Acta Pharmaceutica Sinica B 2014;4(3):202–207

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

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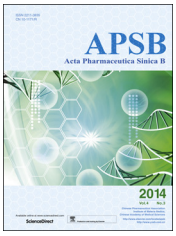

ORIGINAL ARTICLE

PD173074, a selective FGFR inhibitor, reverses MRP7 (ABCC10)-mediated MDR

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Received 25 December 2013; revised 28 January 2014; accepted 14 February 2014

KEY WORDS

PD173074;
ABCC10;
Fibroblast growth factor receptor;
Multidrug resistance;
Tyrosine kinase inhibitor

Abstract Multidrug resistance protein 7 (MRP7, ABCC10) is a recently identified member of the ATP-binding cassette (ABC) transporter family, which adequately confers resistance to a diverse group of antineoplastic agents, including taxanes, vinca alkaloids and nucleoside analogs among others. Clinical studies indicate an increased MRP7 expression in non-small cell lung carcinomas (NSCLC) compared to a normal healthy lung tissue. Recent studies revealed increased paclitaxel sensitivity in the Mrp7$^{-/-}$ mouse model compared to their wild-type counterparts. This demonstrates that MRP7 is a key contributor in developing drug resistance. Recently our group reported that PD173074, a specific fibroblast growth factor receptor (FGFR) inhibitor, could significantly reverse P-glycoprotein-mediated MDR. However, whether PD173074 can interact with and inhibit other MRP members is unknown. In the present study, we investigated the ability of PD173074 to reverse MRP7-mediated MDR. We found that PD173074, at non-toxic concentration, could significantly increase the cellular sensitivity to MRP7 substrates. Mechanistic studies indicated that PD173074 (1 μmol/L) significantly increased the intracellular accumulation and in-turn decreased the efflux of paclitaxel by inhibiting the transport activity without

Abbreviations: ABC, ATP binding cassette; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; HEK293, human embryonic kidney 293; MDR, multidrug resistance; MRP7, multidrug resistance protein 7; MSDs, membrane-spanning domains; NBDs, nucleotide-binding domains; NSCLC, non-small cell lung carcinomas; RTK, receptor tyrosine kinase; TKI, tyrosine kinase inhibitor

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Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

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http://dx.doi.org/10.1016/j.apsb.2014.02.003

altering expression levels of the MRP7 protein, thereby representing a promising therapeutic agent in the clinical treatment of chemoresistant cancer patients.

1. Introduction

Acquired multidrug resistance (MDR) within the tumor population has been a huge obstacle towards attaining a successful chemotherapy. Overexpression of a class of efflux transporters, known as ATP binding cassette (ABC) transporters, is a vital component of the several factors contributing immensely to the development of MDR[1]. Of the 48 known members of the ABC transporter family, the C subfamily of proteins, alternatively known as the ABCC proteins or the multidrug resistance protein (MRP) subfamily, confers resistance and transports several categories of chemotherapeutic agents including taxanes, vinca alkaloids, camptothecans, nucleoside analogs, and physiologic substrates including leukotrienes and glutathione[2]. The C group of ABC transporter subfamily comprises of nine protein members with a common structural arrangement that includes at least two membrane-spanning domains (MSDs) and another two nucleotide-binding domains (NBDs)[2]. The ABCC subfamily is further classified into two groups, long MRPs and short MRPs, in which long MRPs comprise of ABCC1 (MRP1), ABCC2 (MRP2), ABCC3 (MRP3), ABCC6 (MRP6) and ABCC10 (MRP7), all bearing an additional N-terminal transmembrane domain, and short MRPs include ABCC4 (MRP4), ABCC5 (MRP5), ABCC11 (MRP8) and ABCC12 (MRP9), which lack the additional transmembrane domain[3]. In particular, MRP7 consists of three MSDs and two NBDs and is ubiquitously expressed within the body[4].

