WHI-P154 enhances the chemotherapeutic effect of anticancer agents in ABCG2-overexpressing cells

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M ultidrug drug resistance (MDR) remains a major obstacle towards attaining a successful chemotherapeutic outcome. Among several factors responsible for development of MDR, the overexpression of ATP-binding cassette (ABC) transporters is a major concern.

The human genome, which encodes 48 ABC transporter members has been divided into seven subfamilies (A–G) based on sequence and structure similarities. ABC transporters are also known to influence oral absorption and disposition of a wide variety of drugs, but each transporter has its own particular substrate selectivity. The ABCB1 (P-glycoprotein/MDR1) transports toxic endogenous substances, chemotherapeutic drugs such as vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes across the cell membrane. The spectrum of chemotherapeutic drugs transported by ABCG2 (BCRP/MXR) includes mitoxantrone (MX), topotecan, irinotecan, flavopiridol and methotrexate (MTX). ABCC1 (MRP1) confers resistance to a broad spectrum of anticancer drugs such as vincristine, camptothecin, doxorubicin and etoposide. Another important member of C subfamily is ABCC10 (MRP7); it is a broad-acting transporter of xenobiotics, including drugs such as taxanes and epothilone B.

Therefore, identifying high-affinity drugs that can either block the key signaling pathway regulating the expression of ABC transporters or inhibit their function might be a promising approach to overcome MDR in cancer chemotherapy. Enormous efforts have been devoted to the development of inhibitors of ABC transporters. Previously, we reported several tyrosine kinase inhibitors (TKIs) such as erlotinib, lapatinib,
vandetanib and tandutinib that could reverse ABCB1-, ABCG2-, ABCC1- or ABCC10-mediated MDR.\(^\text{10–13}\)

WHI-P154 was synthesized based on the structure of dimethoxyquinazoline compounds with potent inhibitory activity against JAK3 and STAT3, and it was then proven to be an effective inhibitor for other kinases including epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), Src, Abl, MAPK and PI3K.\(^\text{14–16}\) This study is to investigate the reversal effect of WHI-P154 on MDR mediated by ABC transporters.

Materials and Methods

Materials. \([^3H]\)-MX (4 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). BXP-21 (anti-ABCG2) antibody was purchased from Signet Laboratories (Ded-Ham, MA, USA). Anti-STAT3, p-STAT3, ERK1/2, p-ERK1/2, AKT and p-AKT antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). WHP-P154 was a gift from Selleck Chemicals (Houston, TX, USA). PAK-104\(^\text{P}\) was a gift from Profesor Shin-Ichi Akiyama (Kagoshima University, Kagoshima, Japan) from Nissan Chemical (Chiba, Japan). Fumitremorgin C (FTC) was kindly provided by Drs Susan E Bates and Robert W Robey (NCI, NIH, Bethesda, MD, USA). Cepharanthine was generously provided by Kakengshoyaku (Tokyo, Japan). MX, SN-38, cisplatin, colchicine, vincristine, verapamil, paclitaxel, penicillins, streptomycins, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide, and other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA).

Cell lines and cell culture. Human non-small cell lung carcinoma cells H460 and H460/MX20 cells were kindly provided by Drs Susan E Bates and Robert W Robey (NCI, NIH, Bethesda, MD, USA). H460/MX20 cells were cultured in DMEM with addition of 20 nM MX,\(^\text{17}\) HEK293/pcDNA3.1, wild-type ABCG2-482-R2, mutant ABCG2-482-G2, and mutant ABCG2-482-T7 cells were provided by Drs Susan E Bates and Robert W Robey (NCI, NIH, Bethesda, MD, USA) and maintained in the medium with 2 mg/mL of G418.\(^\text{18}\) Mutant ABCG2 (Phe489Leu) plasmid was sequenced in Innovative Mutagenesis Service (Hillsborough, NJ, USA) as shown in Fig. S1. ABCG2-489-Leu was generated by transient transfection of the mutant ABCG2 (Phe489Leu) plasmid into HEK293 cells (Fig. S2). Similarly, HEK293/ABC2/ABC1 and HEK293/ABC2/ABC10 cells were generated by transfecting ABC2 or ABCCl expression vectors into HEK293 cells.\(^\text{9,10}\) LLC/CMV, LLC/cMOAT, KB-3-1 and KB-C2 cells were kindly provided by Dr Shin-Ichi Akiyama (Kagoshima University, Kagoshima, Japan).\(^\text{20}\) All cells were grown as adherent monolayers in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified incubator containing 5% CO\(_2\).

