mixture. The reaction was stopped by adding 30 μl of 5% SDS solution and the released phosphate or phosphate standards were measured at 800 nm after the reaction mixture was supplemented with 160 μl of the detection reagent (5 ml of 35 mM ammonium molybdate in 15 mM zinc acetate, pH 5.0, mixed with 20 ml of 10% ascorbic acid, pH 5.0) and incubated for 20 min at 37 °C. Vanadate-sensitive assays were performed in the presence of sodium orthovanadate (360 μM) and compared to control assays without the addition of sodium orthovanadate. BCRP-specific ATPase activity was calculated from the difference in the readings between these two values. The amount of inorganic phosphate released from the membrane was quantified against the phosphate standard KH_2PO_4 and was calculated as nmol/(min mg) protein.

2.9. Statistical analysis

Data from control and test groups were analyzed for statistical significance using one-way ANOVA with Dunnett post hoc test and Student's *t*-test using the SPSS 13.0 software (SPSS Inc., Chicago, IL).

3. Results

3.1. Synthesis of chalcones

Table 1 lists the structures of the chalcones investigated in this study. They were synthesized by a base-catalyzed Claisen Schmidt condensation, starting from appropriately substituted acetophenones and benzaldehydes. In the case of the hydroxylated chalcones (**15–17**), the hydroxyl groups on 2,4-dihydroxyacetophenone were protected with 2H-3,4-dihydropyran prior to condensation with the aldehyde. The tetrahydro-2H-pyran-2-yloxy groups were then removed by acid-catalyzed hydrolysis to give the final product. No protection of the hydroxyl group was required for the syntheses of chalcones **12** and **13**, probably due to the anomalous "ortho effect" of the 2-hydroxyl group in the acetophenone. The reaction scheme is given in Fig. 2. Details of the syntheses and characterization of chalcones **1–11** have been described earlier (Liu et al., 2001; Liu and Go, 2006) and chalcones **14–17** were synthesized in a similar manner as reported (Liu et al., 2001).

Since chalcone **6** was found to preferentially inhibit BCRP activity (Liu et al., 2008), it was chosen as the lead structure for modification. The structural variations made on **6** were aimed at understanding the contributions to activity made by the substituents on the chalcone template. On ring A, the number of methoxy groups was systematically varied from one (**1–3**), two (**4–7**, **14**) and three (**8–11**) groups. Replacement of the methoxy groups with hydroxyl groups was also explored

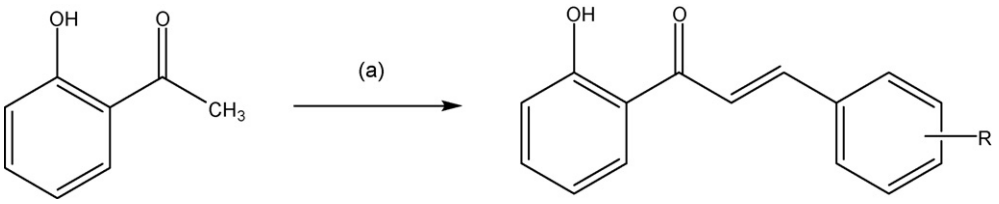


Fig. 2 – Reaction scheme for the synthesis of chalcones 12 (R = H) and 13 (R = 2′-Cl): (a) benzaldehyde or 2-chlorobenzaldehyde, ethanolic KOH, 130 °C, 3–5 min.

(**12**, **13** and **15–17**). Chalcone **16** is the demethylated analogue of chalcone **6** and its activity would highlight the role of the methoxy groups on ring A. The chloro substituent on ring B of **6** was retained in most compounds, but additionally, isomeric 2-chloro, 3-chloro and 3,4-dichloro analogues were included for each class of ring A substituted chalcones.

3.2. *BCRP expression and cytotoxicity profile of stably transfected MDA-MB-231 cells*

To verify the expression levels of BCRP in MDA-MB-231 cells stably transfected with empty vector (MDA-MB-231/V cells), wild-type 482R (MDA-MB-231/R cells) and mutant 482T (MDA-MB-231/T cells) BCRP transporter, western blot analysis was performed. As shown in Fig. 3A, both MDA-MB-231/R and MDA-MB-231/T cells displayed high levels of BCRP, whereas no detectable expression was observed in MDA-MB-231/V cells. To rule out any possible contribution by other transporters in this cell model, cellular protein extracts were also subjected to western blot analysis for the expression of Pgp, MRP1 and MRP2 using cell lysates from transfected MDCKII/MDR1, MDCKII/MRP1 and MDCKII/MRP2 cells as positive controls. As expected, no detectable levels of these proteins were found in all three cell lines (Fig. 3A). MTT assays using MX, a typical BCRP substrate, were then performed to evaluate the cytotoxicity profiles of these cells. As shown in Fig. 3B, the forced expression of both wild-type 482R and mutant 482T BCRP transporters in MDA-MB-231 cells conferred a 34–36-fold increase in resistance to MX, as compared to that transfected with an empty vector. We thus confirmed that this cell model is suitable for evaluating chalcone interactions with the BCRP transporter in the present study.