Membranous MRP7 confers resistance to a wide range of clinically used drugs, including taxanes, vinca alkaloids, nucleoside analogs and epothilone B[5]. Clinical studies indicated that MRP7 expression level was increased in non-small cell lung cancer (NSCLC) when compared to normal lung tissue. A recent report demonstrated that increased paclitaxel sensitivity in Mrp7$^{-/-}$ mouse model, compared to their wild-type counterparts, resulted in neutropenia and bone marrow hypoplasia.

Altering the expression of the transporter proteins or their functions can surmount ABC transporter-mediated MDR. It was hypothesized that inhibiting the transporter activity could restore the cytotoxicity of anticancer drugs against resistant cells. A substantial number of compounds have been identified to reverse ABC transporter-mediated MDR[6]. However, the development of most of these inhibitors has been hampered due to low binding affinity, toxicity and detrimental pharmacokinetic interactions. In addition, very few reversal agents for MRP members have been discovered or advanced to clinical trials. Therefore, there is a constant urge for the discovery and identification of potent and specific inhibitors of MRP transporters. Together, these findings indicate that the modulation of MRP7 activity may have clinical significance in management of human cancers, such as NSCLC.

PD173074 is a small-molecule tyrosine kinase inhibitor (TKI) that disrupts fibroblast growth factor (FGFR) family related signaling. PD173074, a pyrido[2,3-*d*]pyrimidine, was synthesized based on the crystal structure of FGF2-inhibitor complex and was found to exhibit a high degree of complementarity towards the tyrosine kinase domain of FGFR1[7,8]. Early studies demonstrated inhibition of FGFR1 receptor tyrosine kinase (RTK) by PD173074, leading to inhibition of angiogenesis in preclinical murine models. Recently, our group reported that PD173074 could significantly reverse P-gp-mediated MDR[9]. However, the interaction of PD173074 with MRP7 still remains unknown. In the present study, we investigated the characteristics of PD173074 to reverse MRP7-mediated MDR. We found that PD173074 could significantly increase the cellular sensitivity to MRP7 substrates in MRP7-overexpressed cells.

2. Material and methods

2.1. Chemicals

[^{3}H]-paclitaxel (23 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Cepharanthine was generously provided by Kakenshoyaku Co. (Tokyo, Japan). Paclitaxel, vincristine, dimethyl sulfoxide (DMSO) and 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). PD173074 was purchased from Tocris Bioscience (Ellisville, MO).

2.2. Cell lines and cell culture

The previously reported MRP7 expression vector and parental empty vector plasmid were transfected into human embryonic kidney HEK293 cells by electroporation[10]. Transfected cells were selected in DMEM containing 2 mg/mL G418. The parental cell line transfected with empty vector was represented as HEK293 and HEK293 transfected with MRP7 expression vector was represented as HEK293-MRP7. All cell lines were grown as adherent monolayers in DMEM supplemented with 10% fetal bovine serum (FBS), 10,000 IU/mL penicillin and 10,000 μg/mL streptomycin (Hyclone, Logan, UT) in a 5% CO_2 incubator at 37 °C.

2.3. Cytotoxicity assay

An MTT colorimetric assay with minor modifications from that previously described[11] was used to detect the sensitivity of cells to anticancer drugs. Cells were harvested after addition of trypsin and suspended at a concentration of 6×10^3 cells/well. For the reversal experiment, PD173074 (0.25 or 1 μmol/L, 20 μL/well) or cepharanthine (2.5 μmol/L, 20 μL/well) was added, followed by different concentrations of chemotherapeutic drugs (20 μL/well) into designated wells. After 68 h of incubation, 20 μL of MTT solution (4 mg/mL) was added to each well, and the plate was further incubated for another 4 h, allowing viable cells to convert the yellow-colored MTT into dark blue formazan crystals. Subsequently, the medium was aspirated, and 100 μL DMSO was added

to each well to dissolve the formazan crystals. The absorbance was determined at 570 nm by an OPSYS Microplate Reader (DYNEX Technologies, Chantilly, VA). The degree of resistance was calculated by dividing the IC_{50} (concentrations required to inhibit growth by 50%) for the MDR cells by that of the parental sensitive cells. The IC_{50} values were calculated to construct the survival curves using the Bliss method[12].