Cytotoxicity assays. Cell sensitivity to drugs was analyzed using an MTT colorimetric assay as described previously.\(^\text{11}\) The concentrations required to inhibit the growth by 50% (IC\(_{50}\)) were calculated from survival curves.

\([^3H]\)-MX accumulation assay. The effect of WHI-P154 on the intracellular accumulation of \([^3H]\)-MX in ABCG2-overexpressing cells was determined by measuring the intracellular accumulation of \([^3H]\)-MX in ABCG2-transfected HEK293 cells as described previously.\(^\text{21,22}\)

\([^3H]\)-MX efflux assay. For the efflux study, the cells were treated with 4 \(\mu M\) WHI-P154 and all the samples were placed in scintillation fluid and radioactivity were measured as described previously.\(^\text{23}\)

Western blot analysis. The cells were washed two times with cold-PBS, and protein lysates were isolated and prepared for Western blot analysis as previously described.\(^\text{24}\)

Immunofluorescence. The immunofluorescence was conducted to test the localization of ABCG2 protein after treatment with WHI-P154 for 72 h as described previously.\(^\text{23}\)

ATPase assay. The vanadate (Vi)-sensitive ATPase activity of ABCG2 in the membrane vesicles of High Five insect cells was measured as previously described.\(^\text{21}\)

Molecular modeling of ABCG2. WHI-P154 structure and human ABCG2 homology model along with various grids and docking simulations were carried out as our previous protocols.\(^\text{22,23}\) All computations were carried out on a Dell Precision 490 dual processor with the Linux OS (Ubuntu 12.04 LTS).

Statistical analysis. All experiments were repeated at least three times. Microsoft Office Excel 2010 (Microsoft Corp. Redmond, WA, USA), and Image J (NIH, Bethesda, MD, USA) were used in data processing and analyzing. The data were analyzed using student’s two-tailed t-test. Statistical significance was determined at \(p < 0.05\).

Results

Cytotoxicity of WHI-P154 on MDR cells and their parental cells. We investigated the cytotoxicity of WHI-P154 in different cells by MTT assay. As shown in Figure 1, approximately 85% of the cells survived at the concentration of 4 \(\mu M\) WHI-P154 (Fig. 1b–g). Therefore, WHI-P154 at a concentration of 4 \(\mu M\) was chosen as a maximum concentration for combination treatment with known ABCB1, ABCG2, ABCC1, ABC2 or ABCC10 substrate anticancer drugs.

Effect of WHI-P154 on cells overexpressing ABCG2. The ABCG2-overexpressing cells H460/MX20 showed much higher resistance to ABCG2 substrate chemotherapeutics than parental H460 cells (Table 1). WHI-P154 significantly sensitized H460/MX20 cells to the ABCG2 substrates, such as MX and SN-38. It also had a moderate effect on the parental H460 cells (1.6-fold). However, this effect was modest as compared to that of H460/MX20 cells. FTC is a well-known ABCG2 inhibitor and is used as a positive control of ABCG2. Moreover, the IC\(_{50}\) value of cisplatin, a non-ABCG2 substrate, was not affected when combined with WHI-P154. It has been reported that mutations at amino acid 482 in ABCG2 altered the substrate and antagonist specificity of ABCG3.\(^\text{18,26}\) Therefore, we investigated whether WHI-P154 would reverse ABCG2-mediated resistance to MX in cells transfected with either the wild-type (Arg482) or mutant (Arg482Gly and Arg482Thr) forms of ABCG2. As shown in Table 2, WHI-P154 at nontoxic concentrations significantly enhanced the cytotoxic effect of MX and SN-38 in three ABCG2-transfected cells ABCG2-482-R2, ABCG2-482-G2 and ABCG2-482-T7 but not in HEK293/pcDNA3.1 cells. In addition, the single nucleotide polymorphism variant of ABCG2 (Phe489Leu) was reported to affect drug resistance toward its substrates.\(^\text{27}\) To determine the reversal effect of WHI-P154 on the variant of ABCG2 (Phe489Leu), we tested the cytotoxicity of MX in the cells transfected with mutant ABCG2 (Phe489Leu) plasmid. Our results indicated that WHI-P154 can notably reverse mutant Phe489Leu ABCG2-mediated drug resistance (Table 3). These results suggested that WHI-P154 may enhance the cytotoxicity of ABCG2 substrates in cells express-
ing either wild-type or mutant ABCG2 (Arg482Gly/Thr or Phe489Leu).

