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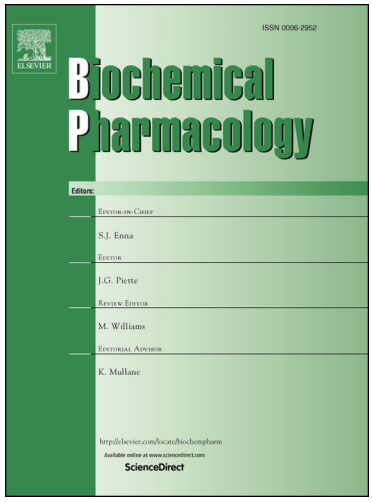

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Biological evaluation of non-basic chalcone CYB-2 as a dual ABCG2/ABCB1 inhibitor

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Abstract

The enhancement of drug efflux caused by ATP-binding cassette (ABC) transporters (including ABCG2 and ABCB1) overexpression is an important factor for multidrug resistance (MDR) in cancers. After testing the reversal activities of 19 chalcone and bis-chalcone derivatives on MDR cancer cell lines, we found that non-basic chalcone **CYB-2** exhibited the most potent reversal activities against both ABCG2- and ABCB1-mediated MDR. The mechanistic studies show that this compound can increase the accumulation of anticancer drugs in both ABCG2- and ABCB1-overexpressing cancer cell lines, resulting from the blocked efflux function of the MDR cancer cell lines. This inhibition is due to the barred ABCG2 and ABCB1 ATPase activities rather than altering the

expression or localization of ABCG2 or ABCB1 transporters. The previous studies showed that non-basic chalcones were ABCG2-specific inhibitors; however, we found that non-basic chalcone **CYB-2** can be developed as an ABCG2/ABCB1 dual inhibitor to overcome MDR in cancers that co-express both ABCG2 and ABCB1. Moreover, non-basic chalcone **CYB-2** has synthetic tractability compared to other chalcone-based derivatives.

Keywords

Multidrug resistance (MDR); Chalcone derivatives; ABCG2; ABCB1; ABC transporters

1. Introduction

The failure of chemotherapy is mainly caused by multidrug resistance (MDR), which involves a variety of cellular pathways. There are many factors that contribute to MDR in cancer, including the reduction of the intracellular accumulation and increase of drug efflux [1], enhanced DNA damage repair [2], cell death inhibition, epithelial-mesenchymal transition [3], drug target alteration [4], and the enhancement of drug inactivation [5]. The increase of drug efflux caused by the overexpression of ATP-binding cassette (ABC) transporters is an important contributor to MDR in cancer cells. ABC transporters with seven subfamilies (ABCA~ABCG) are a superfamily of 48 transporters expressed in humans, of which ABCE and ABCF subfamilies have no membrane transport functions [6]. ABC transporters consist of ABCG2 (breast cancer resistance protein/BCRP; mitoxantrone (MX)-resistant gene/MXR), ABCB1 (P-glycoprotein/P-gp). A large number of anticancer drugs are substrates of ABC transporters; thus these drugs can be pumped out of cancer cells that express ABC

transporters, subsequently decreasing in efficacy. Some well-known substrates for the ABC transporters are as follows: ABCG2 substrates (doxorubicin, mitoxantrone, SN-38, topotecan et. al.) [7]; ABCB1 substrates (colchicine, actinomycin-D, doxorubicin, paclitaxel, vinblastine, vincristine et. al.) [8].

It was also reported that chalcones with α,β-unsaturated carbonyl structure has pharmacological significance exhibiting various biological activities [9,10], e.g., anticancer [11–13], antimicrobial [14,15], anti-inflammatory, and antioxidant [16] properties. The heterocyclic ring system of chalcones can be used as excellent ligands in metal complexes, with various biomedical activities [17]. Several chalcone-based compounds have been approved and marketed [18,19], such as metochalcone (choleretic and diuretic agent), sofalcone (antiulcer and mucoprotective) and hesperidin methylchalcone (venous insufficiency treatment) [20], which reveals the varied utility of chalcone scaffolds in drug development. Some compounds with a chalcone scaffold, such as chalcone- and flavone-based compounds [21], quinazoline-chalcone compounds [22], bifendate-chalcone hybrids [23], and indole-basic chalcone [24], were found to be effective agents that reverse ABCG2- and/or ABCB1-mediated MDR. It was also reported that bis-chalcone with two α, β-unsaturated carbonyl system can reverse ABCG2-mediated MDR [25]. It was also reported that some non-basic chalcones can selectively reverse ABCG2-mediated MDR [26,27]. In previous reports, the chalcone compounds that inhibited both ABCG2 and ABCB1 were chalcone-based derivatives rather than non-basic chalcones.

The previous reports showed that some cancers overexpress both ABCG2 and ABCB1 [28]. The cancers co-expressing multiple transporters may cause more potent resistance to anticancer drugs than cancers overexpressing a single transporter [29]. Herein, we tested some non-basic methoxy and hydroxy chalcones to discover new chalcone derivatives that act as dual inhibitors of potent ABCG2 and ABCB1. We also included bis-chalcones to reveal whether the extended π system is crucial in increasing the reversal effects. We conducted the drug accumulation and efflux assay, Western blotting, immunofluorescence, ATPase assay and docking analysis to reveal the mechanism of chalcone derivatives reversing MDR in cancers.

2. Materials and methods

2.1. Chemistry

The chemicals and reagents for synthesis were purchased from Alfa Aesar (Haverhill, MA.) or Tokyo Chemical Industry (Tokyo, Japan). The synthesis of the chalcone derivatives except **CYB-2**, **CYB-7**, **CYB-15** had been reported in our previous study [30]. **CYB-2**, **CYB-7**, and **CYB-15** were synthesized through the same method as the other chalcone derivatives. The methoxy chalcones **CYB-2** and **CYB-7** were synthesized by the following procedures: benzaldehydes and acetyl benzenes were dissolved in methanol and aqueous KOH (50%) was added. After 3h reaction at 70℃, the solution was poured into water to form precipitates. Methanol was evaporated under reduced pressure. After extraction with CH_2Cl_2 and the evaporation, the raw product was purified by flash chromatography. **CYB-15** was synthesized through the demethylation of **CYB-13** with boron tribromide (BBr_3). The reaction underwent 24 h at room temperature, and

then the mixture was poured into ice water. The precipitate was purified by flash chromatography.

2.2. Cell culture

In this study, we used the human colorectal cancer cell line S1, the human lung cancer cell line NCI-H460 and S1-M1-80 (MX-selected ABCG2-overexpressing cell line of S1) [31], NCI-H460/MX20 (MX-selected ABCG2-overexpressing cell line of NCI-H460) [32]. HEK293/pcDNA3.1 is a human embryonic kidney 293 cell line with a pcDNA3.1 vector, while HEK/ABCG2-482-R2, HEK/ABCB1 were established with a pcDNA3.1 vector containing ABCG2-482-R2 and ABCB1. The transfected cell lines were maintained in medium with G418 (2 mg/mL) [33]. The KB-C2 cell line was selected by colchicine (Alfa Aesar, Haverhill, MA.) (2 μg/mL) with human cervical carcinoma cell line KB-3-1 [34]. The cell lines were cultured with Dulbecco's Modified Eagle Medium (Corning Inc., Corning, NY) under the condition of 37°C and 5% CO_2.

2.3. MTT assay

Cytotoxicity tests, inhibition and reversal effect assays were performed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Biosynth International, Inc., Itasca, IL) assay. Cells were seeded 24h prior to the addition of the drugs. For the MDR reversal activities study, chalcones or positive controls were pre-incubated 2 h prior to the addition of the chemotherapeutic drugs. After 72 h, the MTT reagent (4 mg/mL) was added for 4 h incubation. Subsequently, the supernatant was removed and 100 μL of dimethyl sulfoxide (DMSO) (MilliporeSigma Company, Burlington, MA) was

added to dissolve the purple crystals. The absorbance was measured with a UV/Vis microplate spectrophotometer (Fisher Scientific International, Inc., Hampton, NH).