3.3. *Chalcones inhibited the efflux of mitoxantrone from both wild-type 482R and mutant 482T BCRP-expressing MDA-MB-231 cells*

To investigate the effect of chalcones on the accumulation of BCRP substrates, MX accumulation assays were carried out in the cell lines. MDA-MB-231/V, MDA-MB-231/R and MDA-MB-231/T cells were pre-incubated with DMSO vehicle, 5 μM chalcones or 10 μM FTC for 15 min before incubation with MX at 37 °C for 40 min. FTC, used as a positive control here, is a well-established specific BCRP inhibitor, whose use as a modulator in animal and human studies was precluded by serious neurotoxic effects (Nishiyama and Kuga, 1989). The accumulation of MX in the cells was measured as described in Section 2 by flow cytometry and shown as percentages relative to DMSO vehicle control in Fig. 4. In the presence of FTC, the MX accumulation amount was 300% of the control levels in both MDA-MB-231/R and MDA-MB-231/T cells (Table 2), indicating that FTC was active in inhibiting both wild-type (482R) and mutant (482T) BCRP, thus preventing the efflux of MX. Compared to untreated MDA-MB-231/R or MDA-MB-231/T cells, all compounds except **1**, **3**, **8**, **10** and **12** increased MX accumulation in these cells to a significant degree ($p < 0.01$), indicating that they were also inhibitors of the 482R and 482T BCRP protein. Among these "active" compounds, chalcones **2**, **5–7** and **14–17** elicited comparable or higher levels of MX accumulation compared to 10 μM FTC. These inhibitory effects were observed at low micromolar concentrations of the test chalcones, suggesting that these compounds worked effectively as BCRP inhibitors. MX accumulation assays were concurrently investigated on MDA-MB-231/V cells and neither FTC nor chalcones markedly increased MX accumulation levels in these cells (data not shown). This result further confirmed that the enhanced MX uptake in MDA-MB-231/R and MDA-

Table 2 – Effects of chalcones on MX accumulation in MDA-MB-231/R and MDA-MB-231/T cells

	MX accumulation[a]	
	MDA-MB-231/R	MDA-MB-231/T
Control	100 ± 9.2	100 ± 36.0
FTC (10 μM)	346.9 ± 18.6*	409.27 ± 15.6*
Chalcone (5 μM)		
1	204.6 ± 20.4	257.9 ± 36.7
2	332.7 ± 19.9*	432.5 ± 85.7*
3	211.3 ± 29.4	247.2 ± 33.8
4	295.4 ± 37.4*	406.3 ± 92.5
5	315.3 ± 38.6*	551.9 ± 49.0*
14	323.9 ± 61.7*	575.9 ± 48.6*
6	370.4 ± 42.0*	457.3 ± 82.0*
7	315.5 ± 25.4*	403.1 ± 20.4
8	231.2 ± 8.9	273.2 ± 18.8
9	265.7 ± 42.6*	447.9 ± 67.3*
10	254.2 ± 19.3*	282.8 ± 57.6
11	286.0 ± 50.1*	460.1 ± 135.9*
12	184.5 ± 31.3	408.1 ± 122.9
13	270.4 ± 29.3*	432.6 ± 133.8*
15	344.1 ± 77.7*	745.5 ± 119.7*
16	359.0 ± 79.8*	665.7 ± 108.2*
17	354.4 ± 69.1*	774.5 ± 206.7*

*$p < 0.01$.

[a] MX accumulation was expressed as percentage of control. Data from three independent experiments were expressed as mean ± S.E.M.