Effects of WHI-P154 on cells overexpressing ABCB1, ABCC1, ABCC2 and ABCC10. In order to confirm whether WHI-P154 could modulate the activity of ABCB1, we treated parental KB-3-1 and ABCB1-overexpressing KB-C2 cells with 4 μM WHI-P154 and used paclitaxel as a substrate. Our results indicated that WHI-P154 shows moderate inhibitory effect on ABCB1 (Table 4). Our results also showed that WHI-P154 could not reverse ABCC1-, ABCC2- or ABCC10-mediated MDR (Table 4). Overall, our data suggested that WHI-P154 mainly modulate ABCG2-mediated drug efflux.

Effect of WHI-P154 on intracellular accumulation of [3H]-MX in ABCG2-overexpressing cells. To investigate the potential reversal mechanism of ABCG2-mediated MDR by WHI-P154, we determined the effect of WHI-P154 on the accumulation of [3H]-MX in ABCG2-overexpressing cells. The intracellular level of [3H]-MX was measured in the presence or absence of WHI-P154 in ABCG2-482-R2 and ABCG2-482-G2 cells. The intracellular accumulation of [3H]-MX was lower in the drug resistant cells than that in the parental HEK293/pcDNA3.1 cells. In the presence of WHI-P154 (1 and 4 μM) and FTC (2.5 μM), the intracellular level of [3H]-MX was significantly increased in ABCG2-overexpressing cells (Fig. 2a). However, neither WHI-P154 nor FTC increased the accumulation of [3H]-MX in HEK293/pcDNA3.1 cells (Fig. 2a).

Effect of WHI-P154 on efflux of [3H]-MX in ABCG2-overexpressing cells. In order to confirm the increased accumulation of [3H]-MX was due to inhibition of drug efflux, a time course study was performed to measure intracellular [3H]-MX levels in the presence or absence of WHI-P154. In the absence of WHI-P154, the intracellular [3H]-MX was less in ABCG2-482-R2 cells than the parental cells due to the active ABCG2 efflux. Treatments of 4 μM WHI-P154 in both cells, lead to the blocking of efflux function of ABCG2 thereby the intracellular accumulation of [3H]-MX increased at different time points (30, 60 and 120 min) (Fig. 2b). FTC, at a concentration of 2.5 μM, showed a similar effect. These results herein further validated the ability of WHI-P154 to inhibit the efflux activity of ABCG2.

Effect of WHI-P154 on the expression levels and localization of ABCG2. The reversal effect of ABCG2-mediated MDR could be achieved by either inhibiting ABCG2 function or decreasing the expression levels of ABCG2 protein. Therefore, Western blot analysis was performed to further ascertain the effect of WHI-P154 on the expression levels of ABCG2. Our results showed the expression levels of ABCG2 protein (Fig. 3) were not significantly altered in H460/MX20 cells after treatment
Table 2. WHI-P154 reverses ABCG2-mediated drug resistance in ABCG2-overexpressing cancer cells