2.4 Tritium-labeled drugs accumulation and efflux assay

The drug accumulation assay was conducted as the previous report [35]. The reversal agents were pre-incubated for 2 h, then the [^{3}H]-MX (Moravek Biochemicals Inc., Brea, CA) or [^{3}H]-paclitaxel-containing (Moravek Biochemicals Inc., Brea, CA) medium was added, in the presence or absence of reversal agents. After 2 h of incubation, the cell lysates were added into scintillation fluid (5 mL). For the efflux assay, the procedures of drug accumulation study are performed. Subsequently, the tritium-labeled drug and reversal agents containing medium was removed and the cells were incubated with medium containing only reversal agents. And then the cells were lysed at various time points (0, 30, 60, and 120 min) and the cell lysates were added into 5 mL of scintillation fluid. The radioactivity of the samples was measured with the Packard TRI-CARB1 190`A liquid scintillation analyzer.

2.5. Protein immunoblotting

The proteins were extracted from the cell lysates as described in previous reports [36]. Then the proteins were loaded into sodium dodecyl sulfate polyacrylamide gel and resolved by electrophoresis, and then the resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked and then incubated with the primary monoclonal antibodies against β-actin (Thermo Fisher Scientific, Inc., Waltham, MA), GAPDH (Cell Signaling Technology, Inc., Danvers, MA), ABCG2 (BXP 21) (Gene Tex Inc., Irvine, CA) or ABCB1 (F4) (Sigma-Aldrich,

Inc., St. Louis, MO) overnight at 4°C, followed by 2h incubation at room temperature with anti-mouse IgG, horseradish peroxide (HRP)-linked antibody (Cell Signaling Technology, Inc., Danvers, MA). The chemiluminescent signal was detected using ECL detection reagents (MilliporeSigma Company, Burlington, MA) and captured on the film.

2.6. Immunofluorescence analysis

As described in the previous reports [37], the NCI-H460/MX20 and KB-C2 cells were incubated with or without reversal agents. Thereafter, cells were fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 1% triton X-100 for 10 min. The cells were blocked with 6% BSA for 1 h at 37 °C, and then were incubated with monoclonal anti-ABCG2 protein (BXP 21) (Gene Tex Inc., Irvine, CA) or monoclonal anti-ABCB1 antibody (F4) (Sigma-Aldrich Inc., St. Louis, MO) overnight at 4°C, followed by 2 h incubation with Alexa flour 488 goat anti-mouse IgG (Abcam plc., Cambridge, United Kingdom) at 37°C. After that, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). The images were captured with a Nikon TE-2000S fluorescence microscope (Nikon Instruments Inc., Melville, NY).

2.7. ABCG2/ABCB1 ATPase assay

The ATPase assay was conducted with a PREDEASY ATPase assay kit (Sigma-Aldrich Inc., St. Louis, MO) [38].

2.8. Molecular modeling

Molecular docking was performed in Maestro v11.1 (Schrödinger, LLC, New York, NY, 2017) software [39]. The protein preparation of the wild-type human ABCG2 (PDB:

6FFC) [40] was performed and the grid was generated. The protein preparation and grid generation for ABCB1 (PDB: 6FN1) [41]were performed with the same protocol. The ligand was prepared and Glide docking was conducted and the best-scored conformation of the ligand and protein were obtained. After that, induced-fit docking (IFD) was performed with default parameters.

2.9. Statistical analysis

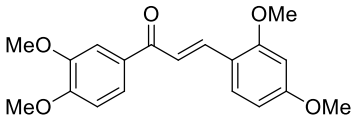
All experiments were conducted at least three times. The significance was analyzed by two-way ANOVA, and P value <0.05 represents significant difference.

3. Results and discussion

3.1 Chemistry

The structural characterization of **CYB-2**, **CYB-7**, and **CYB-15** are shown as follows. **CYB-15** is a new compound which has not been reported in the previous reports. The methoxy chalcones can be synthesized easily via one-step reactions compared to the other chalcone-based derivatives, which is an advantage for methoxy chalcones to be developed as ABCG2/ABCB1 transporters inhibitors.

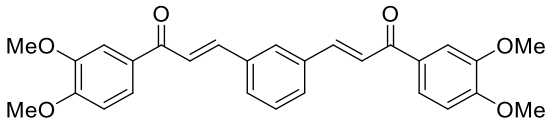
(E)-3-(2, 4-dimethoxyphenyl)-1-(3, 4-dimethoxyphenyl) prop-2-en-1-one (**CYB-2**)



Scheme 1. The structure of **CYB-2**

Yield 70.6 %, ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (d, J = 15.7 Hz, 1H), 7.71 – 7.61 (m, 2H), 7.60 – 7.53 (m, 2H), 6.92 (d, J = 8.4 Hz, 1H), 6.53 (dd, J = 8.6, 2.3 Hz, 1H), 6.47 (d, J = 2.3 Hz, 1H), 3.96 (d, J = 7.2 Hz, 6H), 3.90 (s, 3H), 3.85 (s, 3H), ^{13}C NMR (126 MHz, $CDCl_3$) δ 189.3, 162.9, 160.3, 152.9, 149.1, 139.7, 131.9, 130.8, 122.8, 120.1, 117.3, 110.9, 110.0, 105.4, 98.4, 56.0, 56.0, 55.5, 55.5

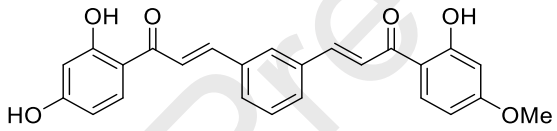
(2E, 2'E)-3, 3'-(1, 3-phenylene) bis (1-(3, 4-dimethoxyphenyl) prop-2-en-1-one) (**CYB-7**)



Scheme 2. The structure of **CYB-7**

Yield 86.0 %, mp 139~140℃. ^{1}H NMR (400 MHz, DMSO) δ 8.45 (s, 1H), 8.09 (d, J = 15.6 Hz, 2H), 7.98 (d, J = 8.0 Hz, 4H), 7.79 (d, J = 15.6 Hz, 2H), 7.64 (s, 2H), 7.56 (t, J = 8.0 Hz, 1H), 7.14 (d, J = 8.4 Hz, 2H), 3.89 (d, J = 5.2 Hz, 12H).^{13}C NMR (101 MHz, $CDCl_3$) δ 188.3, 153.5, 149.4, 143.0, 135.9, 131.2, 129.8, 129.5, 128.3, 123.1, 122.6, 110.8, 110.0, 56.1, 56.1

(E)-1-(2, 4-dihydroxyphenyl)-3-(3-((E)-3-(2-hydroxy-4-methoxyphenyl)-3-oxoprop-1-en-1-yl)phenyl) prop-2-en-1-one (**CYB-15**)