2.4. Immunoblotting and cell lysate

Total cell lysates were prepared by harvesting the cells and rinsing three times with ice-cold PBS. Cell extracts were prepared by incubating cells for 30 min on ice with radioimmunoprecipitation assay (RIPA) buffer (PBS with 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 100 mg/mL *p*-aminophenyl-methylsulfonyl fluoride) with occasional rocking, followed by centrifugation (12,000 rpm, 4 °C for 20 min). The supernatant containing total cell lysates was stored at 80 °C prior to experiments. Cell lysates containing identical amounts of total protein (40 μg) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. After incubation in a blocking TBST buffer (10 mmol/L pH 8.0 Tris–HCl, 150 mmol/L NaCl, and 0.1% Tween 20), and 5% non-fat milk for 2 h at room temperature, the membranes were immunoblotted overnight with primary antibodies against MRP7 (1:200 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) or ß-actin (1:200 dilution) (Cell Signaling, Danvers, MA) at 4 °C, and then incubated at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000 dilution) for 2 h. The protein-antibody complex was detected by chemiluminescence.

2.5. [^{3}H]-paclitaxel accumulation and efflux

Intracellular paclitaxel accumulation and the time-dependent efflux of [^{3}H]-paclitaxel were measured in HEK293 and HEK293-MRP7 cells. For the 4 h accumulation assay, cells were trypsinized and three aliquots (5×10^6 cells) from each cell lines were resuspended in fresh medium. To measure drug accumulation, cells were pre-incubated in DMEM in the presence or absence of PD173074 (0.25 and 1 μmol/L) or cepharanthine (2.5 μmol/L) for 1 h, washed and then incubated with 0.01 μmol/L [^{3}H]-paclitaxel with or without PD173074 (0.25 μmol/L and 1 μmol/L) or cepharanthine (2.5 μmol/L) for another 2 h at 37 °C. The cells were then pelleted at 4 °C, washed twice with 10 mL ice-cold PBS, lysed in lysis buffer, and measured for radioactivity in a liquid scintillation counter, Packard TRI-CARB 1900CA liquid scintillation analyzer (Packard Instrument Company, Downers Grove, IL). For the 68 h accumulation assay, cells were cultured in DMEM in the presence or absence of PD173074 for 68 h. Cells were then trypsinized and resuspended with the same cell number in each group, and incubated with 0.01 μmol/L [^{3}H]-paclitaxel with or without PD173074 (1 μmol/L) for another 2 h at 37 °C. The cells were then collected, washed with ice-cold PBS, lysed and measured for radioactivity in a liquid scintillation counter. For the efflux study, cells were first pre-incubated with or without PD17304 at 1 μmol/L for 1 h and later incubated with 0.01 μmol/L [^{3}H]-paclitaxel as previously described. After washing twice with ice-cold PBS, the cells were cultured in fresh DMEM with or without 1 μmol/L of PD173074 at 37 °C. After 0, 30, 60 or 120 min, aliquots of cells were removed and immediately washed with ice-cold PBS. The cell pellets were collected for radioactivity measurement as described earlier.

2.6. Statistical analysis

All experiments were repeated for at least three times. Statistical differences were determined by the two-tailed student's *t*-test, and were deemed significant if $P < 0.05$.

3. Results

3.1. The effect of PD173074 on the drug sensitivity of MRP7-transfected HEK293 cells

The colorimetric sensitivity assay revealed that HEK293-MRP7 cells, compared to HEK293 cells, displayed significant resistance to various MRP7 substrates such as paclitaxel (11.7-fold) and vincristine (5.4-fold), but showed no significant sensitivity difference to cisplatin (0.9-fold), which is not a substrate of MRP7 (Table 1).