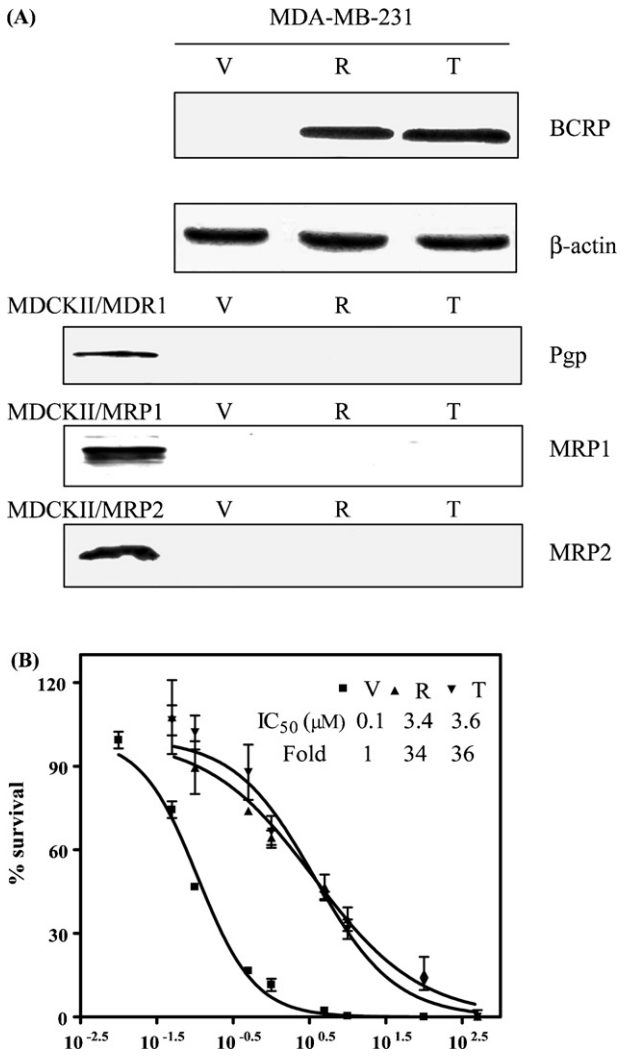


Fig. 3 – (A) Western blot analyses of BCRP, Pgp, MRP1 and MRP2 expressions and (B) IC_{50} of MX in MDA-MB-231/V, MDA-MB-231/R and MDA-MB-231/T cells. In (A), 20 μg of cell lysates from each cell type were subjected to SDS-PAGE and probed with anti-BCRP (BXP-21), anti-Pgp (C219), anti-MRP1 (MRPr-1) and anti-MRP2 (M2III-6) antibodies. Cell lysates derived from MDCKII/MDR1, MDCKII/MRP1 and MDCKII/MRP2 cells, overexpressing Pgp, MRP1 and MRP2, respectively, were used as positive controls for the corresponding transporters. β-Actin was used as loading control. In (B), cells in 96-well plates were treated with increasing concentrations of MX for 72 h, after which cell survival was determined via the MTT assay. Results were obtained from three independent experiments with at least triplicate measurements.

MB-231/T cells was due to the specific inhibition of BCRP transporter.

Interestingly, we found that the chalcone analogues displayed different modulation profiles on MX accumulation in the MDA-MB-231/R and MDA-MB-231/T cells (Fig. 4 and Table 2), with MDA-MB-231/T cells being more sensitive than MDA-MB-231/R cells. For the "active" compounds, the increase in MX accumulation was comparable to that observed with FTC (300–350%) in MDA-MB-231/R cells (Table 2). In MDA-MB-231/T cells, however, the same compounds induced a much higher increase in MX accumulation levels of up to 600–700%, which was twice that of FTC. Looking closely, we observed in particular that chalcones **5**, **9** and **14–16** caused significantly higher levels of MX accumulation ($p < 0.01$) in MDA-MB-231/T cells than MDA-MB-231/R cells (Fig. 4).

A cursory examination of the "inactive" compounds that did not significantly increased MX accumulation showed that these were chalcones with 2-methoxy, 2,3,4-trimethoxy or 2-hydroxy groups on ring A. No chalcone with 2,4-dimethoxy or 2,4-dihydroxy groups was represented among the "inactive" compounds. In addition, 2,4-dimethoxy and 2,4-dihydroxychalcones which had 2-chloro or 3-chloro on ring B (**5**, **14–16**) showed a greater potency in MDA-MB-231/T cells than MDA-MB-231/R cells. The 4-chloro analogues (**6**, **17**) from each class did not show such a difference.

3.4. *Chalcones do not affect transport activity of calcein-AM by Pgp*

Our recent report showed that various basic chalcones acted as Pgp inhibitors whereas a non-basic member (**6**) was a more potent inhibitor of BCRP than Pgp (Liu et al., 2008). Since this observation was made for only one chalcone (**6**), it was of interest to investigate this effect in other chalcones that are structurally related to **6**. Hence, we tested our chalcone library for their effects on Pgp-mediated uptake using the calcein-AM assay. Calcein-AM is a Pgp substrate, which upon intracellular deesterification is converted to calcein, a florescent substance (Litman et al., 2000). Consequently, fluorescence detected will be markedly increased in the presence of inhibitors of Pgp-mediated efflux of calcein-AM. As shown in Fig. 5, only cyclosporine (positive control) increased calcein-AM uptake to a significant degree ($p < 0.01$). The test chalcones had no significant effect on Pgp-mediated uptake, indicating that this series of compounds do not have the dual functionality reported earlier for the other chalcones (Liu et al., 2008).