| Treatments | I_{50} ± SD \:(\text{nM}) | (Resistence fold) |
|------------|---------------------|------------------|
|            | H460                | H460/MX20         |
| MX         | 78.68 ± 4.19 (1.00) | 2420.95 ± 72.19 (30.77) |
| +WHI-P154  | 77.94 ± 4.79 (0.99) | 690.64 ± 108.88 (8.78)* |
| (1 μM)     |                     |                  |
| +WHI-P154  | 54.65 ± 5.22 (0.69)*| 181.03 ± 21.06 (2.30)* |
| (4 μM)     |                     |                  |
| +FTC (2.5 μM) | 48.96 ± 6.03 (0.60)*| 82.96 ± 6.10 (1.05)* |
| SN-38      | 28.03 ± 3.49 (1.00) | 2899.48 ± 289.60 (103.44) |
| +WHI-P154  | 20.14 ± 1.47 (0.72) | 473.50 ± 58.00 (16.89)* |
| (1 μM)     |                     |                  |
| +WHI-P154  | 16.71 ± 2.17 (0.60)*| 66.76 ± 7.31 (2.38)* |
| (4 μM)     |                     |                  |
| +FTC (2.5 μM) | 14.98 ± 0.62 (0.53)*| 38.27 ± 4.76 (1.37)* |
| Cisplatin  | 1443.04 ± 163.44 (1.00) | 2169.08 ± 237.06 (1.05) |
| +WHI-P154  | 1427.16 ± 121.94 (0.99) | 2089.10 ± 153.74 (1.45) |
| (1 μM)     |                     |                  |
| +WHI-P154  | 1373.42 ± 123.73 (0.95) | 2137.03 ± 240.22 (1.48) |
| (4 μM)     |                     |                  |
| +FTC (2.5 μM) | 1309.20 ± 147.27 (0.91) | 2047.32 ± 172.09 (1.42) |

*P < 0.01 versus that obtained in the absence of WHI-P154. *Values represent mean ± SD of at least three independent experiments, each performed in triplicate. †Resistance fold was determined by dividing the I_{50} values of substrate in H460/MX20 cells in the absence or presence of reversal agents, or H460 cells with reversal agents, by the I_{50} of substrate H460 cells without reversal agents. The resistance fold for HEK293/pCNA3.1 and its resistance cell was obtained in a similar manner.

With 4 μM WHI-P154 for 0, 24, 48 and 72 h, and the data also showed low-level expression of ABCG2 in parent H460 cells (Fig. 3a, c). The grayscale ratios of ABCG2/β-actin were proportional to the ABCG2 protein levels (Fig. 3c). Similarly, WHI-P154 did not significantly alter the expression levels of ABCG2 in ABCG2-489-R2 cells (Fig. 3b, d). In addition, we performed immunofluorescence to determine the effect of WHI-P154 on the cellular localization of ABCG2. As shown in Figure 3(e), WHI-P154 did not alter the localization of ABCG2. These results indicated that neither the expression levels nor localization of ABCG2 was significantly altered by WHI-P154.

Table 3. Effect of WHI-P154 on cells transfected with mutant ABCG2 (Phe489Leu)

| Treatments | I_{50} ± SD \:(\text{nM}) | (Resistence fold) |
|------------|---------------------|------------------|
|            | HEK293/pCNA3.1      | ABCG2-489-Leu    |
| MX         | 18.22 ± 2.30 (1.00) | 55.12 ± 6.02 (3.03) |
| +WHI-P154  | 19.30 ± 3.08 (1.06) | 32.70 ± 4.11 (1.79)* |
| (1 μM)     |                     |                  |
| +WHI-P154  | 16.26 ± 2.66 (0.89) | 22.03 ± 3.22 (1.21)* |
| (4 μM)     |                     |                  |
| +FTC (2.5 μM) | 16.90 ± 1.70 (0.93) | 20.12 ± 2.94 (1.10)* |
| SN-38      | 2423.35 ± 290.10 (1.00) | 1737.74 ± 200.66 (0.72) |
| +WHI-P154  | 2268.60 ± 330.21 (0.94) | 1667.89 ± 190.40 (0.69) |
| (1 μM)     |                     |                  |
| +WHI-P154  | 2350.28 ± 250.65 (0.97) | 1857.01 ± 251.43 (0.77) |
| (4 μM)     |                     |                  |
| +FTC (2.5 μM) | 2205.04 ± 209.38 (0.91) | 1604.22 ± 220.30 (0.66) |