We tested PD173074 in combination with MRP7 substrates to ascertain if it would reverse MRP7-mediated MDR. The highest concentration of PD173074 used in the reversal experiments was 1 μmol/L, a concentration that resulted in < 10% growth inhibition in all the cell lines used in the present study (data not shown). PD173074 at 0.25 and 1 μmol/L, had demonstrated dose-dependently and significantly decreased the IC_{50} values of HEK293-MRP7 cells (Table 1). However, PD173074 at 1 μmol/L did not significantly alter the sensitivity of the parental HEK293 cells. In contrast, PD173074 did not significantly reverse the resistance of cells to cisplatin, a non-MRP7 substrate (Table 1). Previously, we reported that cepharanthine could reverse MRP7-mediated resistance to paclitaxel in a competitive manner[13]. Hence, to compare PD173074, we used cepharanthine as a positive control in the present experiment, and we demonstrated that the effect of cepharanthine was comparable to that of PD173074 (Table 1).

3.2. The effect of PD173074 on the intracellular accumulation of [^{3}H]-paclitaxel

To determine the function of MRP7 in mediating the effect of PD173074, we evaluated the accumulation of [^{3}H]-paclitaxel in the presence or absence of PD17307 in HEK293 and HEK293-MRP7 cells. The intracellular concentration of [^{3}H]-paclitaxel in HEK293-MRP7 cells was 32% of that in HEK293 cells. After 1 h treatment, PD173074 at 0.25 and 1 μmol/L significantly enhanced the intracellular [^{3}H]-paclitaxel accumulation in HEK293-MRP7 cells (Fig. 1, $P < 0.05$) while PD173074 did not alter the intracellular accumulation of [^{3}H]-paclitaxel in the parental HEK293 cells, indicating that the action of PD173074 is only related to MRP7 overexpression.

3.3. The effect of PD173074 on the efflux of [^{3}H]-paclitaxel

To ascertain whether the elevated intracellular [^{3}H]-paclitaxel accumulation, caused by PD173074, was due to an inhibition of [^{3}H]-paclitaxel efflux by the MRP7 transporter, we conducted a time-course study to determine [^{3}H]-paclitaxel efflux in the presence of PD173074. Our results indicated that HEK293-MRP7

Table 1 PD173074 reverses the ABCC10-mediated drug resistance to paclitaxel and vincristine.

Compound	HEK293		HEK293-MRP7	
	$IC_{50} \pm SD$[a] (nmol/L)	FR[b]	$IC_{50} \pm SD$[a] (nmol/L)	FR[b]
Paclitaxel	10.37 ± 0.03	[1.0]	122.11 ± 2.24	[11.7]
+PD173074 (0.25 μmol/L)	12.32 ± 0.15	[1.2]	19.27 ± 0.69	[1.9] **
+PD173074 (1 μmol/L)	11.43 ± 0.25	[1.1]	15.78 ± 0.15	[1.5] **
+Cepharanthine (2.5 μmol/L)	11.15 ± 0.91	[1.1]	15.20 ± 0.92	[1.5] **
Vincristine	4.96 ± 0.35	[1.0]	30.28 ± 1.34	[6.1]
+PD173074 (0.25 μmol/L)	4.55 ± 0.11	[0.9]	6.76 ± 0.12	[1.4] **
+PD173074 (1 μmol/L)	4.46 ± 0.28	[0.9]	4.69 ± 0.15	[0.9] **
+Cepharanthine (2.5 μmol/L)	4.53 ± 0.07	[0.9]	4.78 ± 0.05	[1.0] **
Cisplatin	3237.67 ± 107.05	[1.0]	4307.34 ± 28.86	[1.3]
+PD173074 (0.25 μmol/L)	3261.67 ± 15.63	[1.0]	4797.43 ± 145.77	[1.5]
+PD173074 (1 μmol/L)	3421.21 ± 45.03	[1.0]	4425.14 ± 51.50	[1.4]
+Cepharanthine (2.5 μmol/L)	3257.34 ± 39.95	[1.0]	4398.67 ± 62.93	[1.4]

Values in table are representative of at least three independent experiments performed in triplicate.

[a] IC_{50}, concentration that inhibited cell survival by 50% (means ± SD).