Scheme 3. The structure of **CYB-15**

Yield 17.3%, mp 232~234℃. IR 3387, 3225, 2921, 2848, 2693, 1639, 1573, 1513, 1439, 1359, 1313, 1282, 1226, 1174, 1131, 1021, 974, 959, 851, 832,783, 750, 736, 691, 664, 629, 584, 560, 524, 504, 482, 450. ^{1}H NMR (500 MHz, DMSO) δ 13.43 (s, 1H), 13.39 (s, 1H), 10.79 (s, 1H), 8.48 (s, 1H), 8.33 (d, J = 9.1 Hz, 1H), 8.25 (d, J = 9.0 Hz, 1H), 8.15-8.08(m, 2H) 8.00 (t, J = 7.0 Hz, 2H), 7.91-7.85 (m, 2H), 7.58 (t, J = 7.5 Hz, 1H), 6.62 (dd, J = 9.0, 2.0 Hz, 1H), 6.55 (d, J = 2.5 Hz, 1H), 6.46 (dd, J = 9.0, 2.5 Hz, 1H), 6.32 (d, J = 2.0 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 191.8, 191.4, 166.2, 165.9, 165.8, 165.4, 143.4, 142.9, 135.4, 135.3, 133.2, 132.8, 131.1, 131.1, 129.5, 129.3, 122.1, 122.1, 113.9, 113.0, 108.3, 107.6, 102.6, 101.0, 55.8; HRMS m/z 415.11860 (calcd for $C_{25}H_{19}O_6$, 415.11871)

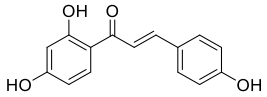
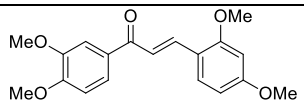
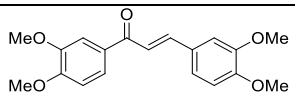
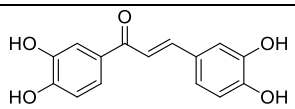
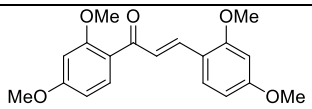
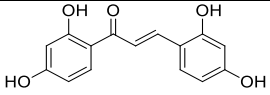
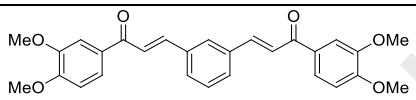
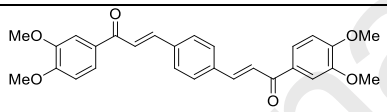
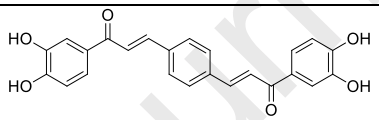
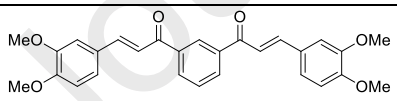
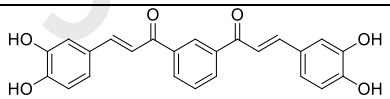
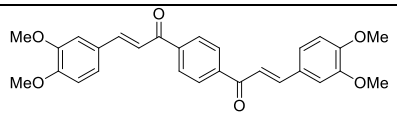
3.2 The effects of chalcones on the ABCG2-mediated MDR cell lines

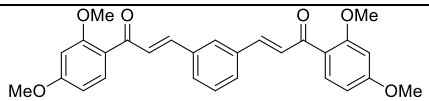
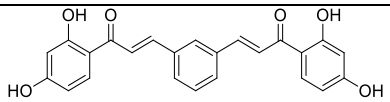
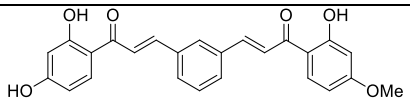
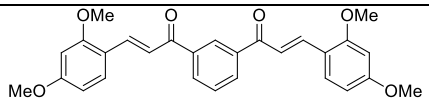
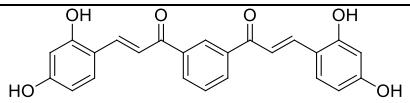
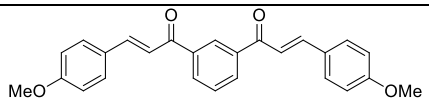
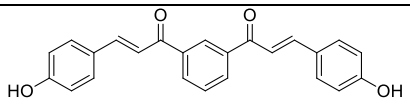
The cytotoxicity of the chalcones and bis-chalcones towards S1 and S1-M1-80 cells are shown in **Table 1**. S1-M1-80 is a MX-selected ABCG2-overexpressing cell line from human colon cancer cell line S1 [31]. Thus, we determined the concentration at which chalcones and bis-chalcones exhibit less than 20% inhibition on the proliferation of S1 and S1-M1-80 cells (**Table 1**). From **Figure 1**, MX, at a concentration of 2.5 μM, showed only 13% inhibition on the proliferation of S1-M1-80 cells. However, the cytotoxicity of MX increased when combined with some chalcone derivatives. the chalcones or bis-chalcones **CYB-2**, **CYB-3**, **CYB-7**, **CYB-10**, **CYB-13** with four methoxy groups, and **CYB-15** with one methoxy group and three hydroxy groups showed more than 30% inhibition on the ABCG2-mediated MDR. When MX was combined with chalcone **CYB-2**, the proliferation of S1-M1-80 cells was inhibited by 65%, which was higher than its demethylation product, **CYB-4**, which had 24% inhibition. When in combination with bis-chalcone **CYB-13**, MX inhibited the proliferation of S1-M1-80 cells by 44%, which was higher than **CYB-14** and **CYB-15** with 13% and 33% inhibition, respectively. In addition, **CYB-15** with one methoxy group showed higher inhibitory effect than **CYB-14** without a methoxy group. Similarly, methoxy bis-chalcone **CYB-16** showed lower inhibitory effect than the corresponding hydroxy bis-chalcone **CYB-17** on the proliferation of S1-M1-80 cells. The position of the methoxy groups on chalcones and the meta or para position of α, β-unsaturated carbonyl groups on bis-chalcones have effects on the reversal activities of chalcones derivatives. 3, 4, 3', 4'-methoxy chalcone **CYB-3** has stronger inhibition on ABCG2 than 2, 4, 2', 4'-methoxy chalcone **CYB-5**. Bis-chalcones **CYB-7** and **CYB-10** at meta position of the α, β-

unsaturated carbonyl groups showed stronger inhibitory activity than **CYB-8** and **CYB-12** at para position, respectively. Among all the compounds, methoxy chalcones **CYB-2** and **CYB-3** exhibited the most potent inhibitory effect on the proliferation of S1-M1-80 cells with survival rates of 65% and 66%, respectively, while methoxy bis-chalcone **CYB-7** also showed effective reversal activity with 44% inhibition. The results indicated that chalcones exhibit better reversal activities than the bis-chalcones. Most of the bis-chalcones did not show significant reversal effects on ABCG2-mediated MDR. In the previous report, the bis-chalcone showed a synergistic effect with an ABCG2 inhibitor, chromones, to strength the inhibition of ABCG2 transporter [25]. Nevertheless, our study showed that chalcones are more potent than the bis-chalcones, thus the extended π system might not be promising to increase the reversal activities of chalcone derivatives. There were similar findings in the investigation of indole derivatives as reversal agents for ABCG2-mediated MDR [35]. Since the two compounds **CYB-2** and **CYB-3** showed potent reversal effects on ABCG2-mediated MDR, the two compounds were selected for further study. As shown in **Figure 2**, when combined with **CYB-2** and **CYB-3** at a concentration of 5 μM, the IC_{50} values of MX on S1-M1-80 cells were 1.86 ± 0.10 and 3.29 ± 0.15 μM, respectively, indicating that **CYB-2** is more potent than **CYB-3**. Thus, **CYB-2** was used for further mechanistic study. Fumitremorgin C (FTC), a well-known ABCG2 inhibitor, was used as a positive control inhibitor.

Table 1. The cytotoxicity of chalcones and bis-chalcones, and the selected concentrations for reversal study.