3.5. *Reversal of mitoxantrone resistance by chalcone treatment*

To determine the functional effect of chalcone inhibition of the BCRP transporter, MX toxicity profile in MDA-MB-231/R and MDA-MB-231/T cells were evaluated using MTT assays. Initial experiments performed with chalcones alone in these cells yielded IC_{50} values of above 5 μM (data not shown). As such, various concentrations at 0.5, 1 and 2 μM were chosen for the following experiments. Table 3 shows the IC_{50} values of MX determined in the absence and presence of the chalcones on both cell lines. In the absence of the chalcones, the IC_{50} values of MX in MDA-MB-231/R and MDA-MB-231/T cells were 3.40 and 3.55 μM, respectively. These values were 34–36-fold higher than that of MDA-MB-231/V cells (IC_{50} 0.10 μM). In the presence of chalcones, the IC_{50} values of MX in MDA-MB-231/R and MDA-MB-231/T cells were significantly decreased in a concentration-dependent

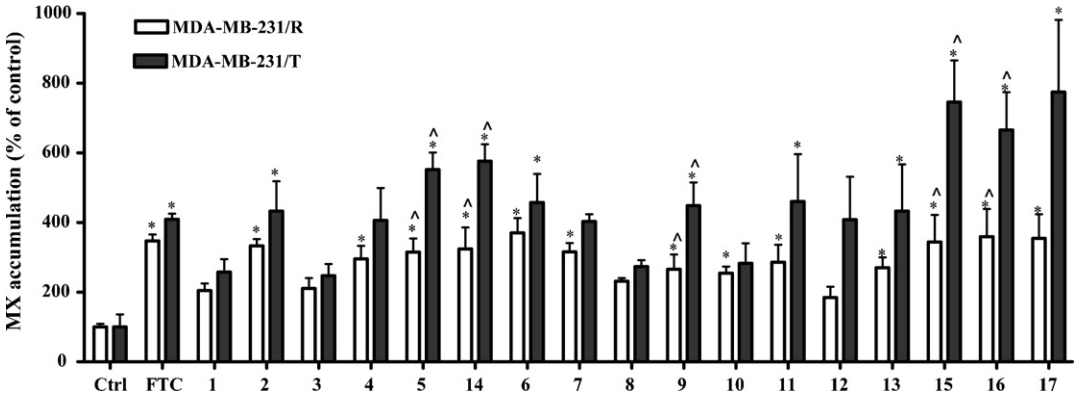


Fig. 4 – Effect of chalcones 1–17 on intracellular MX accumulation in MDA-MB-231/R and MDA-MB-231/T cells as measured by flow cytometry. Cells were pre-incubated with 10 μM FTC (positive control) or 5 μM chalcones or vehicle (0.1% DMSO) for 15 min, followed by incubation with 3 μM MX for 40 min. The level of intracellular fluorescence was then analyzed by flow cytometry and normalized with control fluorescence as percentage values after corrections for cell autofluorescence. Data were representative of 3–4 independent experiments and expressed as mean ± S.E.M. Ctrl, control; 1–17, chalcones 1–17. *Open bar*, MDA-MB-231/R cells; *closed bar*, MDA-MB-231/T cells. Data from vehicle control group and chalcone-treated groups were analyzed using one-way ANOVA for statistical significance. *$p < 0.01$. Statistical difference was also compared between MDA-MB-231/R and MDA-MB-231/T cells for each effective compound using Student's *t*-test. ^$p < 0.01$.

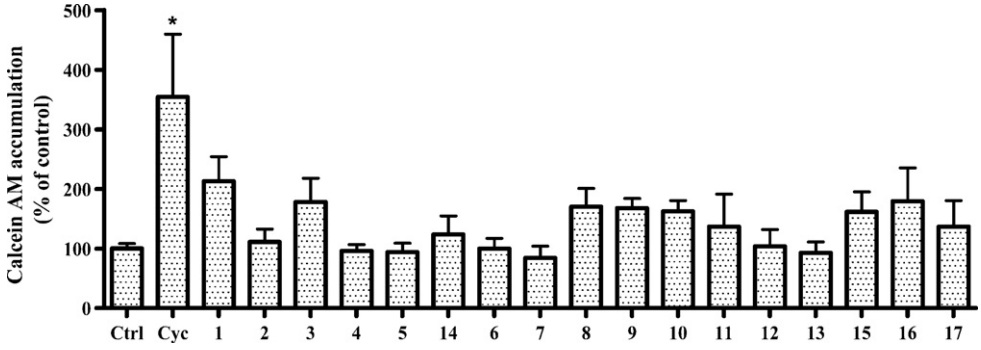


Fig. 5 – Effect of chalcones 1–17 on calcein-AM accumulation in Pgp-overexpressing MDCKII/MDR1 cells. Cells were incubated with 10 μM cyclosporine A (positive control) or 10 μM chalcones or vehicle control (1% DMSO) for 30 min, after which an aliquot of calcein-AM was added to each well to give a final concentration of 2 μM. After 10 min, the fluorescence of each well was measured on a microplate reader with $\lambda_{excitation}$ of 485 nm and $\lambda_{emission}$ of 535 nm. Ctrl, control; Cyc, cyclosporine A; 1–17, chalcones 1–17. Data were obtained from four independent experiments and were expressed as mean ± S.E.M. Data from control group and chalcone-treated groups were analyzed using one-way ANOVA for statistical significance. *$p < 0.01$.