[b] FR, fold-resistance was determined by dividing the IC_{50} values of substrate in HEK293-MRP7 cells by the IC_{50} of substrate in HEK293 cells in the absence of PD173074; or the IC_{50} of substrate in HEK293 cells in the presence of PD173074 divided by the IC_{50} of substrate in HEK293 cells in the absence of PD173074.

**Indicates significantly different from IC_{50} of HEK293-MRP7 without reversal drug ($^{**}P < 0.01$).

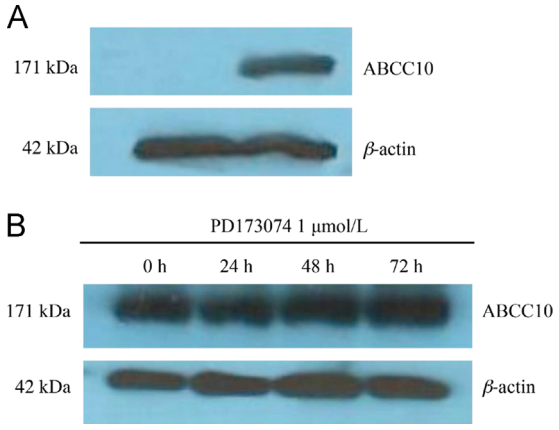

Figure 1 Immunoblot analysis showing the expression of ABCC10. Immunoblot analysis on the expression of ABCC10 efflux transporter. (A) Expression of ABCC10 in HEK293 and HEK293-MRP7 cells. Representative result is shown here and similar results were obtained in two other trials. (B) Cell lysates of ABCC10 protein overexpressing HEK293-MRP7 cells exposed to PD173074 at 1 μmol/L were prepared at different time points (0, 24, 48 and 72 h) and equal amounts (40 μg) were loaded into each well and subjected to immunoblot analysis, later they were exposed to the same amount and the same antibody for ABCC10 as discussed in Section 2. Representative result is shown here and similar results were obtained in two other trials.

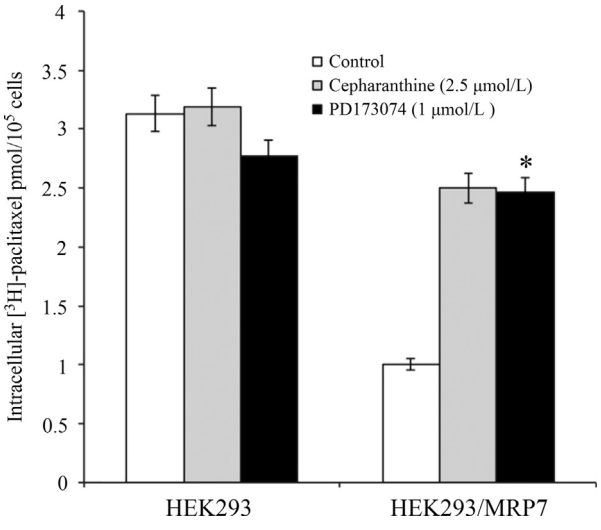

Figure 2 Effect of PD173074 on the accumulation of [^{3}H]-paclitaxel. The accumulation of [^{3}H]-paclitaxel was measured after the cells (parental HEK293 and HEK293-MRP7) were pre-incubated with or without PD173074 at 1 μmol/L or cepharanthine for 1 h at 37 °C and then incubated with 0.1 μmol/L [^{3}H]-paclitaxel for another 2 h at 37 °C. Columns are the mean of triplicate determinations; bars represent SD. $^*P < 0.05$ *versus* the control group. The figure is a representative of three independent experiments each performed in triplicates.

cells extruded a significantly higher percentage of [^{3}H]-paclitaxel than HEK293 cells (Fig. 2, $P < 0.05$). When the cells were incubated with 1 μmol/L of PD173074, the HEK293-MRP7 cells, but not the parental HEK293 cells, significantly blocked the intracellular [^{3}H]-paclitaxel efflux at different time periods (0, 30, 60 and 120 min).