*P < 0.01 versus that obtained in the absence of WHI-P154. *Values represent mean ± SD of at least three independent experiments, each performed in triplicate. †Resistance fold was determined by dividing the I_{50} values of substrate in ABCG2-489-Leu cells in the absence or presence of reversal agents, or HEK293/pCNA3.1 cells with reversal agents, by the I_{50} of substrate HEK293/pCNA3.1 cells without reversal agents.
Table 4. The effect of WHI-P154 on cells overexpress ABCB1, ABCB1, ABCC2 or ABCC10

| Treatments | IC50 ± SD† (nM) (Resistance fold) |
|------------|----------------------------------|
|            | KB-3-1                           | KB-C2                           |
| Paclitaxel | 0.62 ± 0.05 (1.00)‡                | 78.71 ± 8.20 (126.95)           |
| +WHI-P154 (4 μM) | 0.68 ± 0.07 (1.10)                | 34.52 ± 3.34 (55.68)           |
| +Verapamil (4 μM) | 0.65 ± 0.07 (1.05)               | 2.46 ± 0.21 (3.97)             |
| Vincristine | 1.19 ± 0.14 (1.00)‡                | 20.48 ± 2.66 (17.47)           |
| +WHI-P154 (4 μM) | 1.16 ± 0.08 (0.97)                | 17.68 ± 2.35 (14.85)           |
| +PAK-104P (2.5 μM) | 0.49 ± 0.06 (0.41)*              | 2.81 ± 0.31 (2.35)*            |
| LLC/CMV     |                                  |                                 |
| Vincristine | 214.65 ± 23.22 (1.00)‡             | 888.19 ± 100.92 (4.14)          |
| +WHI-P154 (4 μM) | 256.42 ± 31.89 (1.19)             | 714.98 ± 139.43 (3.33)          |
| +PAK-104P (2.5 μM) | 211.75 ± 24.90 (0.99)            | 253.14 ± 22.04 (1.18)*          |
| Paclitaxel | 3.83 ± 1.66 (1.00)‡                 | 77.80 ± 4.21 (9.28)             |
| +WHI-P154 (4 μM) | 7.02 ± 1.19 (0.84)               | 73.69 ± 8.65 (8.79)            |
| +Cepharanthine | 6.46 ± 1.30 (0.77)               | 6.53 ± 1.01 (0.78)*             |
| (2.5 μM)    |                                  |                                 |

*P < 0.01 versus that obtained in the absence of WHI-P154. †Values represent mean ± SD of at least three independent experiments, each performed in triplicate. Resistance fold was determined by dividing the IC50 values of substrate in KB-C2 cells in the absence or presence of reversal agents, or KB-3-1 cells with reversal agents, by the IC50 of substrate KB-3-1 cells without reversal agents; the resistance fold for HEK293/pcDNA3.1, HEK293/ABCC1, LLC/cMOAT and HEK293/ABCC10 cells was obtained in the similar manner.

ABCG2, the membrane vesicles of High Five insect cells overexpressing ABCG2 were used in the presence of various concentrations of WHI-P154 under conditions that suppressed the activity of other major membrane ATPases. As shown in Figure 4, WHI-P154 produced a significantly stimulation of ABCG2 ATPase activity at low concentrations (~10 μM). The results suggested that WHI-P54 may be a substrate for ABCG2.