manner (Table 3), showing that the chalcones potentiated MX cytotoxicity in the cells. Although such a modulation effect was observed for most of the chalcones evaluated, compounds **5** and **14–16** stood out for their potency on both MDA-MB-231/R and MDA-MB-231/T cells. In contrast, such modulation effect was not observed in the BCRP-deficient MDA-MB-231/V cells (data not shown). Our results clearly demonstrated the reversal of BCRP mediated-MDR to MX by the co-administration of chalcones. In correlation with the results obtained in MX accumulation studies, chalcones with 2,4-dimethoxy or 2,4-dihydroxyl groups on ring A (**4–6**, **14–17**) exhibited higher reversal activities than other chalcones (Table 3). However, no significant difference in the reversal of MX resistance by these compounds was observed between MDA-MB-231/R and MDA-MB-231/T cells.

3.6. *Effect of chalcones on BCRP ATPase activity*

BCRP transports substrates by utilizing ATP hydrolysis as energy source. As sodium orthovanadate inhibits only BCRP-specific ATPase activity (Xia et al., 2007), the vanadate-sensitive ATPase assay allows us to exclude contributions by other ATPases and quantify BCRP-specific ATPase activity. The difference between values obtained in the presence and absence of sodium orthovanadate will represent net ATPase activity contributed by BCRP. As shown in Fig. 6, the ATPase activities of the wild-type 482R and mutant 482T BCRP transporters were 7.1 and 8.2 nmol ATP hydrolyzed/(min mg) protein, respectively. The presence of 10 μM of FTC led to a significant reduction of BCRP ATPase activity to 4–5 nmol ATP hydrolyzed/(min mg) protein in T cell membranes ($p = 0.011$).

Table 3 – IC_{50} values of MX in MDA-MB-231/R and MDA-MB-231/T cells in the absence and presence of chalcones (0.5, 1 and 2 μM)

Chalcone[a]	IC_{50} values of MX[b]					
	MDA-MB-231/R (control[c]: 3.40 ± 0.16)			MDA-MB-231/T (control[c]: 3.55 ± 0.84)		
	0.5 μM	1 μM	2 μM	0.5 μM	1 μM	2 μM
1	1.52 ± 0.15*	1.51 ± 0.11*	1.24 ± 0.49*	2.92 ± 0.13	1.94 ± 0.20	1.20 ± 0.26*
2	1.83 ± 0.70	1.46 ± 0.19*	0.86 ± 0.21*	1.59 ± 0.63*	1.08 ± 0.43*	0.77 ± 0.26*
3	3.10 ± 1.08	1.97 ± 0.30*	1.67 ± 0.28*	3.19 ± 0.13	2.41 ± 0.81	2.02 ± 0.40
4	2.12 ± 0.50	1.14 ± 0.26*	0.87 ± 0.19*	2.49 ± 0.50	1.16 ± 0.15*	1.06 ± 0.30*
5	1.64 ± 0.27*	1.16 ± 0.34*	0.78 ± 0.27*	1.89 ± 0.37	0.88 ± 0.21*	0.60 ± 0.06*
14	1.33 ± 0.65*	0.82 ± 0.25*	0.45 ± 0.12*	1.52 ± 0.34	0.99 ± 0.29*	0.66 ± 0.29*
6	2.72 ± 0.37	2.72 ± 0.38	1.00 ± 0.24*	2.03 ± 0.15*	1.57 ± 0.67*	1.28 ± 0.25*
7	2.25 ± 0.40	2.14 ± 0.39*	1.96 ± 0.52*	2.97 ± 0.38	1.58 ± 0.44*	1.11 ± 0.25*
8	2.80 ± 0.54	2.04 ± 0.33*	1.53 ± 0.44*	3.18 ± 0.70	1.88 ± 0.26*	1.88 ± 0.29*
9	2.25 ± 0.40*	1.64 ± 0.26*	1.67 ± 0.33*	2.78 ± 0.06	1.80 ± 0.40*	1.23 ± 0.22*
10	3.01 ± 0.29	2.60 ± 0.19*	1.99 ± 0.23*	3.21 ± 0.74	2.03 ± 0.50	1.63 ± 0.62
11	2.61 ± 0.19*	3.12 ± 0.67	2.63 ± 0.54	2.86 ± 0.17	1.74 ± 0.41	1.28 ± 0.10*
12	2.33 ± 0.34*	2.56 ± 0.51	1.72 ± 0.29*	2.82 ± 0.44	1.87 ± 0.02	1.22 ± 0.39
13	2.50 ± 0.32*	1.73 ± 0.17*	1.31 ± 0.10*	1.70 ± 0.13	1.47 ± 0.30	0.67 ± 0.15*
15	1.10 ± 0.30*	1.13 ± 0.06*	0.70 ± 0.28*	2.86 ± 0.16	0.81 ± 0.11*	0.55 ± 0.10*
16	0.85 ± 0.27*	0.64 ± 0.16*	0.30 ± 0.07*	1.93 ± 0.27	1.02 ± 0.18*	0.64 ± 0.25*
17	2.22 ± 0.31*	1.42 ± 0.30*	1.03 ± 0.29*	1.72 ± 0.35	1.00 ± 0.22*	0.61 ± 0.16*

*$p < 0.01$.