Considering the accumulation of [^{3}H]-paclitaxel in HEK293-MRP7 cells in the absence of PD173074 at 0 min as 100%, the percentages observed at 30, 60 and 120 min were 44.12%, 27.64% and 25.24%, respectively. When HEK293-MRP7 cells were incubated with PD173074, the percentages at 30, 60 and 120 min increased to 78.69%, 69.36%, and 56.77%, respectively (Fig. 3,

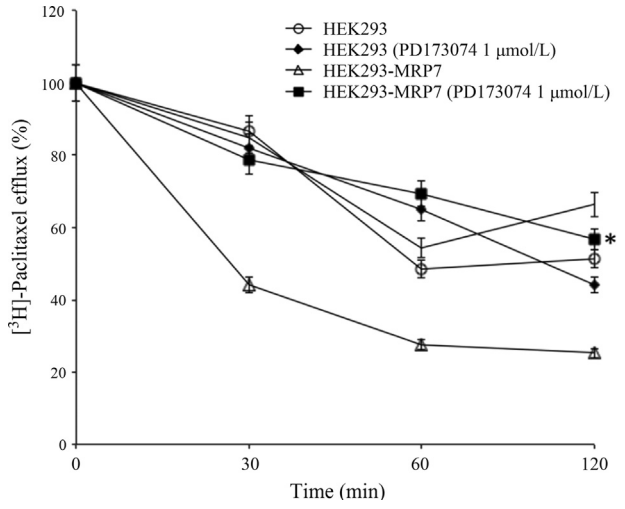

Figure 3 Effect of PD173074 on the efflux of [^{3}H]-paclitaxel. Cells were pre-treated with or without PD173074 at 1 μmol/L for 1 h at 37 °C and further incubated with 0.1 μmol/L [^{3}H]-paclitaxel at 37 °C for 2 h. Cells were then incubated in fresh medium with or without the reversal agents for different time periods at 37 °C. Cells were then collected and the intracellular levels of [^{3}H]-paclitaxel were determined by scintillation counting. A time course *versus* percentage of intracellular [^{3}H]-paclitaxel was plotted (0, 30, 60 and 120 min). Cepharanthine (2.5 μmol/L) was used as a positive control. Error bars represent SD. *$P<0.05$ *versus* the control group. The figure is a representative of three independent experiments each done in triplicates.

$P<0.05$ for the same time point comparison). Cepharanthine at 2.5 μmol/L effectively blocked MRP7 efflux function and significantly increased the levels of paclitaxel in HEK293-MRP7 cells.

3.4. *The effect of PD173074 on the expression of MRP7*

In HEK293-MRP7 cells, the reversal of MRP7-mediated MDR could be achieved by either decreasing MRP7 expression or blocking the efflux function of the transporter. To evaluate the effect of PD173074 on MRP7 expression, HEK293-MRP7 cells were treated with PD173074 and the levels of MRP7 expression were examined by Western blot analysis. We found that the protein level of MRP7 in HEK293-MRP7 cells remained unaltered after treatment with PD173074 at 1 μmol/L for 0, 24, 48 and 72 h (Fig. 3). These data suggest that PD173074 blocks the function of the transporter without affecting its expression levels.

4. Discussion

In spite of limited reports indicating the widespread tissue expression of MRP7[4], it remains as one of the least characterized ABC family members. MRP7 expression level is up-regulated in NSCLC as compared to normal lung tissues, and its higher expression is correlated to advanced pathological grades in adenocarcinoma[14]. In hepatocellular carcinoma, MRP7 expression level is augmented when compared with normal adjacent healthy liver tissues[15], and *MRP7* gene expression levels in colorectal tumors correlate with tumor grade[16]. Absence of MRP7 *in vivo* sensitizes animals to paclitaxel, with Mrp7$^{-/-}$ mice exhibiting enhanced sensitivity compared to their wild-type counterparts following paclitaxel treatment[17], entailing that increased MRP7 expression might be a biomarker for and regulator of treatment response in certain cancers. In a recent study, intermittent and continuous docetaxel chemotherapy in chemosensitive and chemoresistant ovarian mice shows that MRP7 gene expression is increased along with MDR1 in chemoresistant ovarian tumors during intermittent docetaxel treatment. This implies that chemotherapy-dosing schedule affects the development, further worsening, or circumvention of drug resistance in chemosensitive and chemoresistant ovarian cancer[18].