WHI-P154 docking analysis with human ABCG2 homology models. The Glide predicted docked model of WHI-P154 at Arg482 centroid-based grid of ABCG2 is shown in Figure 5. The aniline ring of WHI-P154 formed hydrophobic interactions with the side chains of Phe489, Ile573, and Pro574. The hydroxyl substituent of the aniline ring may form a hydrogen bond with the side chain of Tyr464 (HO······NH-Ser486, 1.8 Å). The bromine atom present on the aniline ring entered into electrostatic interaction with the hydroxyl group of Tyr464 (BR······HO-Tyr464, 2.9 Å). The 6,7-dimethoxyquinazoline ring was stabilized in a large hydrophobic cavity formed by Phe489, Phe507, Phe511, Ala580, Leu581, Trp527 and Val631. The oxygen atom of the 7-methoxy group formed electrostatic contacts with the imidazole ring NH of His630 (CH2O······HN-His630, 4.1 Å) and side chain amino group of Asn629 (CH2O······H2N-Asn629, 4.0 Å). The N1 of the quinazoline ring was stabilized by electrostatic interaction with the side chain -NH of His630 (-N1······HN-His630, 3.7 Å).

Discussion

ABCG2 is a known molecular cause of MDR in various cancer cells. Therefore, strategies to block ABCG2-mediated active efflux may provide a therapeutic benefit for overcoming MDR. WHI-P154, a potent inhibitor of JAK3 and EGFR, displays antitumor activity by directly inhibiting tumor cell proliferation and survival, causing apoptotic cell death at low concentrations. WHI-P154 was proven to be an effective agent against acute lymphoblastic leukemia, and it inhibited glioblastoma cell adhesion and migration in the context of ECM.

Here we reported for the first time that WHI-P154 could specifically inhibit the ABCG2 transporter in ABCG2 overexpressing cells. As demonstrated by MTT assay, WHI-P154 enhanced the cytotoxicity of MX and SN-38 in ABCG2-overexpressing cells, and also moderately increased the cytotoxicity of these two drugs in H460 cells with low level of ABCG2 as seen in Figure 3(a,c). As reported that amino acid at position 482 is a hot spot for mutation in ABCG2, Arg482 to mutant Gly482 or Thr482 may result in reduced function of ABCG2 (Phe489Leu). As shown in Table 3, WHI-P154 enhanced the cytotoxicity of MX in the cells transfected with mutant ABCG2 (Phe489Leu). Additionally, WHI-
P154 also moderately increased the sensitivity of ABCB1-overexpressing cells to its substrate paclitaxel. In contrast, it had no effect on ABCC1-, ABCC2-, or ABCC10-mediated resistance. These data indicated that WHI-P154 acted as a potent inhibitor of ABCG2.

To investigate the mechanism of the reversal effect, we examined the effects of WHI-P154 via the accumulation assay of [3H]-MX. Our results showed that WHI-P154 could significantly enhance the intracellular concentration of [3H]-MX in ABCG2-482-R2 cells and ABCG2-482-G2 cells, but had no effect on the parental cells. This was confirmed by a time course efflux study of [3H]-MX cellular levels where WHI-P154 at 4 μM significantly reduced the efflux of [3H]-MX from ABCG2-482-R2 cells. Moreover, our results showed that WHI-154 did not alter the expression level or the cellular localization of ABCG2 protein in MDR cells. These results collectively indicated that WHI-P154 could inhibit the transport function of ABCG2, thereby increasing the intracellular accumulation of its substrates.

A previous report showed that the phosphorylation of AKT and ERK1/2 pathways might be related to the sensitivity of chemotherapeutic agents in cancer cells. In addition, JAK3 was involved in the downstream activation of STAT3, which often correlated with chemotherapy drug resistance. However, low concentrations of WHI-P154 (1, 2 μM) did not block the phosphorylation of STAT3, AKT or ERK1/2 pathways.
Values are plotted and error bars depict SD (ATPase assay buffer as described in Materials and Methods. The mean green, hydrogenating ABCG2 were incubated with increasing concentrations of WHI-P154 (0–50 μM) in the presence and absence of 10 mM vandetanib, in ATPase assay buffer as described in Materials and Methods. The mean values are plotted and error bars depict SD (n = 3).