Compd.	**Structure**	**S1**	**S1-M1-80**	

		IC_{50} [a] / μM	95% confidence intervals [b]	IC_{50} [a] / μM	95% confidence intervals [b]	Concentration for reversal study [c] / μM
CYB-1		40.6±1.9	20.4 ~ 54.6	42.3±6.3	28.2~58.3	5
CYB-2		27.9±1.0	18.5~36.3	18.0±1.6	12.8 ~ 33.0	5
CYB-3		16.9±0.1	11.9 ~ 18.6	19.1±4.0	18.5 ~ 22.9	5
CYB-4		34.5±4.3	26.7 ~ 40.6	>100	NA [d]	10
CYB-5		36.3±6.7	23.9 ~ 46.2	29.9±4.8	17.6~36.8	5
CYB-6		>100	NA [d]	>100	NA [d]	10
CYB-7		3.2±0.8	2.2 ~ 3.6	4.0±0.5	2.9 ~ 4.6	1
CYB-8		>100	NA [d]	>100	NA [d]	10
CYB-9		24.5±6.9	18.6 ~ 32.5	>100	NA [d]	5
CYB-10		24.9±2.4	20.3 ~ 38.8	30.0±4.9	19.2 ~ 31.5	10
CYB-11		11.0±0.3	9.2 ~ 13.6	35.7±2.1	28.1 ~ 41.6	5
CYB-12		83.9±7.6	50.4 ~ 96.3	>100	NA [d]	10

CYB-13		2.7±0.9	2.1 ~3.3	3.5±0.9	2.8 ~ 4.5	1
CYB-14		2.5±0.4	2.0 ~ 3.4	4.5±0.2	3.5 ~ 5.1	1
CYB-15		8.4±0.07	7.1 ~ 9.2	8.6±0.8	7.4 ~9.6	1
CYB-16		98.0±0.6	NA [d]	>100	NA [d]	10
CYB-17		83.2±3.4	57.6 ~ 98.8	>100	NA [d]	10
CYB-18		33.5±8.2	29.2 ~40.5	39.1±1.8	31.6 ~ 48.2	10
CYB-19		13.7±0.1	11.5 ~ 14.5	19.1±1.8	11.3 ~ 20.4	5

[a] IC_{50} values were obtained by MTT assay, indicating the concentration at which the compounds inhibited the cell proliferation by 50%. [b] 95% confidence intervals for IC_{50} values. [c] The concentration used for reversal study showed less than 20% inhibition on the proliferation of S1 and S1-M1-80 cells. [d] The highest concentration used for cytotoxicity assay was 100 μM. When IC_{50} value is closed to or higher than 100 μM, the 95% confidence intervals for IC_{50} value can not be obtained.

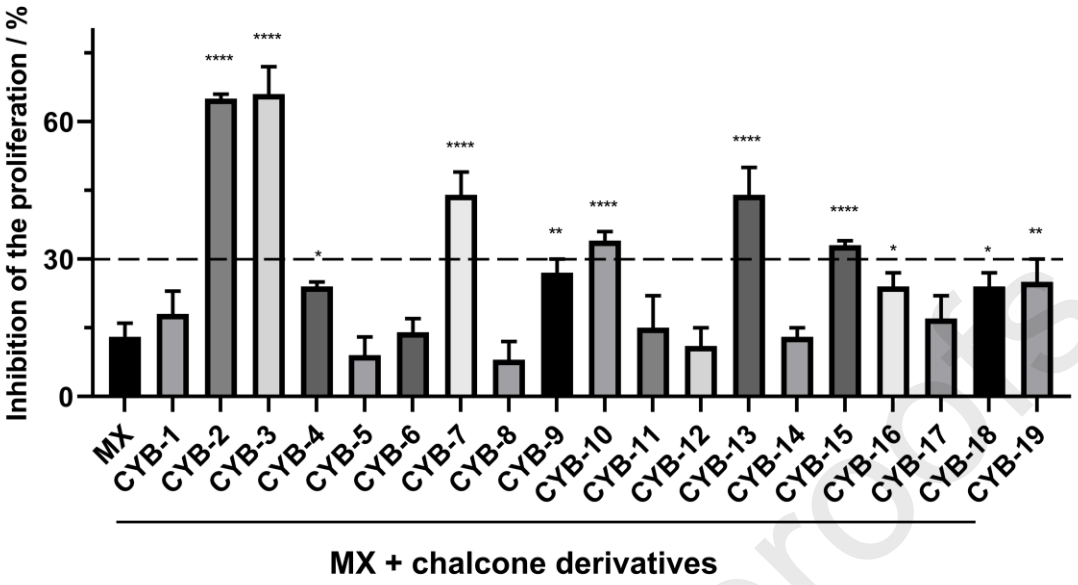


Figure 1. The inhibition of S1-M1-80 cell proliferation by the combination of the chalcone derivatives and MX (2.5 μM). The inhibition of the proliferation was conducted with MTT assay. The statistical significances between the treatment groups and the control group were indicated as follows: *, P < 0.05, **, P < 0.01, ****, P < 0.0001, one-way ANOVA.

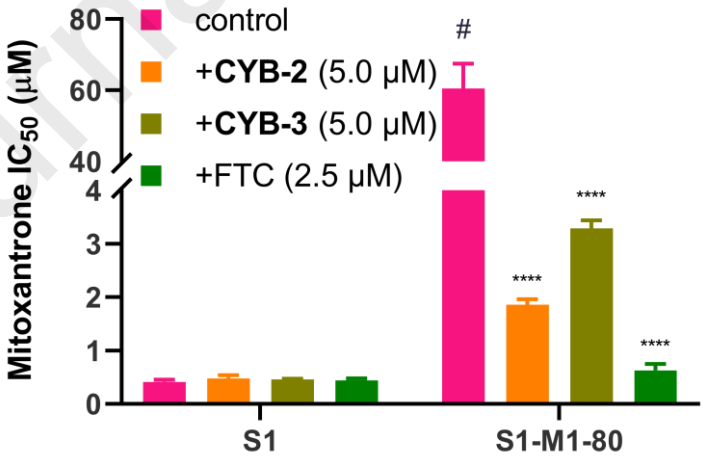


Figure 2. The reversal effect of the chalcone derivatives **CYB-2** and **CYB-3** on S1-M1-80 cells. IC50 values were obtained by MTT assay, indicating the concentration at which

the compounds inhibited the cell proliferation by 50%. The statistical significances between the treatment groups and the control group were indicated as follow: ****, $P < 0.0001$, two-way ANOVA. #, $P < 0.0001$, indicating the statistical significances between the multidrug resistant cell control groups and the parental cell groups, two-way ANOVA.

3.3 The effects of CYB-2 on the ABCG2- and ABCB1--mediated MDR cell lines

The representative cytotoxic curves of **CYB-2** on the cell lines that were used for reversal activity study are shown in **Figure 3**, which indicates that **CYB-2** exhibits less than 20% inhibition on the proliferation of the cell lines at the tested concentration. As shown in **Figure 4A**, **4B**, **CYB-2** significantly increased the cytotoxicity of MX in ABCG2-overexpressing S1-M1-80 and NCI-H460/MX20 cell lines, and effectively increased the cytotoxicity of doxorubicin in S1-M1-80 cell line (**Figure 4C**). Moreover, **CYB-2** enhanced the cytotoxicity of colchicine in ABCB1-overexpressing KB-C2 cells (**Figure 4D**). However, **CYB-2** cannot sensitize NCI-H460/MX20 or KB-C2 cells to cisplatin, a non-substrate of the ABCG2 or ABCB1 (**Figure 4E**, **4F**). In addition, **CYB-2** can effectively reverse MDR in HEK/ABCG2-482-R2 and HEK/ABCB1 (**Figure 4G**, **4H**), which were transfected with ABCG2 or ABCB1 using human embryonic kidney 293 HEK293/pcDNA3.1 cells. Thus, we concluded that **CYB-2** sensitized both ABCG2- and ABCB1-mediated MDR cell lines to anticancer drugs by interacting with ABCG2 and ABCB1.