[a] Chalcone, in the presence of each compound.

[b] Data from three independent experiments were expressed as mean ± S.E.M. in μM.

[c] Control, in the absence of chalcones.

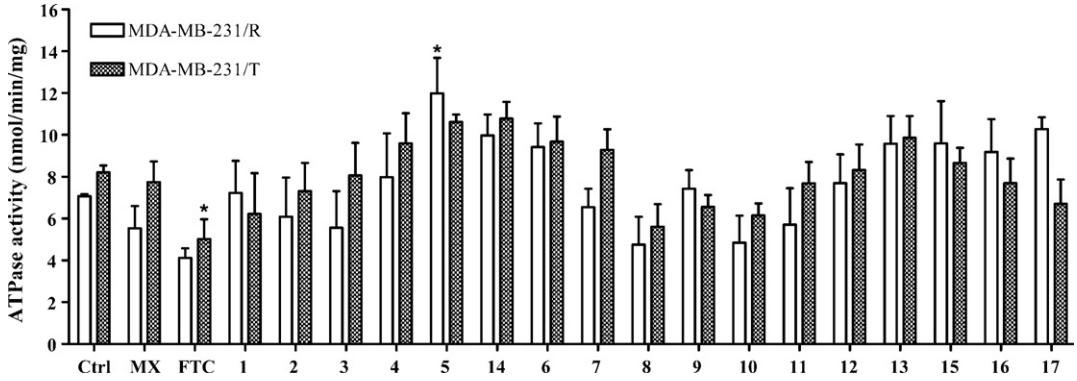


Fig. 6 – Effect of chalcones 1–17 on vanadate-sensitive BCRP ATPase activity in crude membranes taken from MDA-MB-231/R and MDA-MB-231/T cells. Crude membrane protein (5 mg protein/ml) from cells was incubated at 37 °C with 10 μM MX or FTC or chalcones in the presence and absence of sodium orthovanadate (360 μM) in ATPase assay buffer for 20 min. The reaction was stopped by adding 30 μl of 5% SDS solution and the released phosphate or phosphate standards were measured as described in Section 2. *Open bar*, MDA-MB-231/R cells; *shaded bar*, MDA-MB-231/T cells. Data were obtained from three independent experiments and expressed as mean ± S.E.M. Data from control group and chalcone-treated groups were analyzed using one-way ANOVA for statistical significance. *$p < 0.05$.

Although a similar reduction was observed in R cell membranes, there was no statistical significance ($p = 0.166$). MX, on the other hand, did not produce any significant effect.

Several 2,4-dimethoxy chalcones (**5**, **6** and **14**) stimulated BCRP ATP hydrolysis, with the rank order of **6** < **14** < **5**, although only the effect of compound **5** was statistically significant in MDA-MB-231/R cells ($p = 0.002$). These stimulatory effects on BCRP ATPase activity were not compatible with their inhibitory effects on BCRP activity in MX accumulation and toxicity studies, as reported in Sections 3.3 and 3.5. The conflicting findings may be related to the different concentrations of chalcones used for the various assays and this led us to determine if the effect of chalcones on BCRP ATPase activity showed a dose response relationship. Hence, we selected compounds **5** and **14** and evaluated their effects on BCRP ATPase activity on both MDA-MB-231/R and MDA-MB-231/T membranes at varying concentrations of 1, 2, 5, 10 and 20 μM. As seen from Fig. 7, a dose response effect was evident with these compounds. The stimulatory effects of compounds **5** and **14**