**Fig. 4.** Effect of WHI-P154 on the ATPase activity of ABCG2. Crude membranes (10–20 μg protein/reaction) from high-five cells expressing ABCG2 were incubated with increasing concentrations of WHI-P154 (0–50 μM), in the presence and absence of 10 mM vanadate, in ATPase assay buffer as described in Materials and Methods. The mean values are plotted and error bars depict SD (n = 3).

![XP Glide predicted binding mode of WHI-P154 with homology modeled ABCG2. The docked conformation of WHI-P154 as ball and stick model is shown within the large cavity of ABCG2. Important amino acids are depicted as sticks with the atoms colored as carbon, green, hydrogen-white, nitrogen-blue, oxygen-red, whereas WHI-P154 is shown with the same color scheme as above except carbon atoms are represented in orange. Dotted black lines indicate hydrogen-bonding interactions, whereas dotted red lines indicate electrostatic interactions.](image)

**Fig. 5.** (Fig. 3f) in H460/MX20 cells. WHI-P154 at 4 μM could moderately inhibit the phosphorylation of STAT3, AKT and ERK1/2. The phosphorylation of STAT3, AKT and ERK1/2 was not affected by treatment with low concentrations of WHI-P154. Therefore, the antagonism of JAK3 or EGFR receptors is unlikely to play a significant role in the sensitizing effect of WHI-P154 in ABCG2-overexpressing cells.

ABC transporters use the binding and hydrolysis of ATP to power the translocation of diverse substrates, thus ATPase activity is directly proportional to the transport activity. In our previous studies, several reversal agents such as apatinib, lapatinib, erlotinib could stimulate ATPase activity of ABCG2 at extremely low concentrations. Thus we investigated the interaction of WHI-P154 with ABCG2 by ATPase assay. Our results showed that WHI-P154 could stimulate the ABCG2 ATPase activity at low concentrations. These results suggested that WHI-P154 interact directly with ABCG2 and may be a substrate of the transporter.

To identify the molecular binding of WHI-P154 with ABCG2 transporter, we performed docking studies at various sites of human ABCG2. WHI-P154 showed QikProp value of 3.2 and then its inhibition activity on ABCG2 might be explained based on its ability to distribute with biomembrane from which ABCG2 extracts it. Moreover, WHI-P154 appeared to exhibit all of the pharmacophoric features such as hydrophobic groups, aromatic ring centers, hydrogen bond donors and acceptors that had been identified as critical for ABCG2 inhibition. Overall, this docking model of WHI-P154 will form the basis for further optimization of dimethoxyquinazoline derivatives as inhibitors of ABCG2.

In conclusion, here we report for the first time that WHI-P154 could significantly reverse wild-type and mutant ABCG2-mediated MDR by inhibiting the efflux function without affecting expression level or localization of ABCG2. Further studies are warranted to confirm whether WHI-P154 could contribute to improving clinical outcomes in patients receiving chemotherapy.

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**Disclosure Statement**

The authors have no conflict of interest.

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plasmids, Western blot analysis of ABCG2 (Phe489Leu) in HEK293 cells transfected with pcDNA3.1 plasmids, pcDNA3.1-ABCG2 (Phe489Leu) plasmids, \( \beta \)-actin was used as equal loading control.

### Supporting Information

Additional supporting information may be found in the online version of this article:

**Fig. S1.** Basic sequencing to confirm target mutation, the sequence alignment of wild type ABCG2 and mutant ABCG2 (Phe489Leu), TAA is a stop codon.

**Fig. S2.** Western blot analysis of ABCG2 (Phe489Leu) in HEK293 cells transfected with pcDNA3.1 plasmids, pcDNA3.1-ABCG2 (Phe489Leu) plasmids, \( \beta \)-actin was used as equal loading control.

### Table S1.

| Binding energies of WHI P154 within each of the predicted binding sites of ABCG2. | 5% of 3D Model | 5% of 2D Model | 5% of 1D Model | 5% of 0D Model |
|---|---|---|---|---|
| Mean | 1.23 | 1.24 | 1.25 | 1.26 |
| Standard Deviation | 0.12 | 0.13 | 0.14 | 0.15 |

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