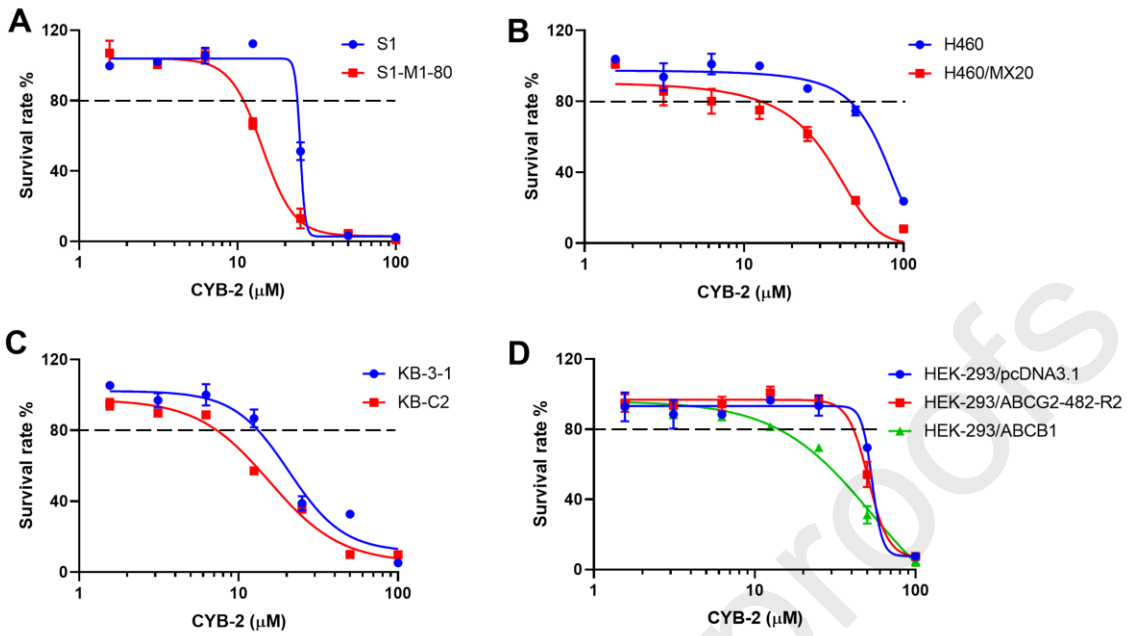


Figure 3. The cytotoxic curves of **CYB-2** on different cell lines.

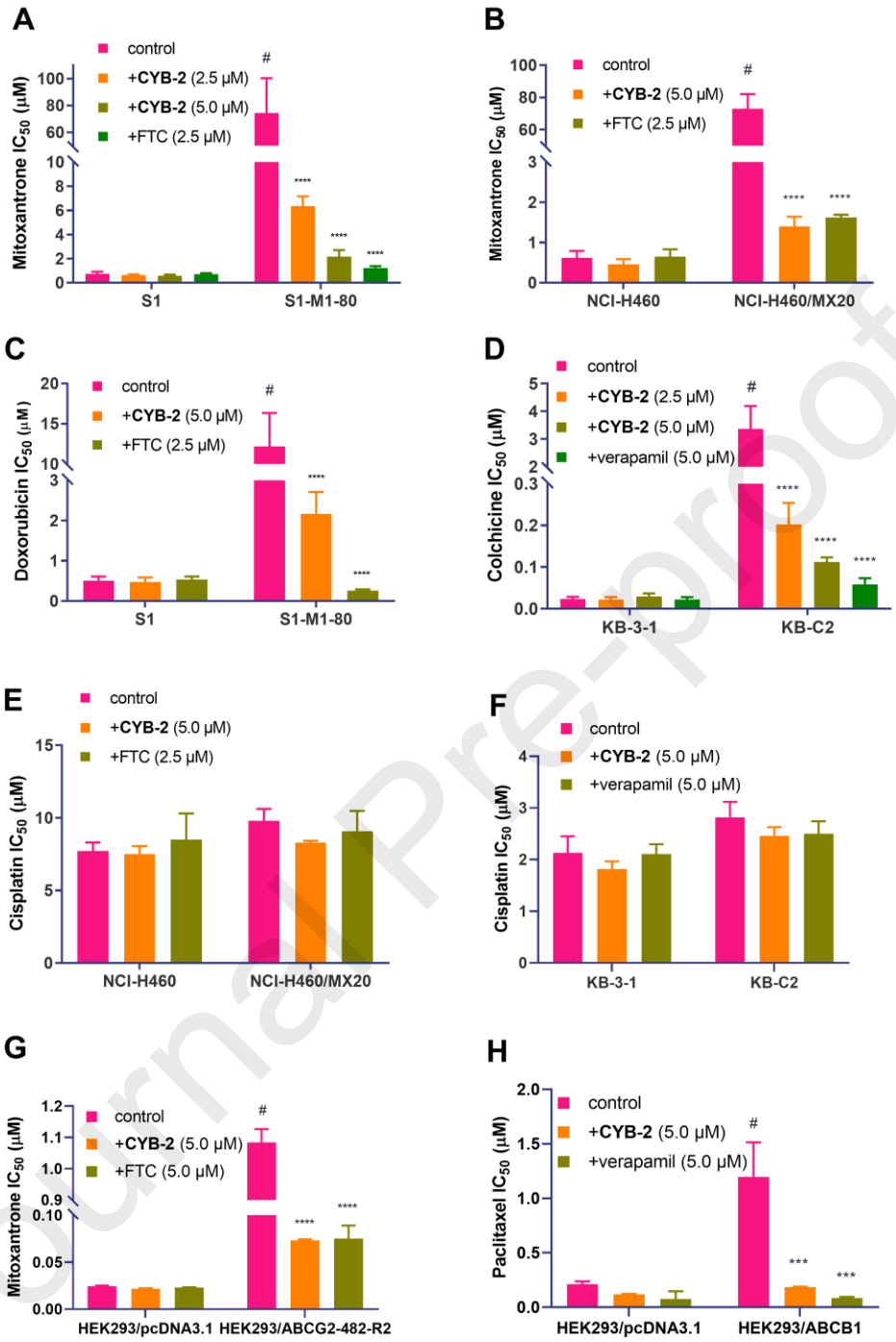


Figure 4. Reversal effect of **CYB-2** on ABCG2- and ABCB1- mediated MDR cell lines. IC_{50} values were obtained by MTT assay, indicating the concentration at which the compounds inhibited the cell proliferation by 50%. FTC and verapamil are used as

positive control inhibitors for ABCG2 and ABCB1, respectively. The statistical significances between the treatment groups and the control group were indicated as follows: ***, $P < 0.001$; ****, $P < 0.0001$, two-way ANOVA. $^{\#}$, $P < 0.0001$, indicating the statistical significances between the multidrug resistant cell control groups and the parental cell groups, two-way ANOVA.

Table 2. The different cell lines used in this study.