Currently, pre-clinical research and clinical trials are investigating the combination of EGFR TKIs with other antineoplastic drugs to ameliorate the therapeutic outcome of cancer patients. Thus, the interaction of EGFR TKIs, with P-gp and/or MRP7 should be addressed when exploring the combined use of EGFR, TKIs with cytotoxic anticancer drugs that are substrates of P-gp, BCRP and/or MRP7. TKIs have demonstrated to act on the catalytic site of the tyrosine kinase domain by competing with ATP binding, thereby blocking the kinase activity. Various *in vitro* studies have described TKIs to interact and modulate the function of the ABC transporters[19,20]. Nilotinib, an HER2/EGFR inhibitor, approved for the use of chronic myelogenous leukemia (CML), has been shown to inhibit P-gp-, BCRP- and MRP7-mediated MDR[21]. The 4-anilinoquinazoline-derived EGFR TKIs, such as lapatinib (Tykerb®) and erlotinib (Tarceva®), have been shown to inhibit the ABCC10-mediated drug resistance[22]. However, no reports of any drug have been clinically approved for the reversal of MDR due to pharmacokinetic interactions or toxicity issues.

PD173074, a selective FGFR TKI, has shown promising results of blocking the growth of small cell lung cancer (SCLC) both *in vitro* and *in vivo*[23]. FGFR signaling has been related to neoangiogenesis[24,25], induction of SCLC cell proliferation, and resistance to cytotoxic drugs[26,27]. When used *in vivo*, PD173074 was shown to inhibit FGF-driven neoangiogenesis, while being exempt of general toxicity[8,28]. Recently, our group reported that PD173074 could significantly reverse P-gp (ABCB1)-mediated MDR[9]. However, the interaction of PD173074 with other MRP members remains unknown.

This is the first report demonstrating the effect of PD173074 on MRP7-mediated MDR. Our data indicated that PD173074 could potently reverse MRP7-mediated MDR. PD173074 significantly sensitized MRP7-overexpressing cells to a variety of MRP7 substrates, including paclitaxel, docetaxel and vincristine. PD173074 at 1 μmol/L was able to reverse MRP7-mediated MDR completely. In coherence with cytotoxicity data, drug accumulation studies demonstrated that PD173074 significantly enhanced the intracellular accumulation of [^{3}H]-paclitaxel in MRP7-overexpressing cells. The efflux data suggested that increased intracellular accumulation of [^{3}H]-paclitaxel was contributed by rapid and direct inhibition of MRP7-mediated drug efflux by PD173074 within a short time period (2–4 h). Therefore, the reversal of MRP7-mediated MDR by PD173074 in HEK293-MRP7 cells involved direct inhibition of MRP7 efflux function without interfering MRP7 protein expression.

5. Conclusions

Our findings indicate for the first time that the FGFR TKI, PD173074, is able to effectively reverse MRP7-mediated MDR. The mechanism of MDR modulation by PD173074 is associated with an increased intracellular drug accumulation by inhibiting drug efflux from MDR cells. These results suggest that PD173074 could

be used to augment the clinical response by established chemotherapeutic agents that are substrates of MRP7. Therefore, the use of PD173074 along with anti-neoplastic agents that are MRP7 substrates warrants further study.

Acknowledgments

This work was supported by funds from NIH (No. 1R15CA143701) and St. John's University Research Seed Grant (No. 579-1110-7002) to Z.S. Chen. The authors would like to thank the late Dr. Gary D. Kruh (University of Illinois at Chicago, USA) for HEK293 cell line and the *MRP7* cDNA.

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