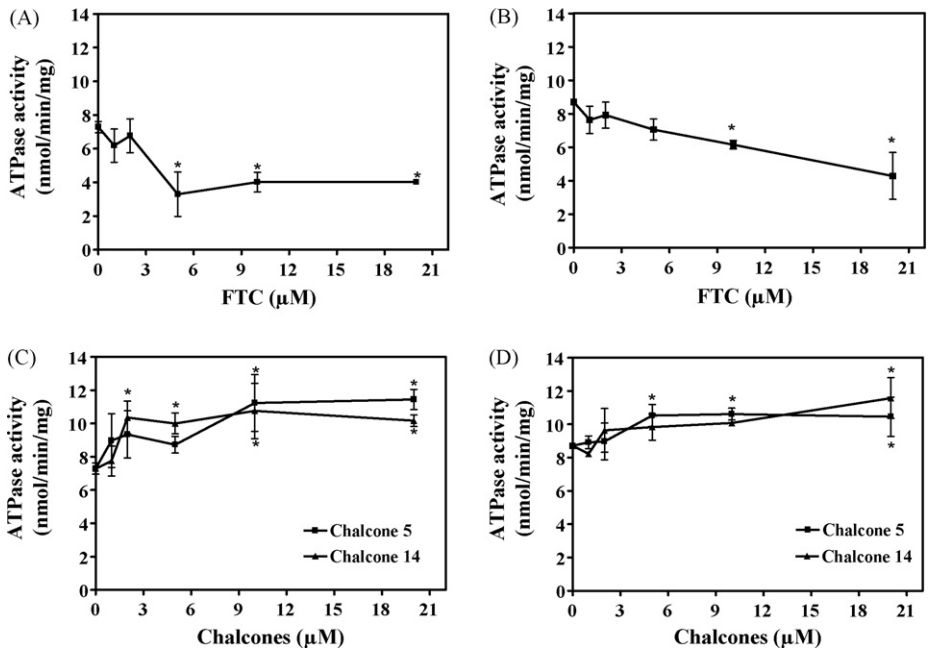


Fig. 7 – Concentration-dependent effects of FTC and selected chalcones (5 and 14) on vanadate-sensitive BCRP ATPase activity in crude membranes taken from MDA-MB-231/R (A and C) and MDA-MB-231/T (B and D) cells. The vanadate-sensitive BCRP ATPase assays were performed with the incubation of crude membrane protein (5 mg protein/ml) from cells with increasing concentrations of FTC or chalcones in the presence and absence of sodium orthovanadate (360 μM) in ATPase assay buffer at 37 °C for 20 min. The reaction was stopped by adding 30 μl of 5% SDS solution and the released phosphate or phosphate standards were measured as described in Section 2. Data were obtained from three independent experiments and expressed as mean ± S.E.M. Data from control group and treated groups were analyzed using one-way ANOVA for statistical significance. *$p < 0.05$.

increased with concentration (more gradually in the case of the MDA-MB-231/T membranes) and reached a plateau at 10 μM. Similar experiments were performed using membranes prepared from MDA-MB-231/V cells but no detectable readings were obtained (data not shown), suggesting that endogenous ATPase activities did not interfere with our assays.

4. Discussion

In this study, we investigated the ability of a small library of methoxylated and hydroxylated chalcones to modulate BCRP activity. The chalcones may be conveniently classified as 2-methoxy, 2,4-dimethoxy, 2,3,4-trimethoxy, 2-hydroxy and 2,4-dihydroxy chalcones, based on the substitution of the A ring. Ring B in the chalcone is either substituted with chloro groups (at 2′, 3′, 4′ or 3′,4′ positions) or left unsubstituted. We found a good representation of chalcones from the 2,4-dimethoxy and 2,4-dihydroxy families that significantly modulated BCRP activity as revealed by MX accumulation and toxicity assays. There was also a preference for a mono-halogenated ring B, with good inhibitory activity associated with 2′ and 3′ chloro groups.

Amino acid at position 482 has been shown to be critical in determining the substrate specificity of BCRP (Honjo et al., 2001). Recent reports also revealed inhibitor specificity on wild-type 482R and mutant 482T BCRP. Gupta et al. (2004) demonstrated a stronger inhibitory potential of HIV protease inhibitors on wild-type 482R BCRP than mutant 482T BCRP. In order to compare the inhibition specificity of the chalcones on wild-type and mutant BCRP, MX accumulation and toxicity assays were performed in the presence of chalcones at 5 μM on both MDA-MB-231/R and MDA-MB-231/T cells. A 3- or 6-fold induction was observed in MDA-MB-231/R and MDA-MB-231/T cells in the MX accumulation studies, respectively, showing that chalcones displayed a stronger BCRP inhibitory activity on MDA-MB-231/T cells than MDA-MB-231/R cells. In contrast, MX IC_{50} data obtained using lower concentrations of chalcones (0.5–2 μM) did not reveal significant differences in the ability of chalcones to reverse MX resistance in both cells. This was especially evident for compounds **5**, **9** and **14–16**, where there was a significant difference in MX accumulation ($p < 0.01$) between MDA-MB-231/R and MDA-MB-231/T cells, but not in the reversal of MX resistance. Furthermore, even though the sensitivity of the cells to MX was increased, it was not restored to levels similar to that of MDA-MB-231/V cells. This suggests that the specificity in inhibition occurred only in an acute manner but was not sustained throughout the 72 h of incubation in the cytotoxicity studies. In this case, we cannot rule out the possibility of rapid cellular metabolism of the chalcones which may have limited their effects over a longer period of time. Moreover, the concentrations used in the cytotoxicity studies were lower to ensure that the toxicity contributed by the chalcones alone did not interfere with the studies and higher concentrations may be used in future studies. In general, it

is thus unclear how the amino acid position 482 on BCRP may affect the recognition and binding of the chalcones to BCRP and further studies are required to elucidate its exact role.