Cell lines	Cell type	Parental / multidrug resistant	ABC transporters overexpressed
S1	Human colon cancer	Parental	/
S1-M1-80	Human colon cancer	Multidrug resistant	ABCG2
NCI-H460	Human lung cancer	Parental	/
NCI-H460-MX20	Human lung cancer	Multidrug resistant	ABCG2
KB-3-1	Human cervical carcinoma	Parental	/
KB-C2	Human cervical carcinoma	Multidrug resistant	ABCB1
HEK293/pcDNA3.1	Human embryonic kidney 293 cell line	Parental	/
HEK/ABCG2-482-R2	Human embryonic kidney 293 cell line	Multidrug resistant	ABCG2
HEK/ABCB1	Human embryonic kidney 293 cell line	Multidrug resistant	ABCB1

3.4 The effects of CYB-2 on the anticancer drugs accumulation and efflux in ABCG2- and ABCB1-mediated MDR cell lines

From the accumulation and efflux assay, the results indicated that **CYB-2** can effectively increase the accumulation of [^{3}H]-MX in ABCG2-mediated NCI-H460/MX20 cells (**Figure 5A**), and it is comparable with fumitremorgin C (FTC). **CYB-2** can also significantly increase the accumulation of [^{3}H]-paclitaxel in the ABCB1-mediated KB-

C2 cells (**Figure 5B**). **CYB-2** did not change the efflux function of the parental cells NCI-H460 and KB-3-1 (**Figure 5C, 5D**), while the efflux of anticancer drugs decreased in multidrug resistant NCI-H460/MX20 and KB-C2 cell lines (**Figure 5E, 5F**). The results revealed that **CYB-2** increased the accumulation of anticancer drugs in NCI-H460/MX20 and KB-C2, resulting from the inhibition of drug efflux, thus re-sensitizing the MDR cell lines to anticancer drugs.

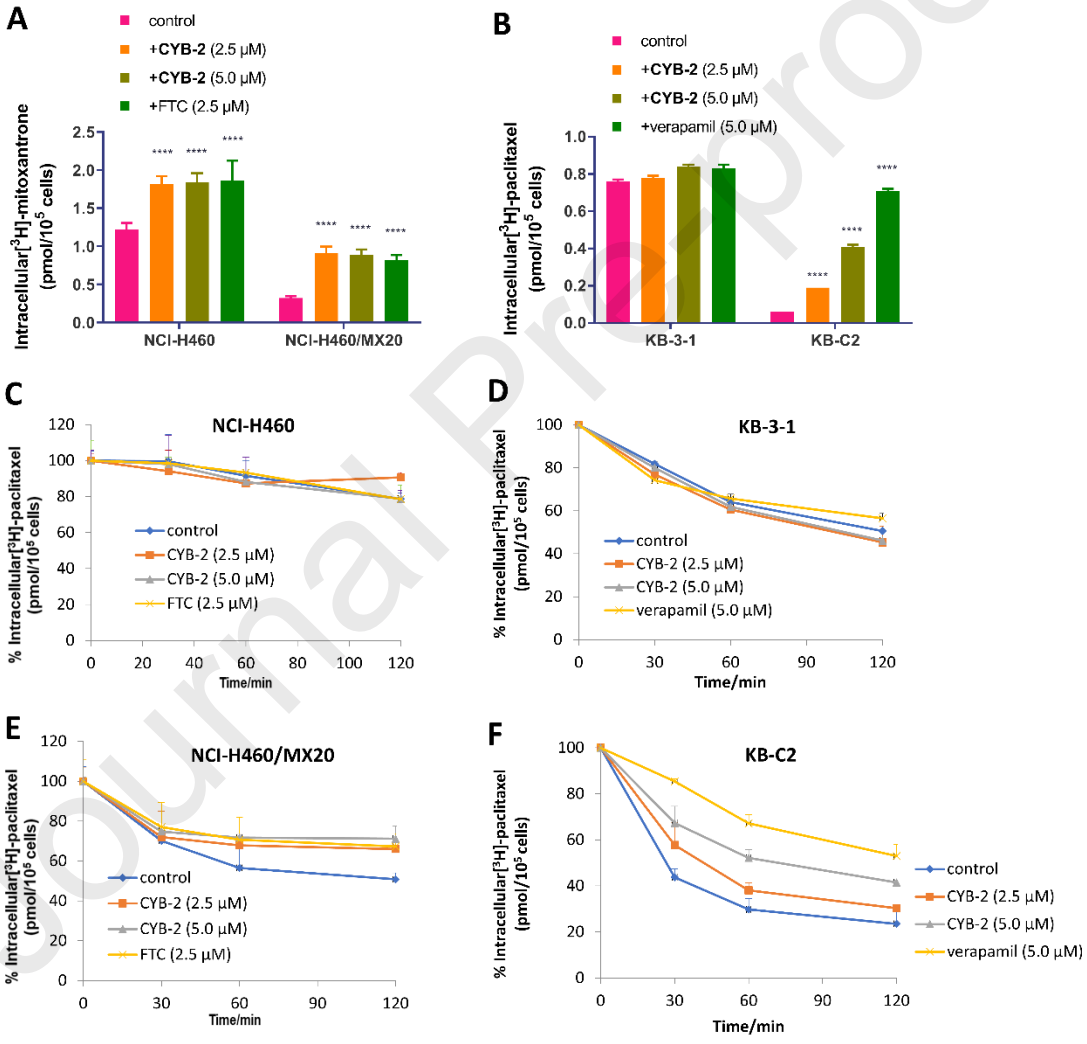


Figure 5. The effects of **CYB-2** on the accumulation and efflux of [^{3}H]-MX and [^{3}H]-paclitaxel in ABCG2- and ABCB1-mediated MDR cell lines. FTC was used as a positive

control inhibitor of ABCG2 and verapamil was used as a positive control inhibitor of ABCB1. The statistical significances between the treatment groups and the control group were indicated as follow: ****, $P < 0.0001$, two-way ANOVA.

3.5 The effects of CYB-2 on the expression and subcellular localization of ABCG2 and ABCB1 in MDR cells

The down-regulation of ABC transporters or translocation of ABC transporters from membrane to cytoplasm can result in the decrease of MDR in cancer cells. The immunoblot results showed that the expression of ABCG2 and ABCB1 did not significantly change after the treatment of **CYB-2** (**Figure 6A**, **6B**). Moreover, the subcellular localization of ABCG2 in NCI-H460/MX20 and ABCB1 in KB-C2 did not alter after the treatment with **CYB-2** (**Figure 6C**, **6D**). Thus, we hypothesized that ABC transporter activities were inhibited by **CYB-2**, which further inhibited the efflux function of the ABC transporters.

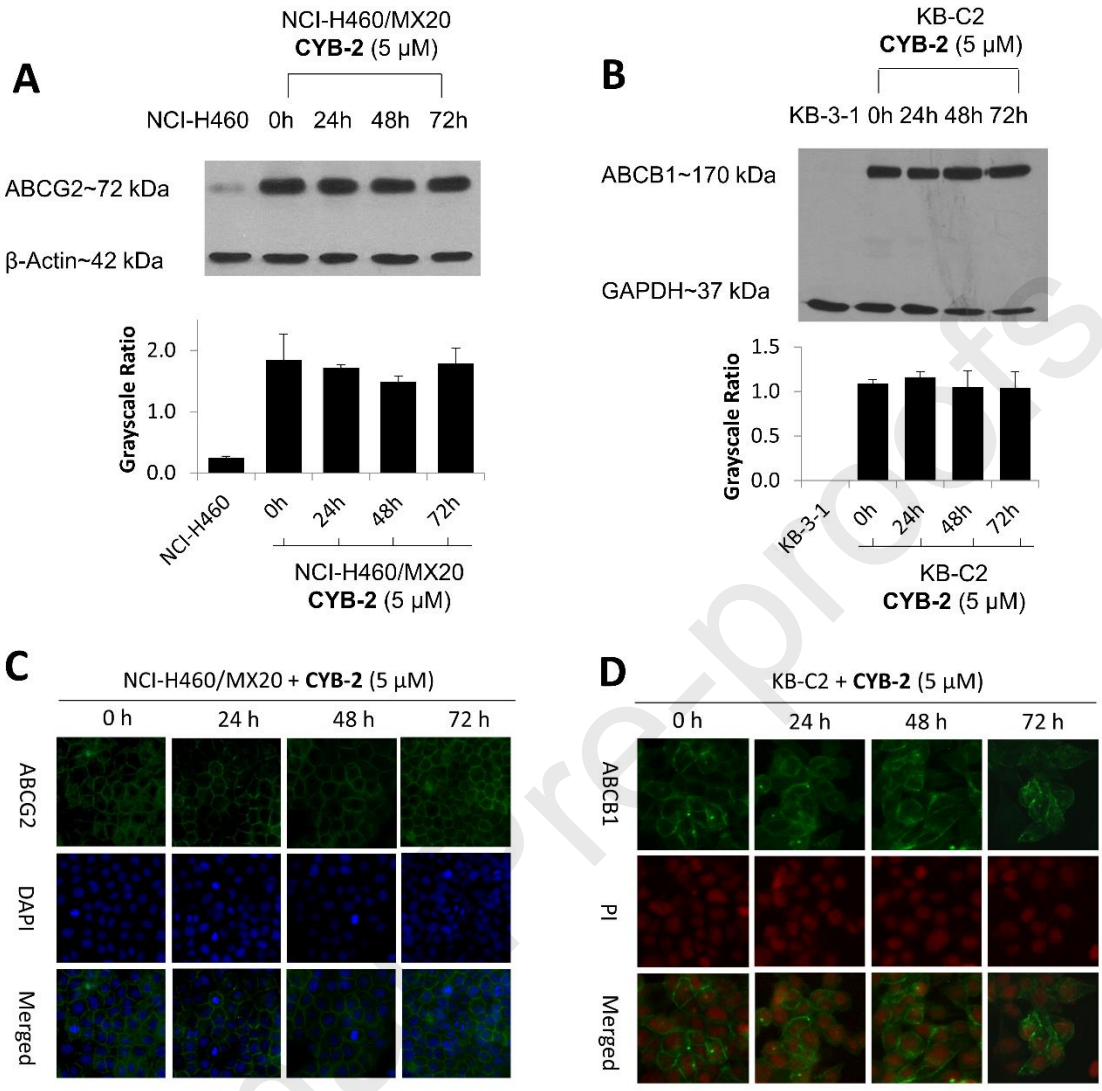


Figure 6. (A) Immunoblot analysis showing the expression of ABCG2 in NCI-H460/MX20 cells, and (B) ABCB1 in KB-C2 cells after the treatment with **CYB-2**. The effects of **CYB-2** on subcellular localization of (C) ABCG2 and (D) ABCB1 in MDR cells.

3.6 The effect of CYB-2 on ABCG2 and ABCB1 ATPase activities

Previous reports have shown that both the inhibition and stimulation of ABC transporters ATPase can result in the reverse of MDR [42,43]. Thus, **CYB-2** blocks the

efflux of anticancer drugs due to inhibition of ABCG2 or ABCB1 ATPase activities, or stimulates the ATPase activity and works as a competitive substrate. To determine whether the activities of ABCG2 and ABCB1 ATPase were inhibited or stimulated, we performed the ATPase assay. The results (**Figure 7**) suggested that **CYB-2** inhibited the activities of ATPase in both ABCG2 and ABCB1 with IC_{50} values of 0.95 μM and 2.01 μM, respectively. **CYB-2** exhibits strong inhibition on ABCG2 and ABCB1 ATPase, thus blocking the efflux function of MDR cancer cells and increasing the accumulation of anticancer drugs.

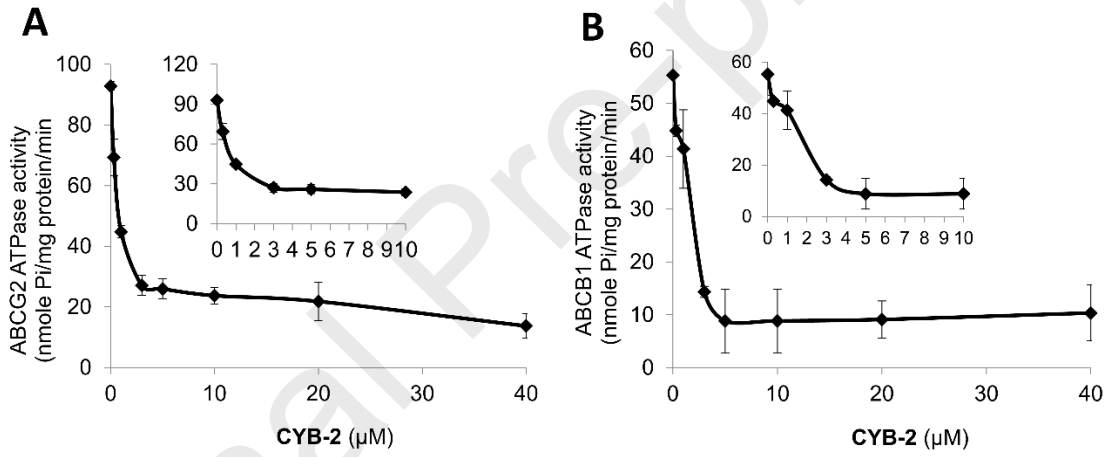


Figure 7. The effects of **CYB-2** on the accumulation and efflux function of [^{3}H]-MX and [^{3}H]-paclitaxel on ABCG2- and ABCB1-mediated MDR cell lines. IC_{50} values indicate the concentration at which **CYB-2** inhibit the cell proliferation by 50%.

3.7 The binding mode of CYB-2 with ABCG2 and ABCB1

The docked positions of **CYB-2** within the binding cavity of human ABCG2 (PDB: 6FFC) (**Figure 8A**) and ABCB1 (PDB: 6FN1) (**Figure 8D**) are shown in **Figure 8** with docking scores -9.607 kcal/mol and -8.028 kcal/mol, respectively. One phenyl ring of

CYB-2 forms a π-π interaction with Phe439 in the B chain of the ABCG2 protein (**Figure 8B**). Apart from the π-π interaction, **CYB-2** also has interactions with the residues Leu405, Phe432, Val546, and Met549 of ABCG2 (**Figure 8C**). For the interaction between **CYB-2** and ABCB1 protein, the residue Tyr982 forms π-π stacking with one phenyl ring of **CYB-2**. In the meantime, a methoxy group forms a hydrogen bond with Gln945 (-OCH_3•••H_2N-Gln945), and the carbonyl group of **CYB-2** has a hydrogen binding with Tyr952 (**Figure 8E**). Moreover, **CYB-2** interacts with a hydrophilic pocket of ABCB1 with residues such as Leu64, Met67, Met68, Phe727, Phe731, Met948, Tyr949, Tyr952, Phe977, and Phe982 (**Figure 8F**).