BCRP is energized by ATP hydrolysis and the ATPase assay has been widely validated as one of the major methods to determine the binding affinity and inhibitory mechanism of BCRP inhibitors. Data in the literature suggested diverse effects and different mechanisms of interaction between BCRP inhibitors and the BCRP protein. Of the compounds observed to inhibit BCRP activity, FTC is used widely as an ATPase inhibitor (Rabindran et al., 2000); curcuminoids significantly stimulated wild-type BCRP ATPase activity with EC_{50} of 7.5–18 nM (Chearwae et al., 2006); 6-prenylchrysin and tectochrysin (belonging to the flavonoid family) showed a stimulation on mutant (482T) BCRP ATPase at a concentration of 5 μM but not on wild-type BCRP ATPase (Ahmed-Belkacem et al., 2005); and dietary phenethyl isothiocyanate was shown to inhibit BCRP ATPase activity (Ji and Morris, 2005). There are contradictory reports, however, on the effects of MX on BCRP ATPase activity in several cellular systems. A stimulatory effect was reported using MX resistant MCF/MX100 cells, which correlated well to its nature of being a BCRP substrate (Ji and Morris, 2005), whereas no significant changes in wild-type BCRP ATPase activity was observed in BCRP-transfected HEK293 cells with MX (Vethanayagam et al., 2005). Our data showed a correlation with the latter in that MX did not produce significant effect in both MDA-MB-231/R and MDA-MB-231/T membranes.

Surprisingly, although all our chalcone compounds inhibited BCRP-mediated drug accumulation and toxicity, they displayed diverse effects on BCRP ATPase activity. A stimulatory effect was mostly observed with chalcones with 2,4-dimethoxy substitution on ring A (**5**, **6** and **14**) which were earmarked as good BCRP inhibitors in the MX accumulation and cytotoxicity assays. A significant effect, however, was observed only in compound **5** although the other two members (**6** and **14**) have also shown high potency in MX accumulation and cytotoxicity. This discrepancy may be a result of low basal ATPase levels and relatively huge variations among the samples. Besides, similar stimulatory effects on BCRP ATPase activity have been observed with newly characterized BCRP inhibitors. Chearwae et al. has reported a concentration dependent stimulatory effect on BCRP ATPase activity with curcumin (Chearwae et al., 2006). Instead of exerting their inhibitory effect directly by inhibiting ATP hydrolysis, the authors proposed that curcumin might have bound to the drug binding site (ATP hydrolysis site) of the BCRP protein, leading to the stimulation of ATPase activity but the impairment of transporter efflux activity of substrate drugs. In our study, although the interactions between chalcones and BCRP remain to be elucidated, their mode of BCRP inhibition may not have involved BCRP ATPase inhibition.

Lastly, in good agreement with our previous study (Liu et al., 2008), we demonstrated that our extended series of non-basic chalcones increased BCRP-mediated drug accumulation and toxicity, with little or no effect on Pgp inhibition. This is further evidence that the removal of basic functionalities from chalcones caused a shift in the inhibitory profile from Pgp to BCRP. Such a finding shows that structural modifications can result in an increase in specificity in the inhibition of ABC transporters. It is also worth noting that besides BCRP and Pgp, several flavonoids have recently been shown to inhibit MRP1 and MRP2 (Van Zanden et al., 2005). Compounds such as diosmetin, chrysoeriol and tamarixetin were observed to inhibit MRP1 while myricetin and robinetin were observed to inhibit MRP2 with IC_{50} values in the micromolar range (Van Zanden et al., 2005). Given their structural similarities, we cannot exclude the possible effect of our chalones on these transporters. Further experiments need to be performed to evaluate their impact on other transporters other than BCRP and Pgp.

In conclusion, our present *in vitro* studies provide strong evidence of chalcones as effective BCRP-reversing agents and suggest that they may be potential candidates for development as BCRP inhibitors. MDR, as conferred by BCRP, has been a difficult barrier to overcome in the treatment of solid tumors and hematological malignancies. A specific BCRP inhibitor which would not interfere with other transporters and cause unwanted drug–drug interactions is highly desired in clinical use. Although our chalcone compounds do not inhibit Pgp, pharmacokinetic interactions with drugs that are BCRP substrates may still occur, resulting in alterations in bioavailability and clearance during concomitant use. In addition, mutant BCRP protein such as 482T BCRP has only been found in drug-induced cell lines, but we cannot exclude the possible expression of such proteins in cancer tissues due to extensive exposure to chemotherapeutic agents. If these conditions are encountered, chalcones may offer an advantage since they inhibit both the wild-type and one mutant form of BCRP.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2008.06.001.

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