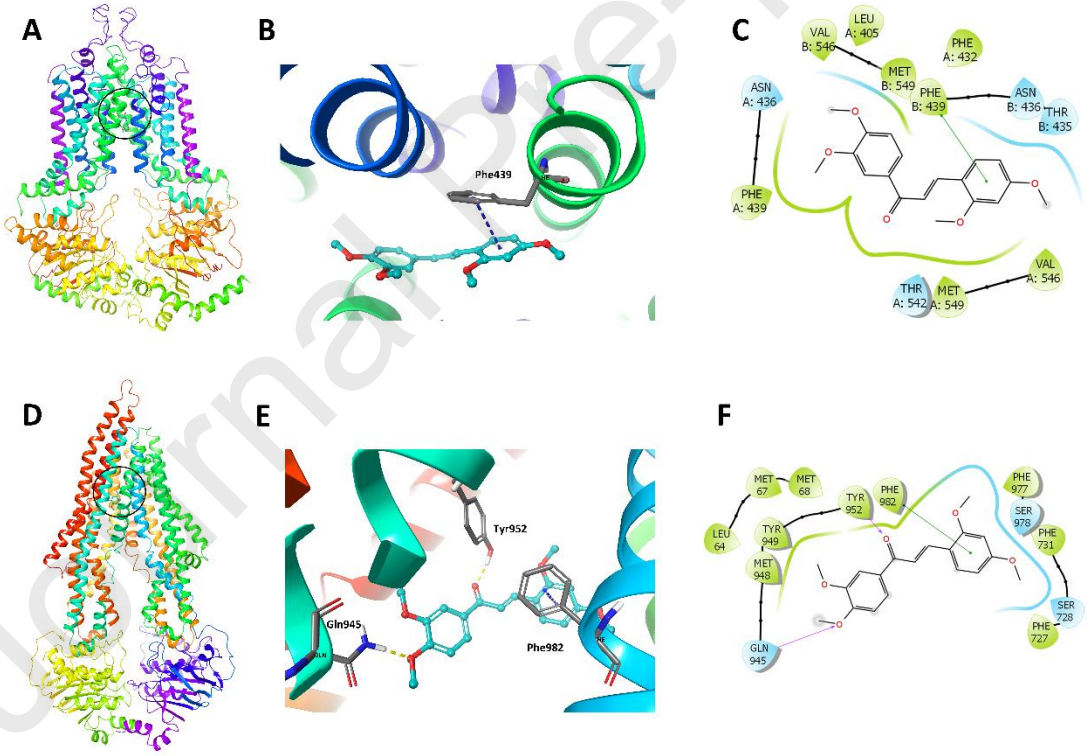


Figure 8. Docked positions of **CYB-2** with the human ABCG2 and ABCB1 transporter proteins. (A) **CYB-2** in the binding site of ABCG2 indicated with a black circle. (B) The binding of **CYB-2** and the human ABCG2 transporter protein in a ball-and-stick model. **CYB-2** is shown with

following color code: carbon - cyan, hydrogen - white, and oxygen - red. The important interaction was indicated: ring centroids - blue dotted line. (C) The interactions of **CYB-2** with human ABCG2. The interaction was indicated as follows: π-π stacking interaction - the green line. (D) **CYB-2** in the binding site of ABCB1 indicated with a black circle. (E) The binding of **CYB-2** and the human ABCB1 protein in a ball-and-stick model. The important interactions were presented: ring centroids - blue dotted lines; hydrogen bonds - dotted yellow lines. (F) The interactions of **CYB-2** with the binding site of the human ABCB1 protein. The interactions were indicated as follows: hydrogen bonds - purple arrow; π-π stacking aromatic interactions - the green lines.

In conclusion, this study investigated the effect of methoxy and hydroxy substitutions and the extended π system on the potencies of chalcones and bis-chalcones. From the structure-activity relationship study, the methoxy chalcones or bis-chalcones showed potent inhibition on ABCG2-mediated MDR, and demonstrated that methoxy groups enhance the reversal activities of chalcones. Moreover, the methoxy chalcones **CYB-2** and **CYB-3** exhibit more potent reversal activities than methoxy bis-chalcones, indicating that the extended π system does not enhance the potency of the chalcone derivatives. In addition, the position of the methoxy groups of chalcones and the α, β-unsaturated carbonyl groups of bis-chalcones have effects on the inhibition of ABCG2. Among all the compounds, methoxy chalcone **CYB-2** showed the best reversal activity on ABCG2-mediated MDR; in addition, it can also reverse ABCB1-mediated MDR. The mechanistic studies indicated that **CYB-2** inhibits the basal ATP hydrolysis of both ABCG2 and ABCB1 transporters, thereby inhibiting the efflux function of the transporters and thus increasing the intracellular accumulation of certain substrates into the MDR cells without affecting ABCG2 or ABCB1 protein expression and localization. The previous report indicates that bis-chalcone can reverse ABCG2-mediated MDR [25], in addition, some

non-basic chalcones can also selectively reverse ABCG2-mediated MDR [26,27]. In this study, non-basic chalcone **CYB-2** show stronger reversal activity on ABCG2 than all the bis-chalcones we tested in this study. **CYB-2** also exhibits inhibitory activity on ABCB1, which is a superiority compared to the reported non-basic chalcones with specific ABCG2 inhibition in the treatment of ABCG2 and ABCB1 co-expressing cancers. Furthermore, as ABCG2/ABCB1 duel inhibitors, non-basic chalcones have synthetic tractability compared to the reported chalcone-based derivatives, such as bifendate-chalcone hybrids and flavone-based chalcones. These results revealed that non-basic methoxy chalcones have potential to be developed as reversal agents to overcome MDR in ABCG2 and ABCB1 co-expressing cancers.

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Conflicts of interest

The authors declared no conflicts of interest.

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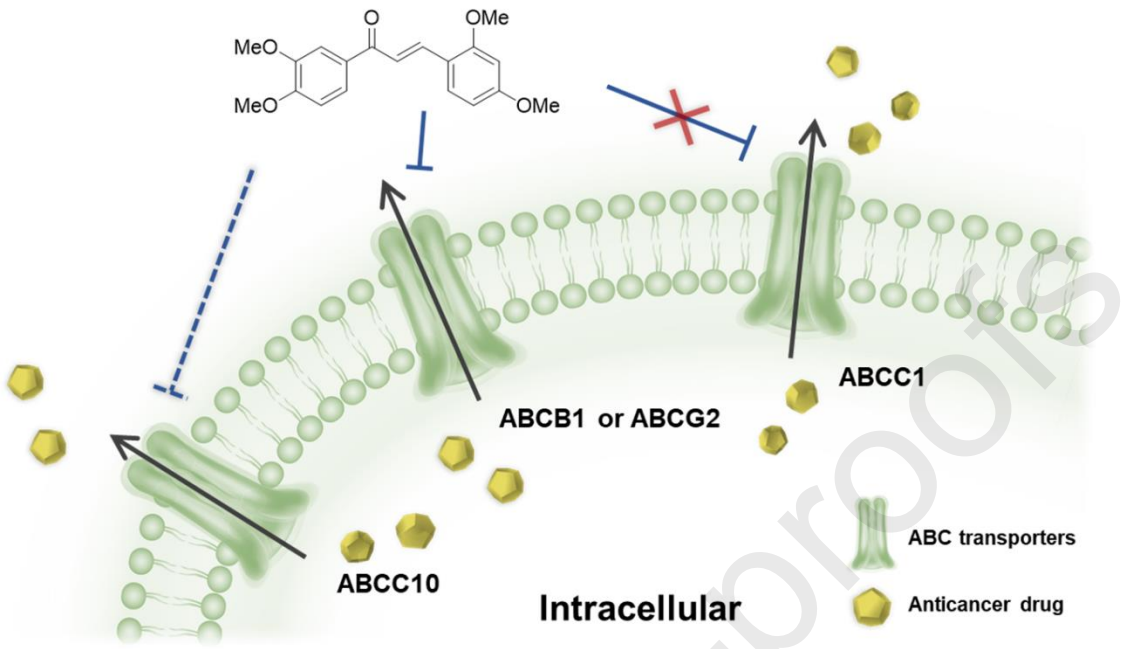
Credit Author Statement

C. C. wrote this paper and conducted the synthesis of the compounds, most of the MTT assays, and docking analysis. W. Z. conducted the Western blotting. J. W. conducted a part of the MTT assays. Z. L. performed the accumulation and efflux assay. Y. Z. was involved in the immunofluorescence assay. Y. W. and P. G. performed the ATPase assay. C. T. was involved in planning the work and revising the manuscript. B. W. and Z. C. supervised and supported the project.



- Anticancer drugs accumulation increased and drug efflux decreased.
- ABCB1 or ABCG2 ATPase activities were inhibited.
- Protein expression and localization were not altered.