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

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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 intracellular accumulation and in-turn decreased the efflux of paclitaxel by inhibiting the transport activity without
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 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)\(^3\). 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 cancer (NSCLC) when compared to normal lung tissue. A recent study demonstrated inhibition of FGFR1 receptor tyrosine kinase (RTK) by PD173074, leading to inhibition of angio genesis in preclinical murine models. Recently, our group reported that PD173074 could significantly reverse P-gp-mediated MDR\(^6\). 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

\(^{[1]}\)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

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

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2.5. [3H]-paclitaxel accumulation and efflux

Detected by chemiluminescence.

To each well to dissolve the formazan crystals. The absorbance was determined at 570 nm by an OPTECH Microplate Reader (DYNEX Technologies, Chantilly, VA). The degree of resistance was calculated by dividing the IC50 (concentrations required to inhibit growth by 50%) for the MDR cells by that of the parental sensitive cells. The IC50 values were calculated to construct the survival curves using the Bliss method.

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 IC50 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.

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 [3H]-paclitaxel

To determine the function of MRP7 in mediating the effect of PD173074, we evaluated the accumulation of [3H]-paclitaxel 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 [3H]-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 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 [3H]-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 [3H]-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.

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

To ascertain whether the elevated intracellular [3H]-paclitaxel accumulation, caused by PD173074, was due to an inhibition of [3H]-paclitaxel efflux by the MRP7 transporter, we conducted a time-course study to determine [3H]-paclitaxel efflux in the presence of PD173074. Our results indicated that HEK293-MRP7...
cells extruded a significantly higher percentage of \([1^H]\)-paclitaxel than HEK293 cells (Fig. 2, \(P<0.05\)). When the cells were incubated with 1 \(\mu\)mol/L of PD173074, the HEK293-MRP7 cells, but not the parental HEK293 cells, significantly blocked the intracellular \([1^H]\)-paclitaxel efflux at different time periods (0, 30, 60 and 120 min).

Considering the accumulation of \([1^H]\)-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).

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

| Compound   | HEK293 IC\(_{50}\) ± SD (nmol/L) | HEK293-MRP7 IC\(_{50}\) ± SD (nmol/L) |
|------------|----------------------------------|--------------------------------------|
| Paclitaxel | 10.37 ± 0.03                     | 122.11 ± 2.24                       |
| +PD173074 (0.25 \(\mu\)mol/L) | 12.32 ± 0.15                     | 19.27 ± 0.69                        |
| +PD173074 (1 \(\mu\)mol/L)   | 11.43 ± 0.25                     | 15.78 ± 0.15                        |
| +Cepharanthine (2.5 \(\mu\)mol/L) | 11.15 ± 0.91                     | 15.20 ± 0.92                        |
| Vincristine | 4.96 ± 0.35                      | 30.28 ± 1.34                        |
| +PD173074 (0.25 \(\mu\)mol/L) | 4.55 ± 0.11                      | 6.76 ± 0.12                         |
| +PD173074 (1 \(\mu\)mol/L)   | 4.46 ± 0.28                      | 4.69 ± 0.15                         |
| +Cepharanthine (2.5 \(\mu\)mol/L) | 4.53 ± 0.07                      | 4.78 ± 0.05                         |
| Cisplatin  | 3237.67 ± 107.05                 | 4307.34 ± 28.86                     |
| +PD173074 (0.25 \(\mu\)mol/L) | 3261.67 ± 15.63                 | 4797.43 ± 145.77                   |
| +PD173074 (1 \(\mu\)mol/L)   | 3421.21 ± 45.03                 | 4425.14 ± 51.50                    |
| +Cepharanthine (2.5 \(\mu\)mol/L) | 3257.34 ± 39.95                 | 4398.67 ± 62.93                    |

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

\(^a\)IC\(_{50}\) concentration that inhibited cell survival by 50% (mean ± SD).

\(^b\)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 (**\(^*P<0.01\)).
expression of MRP7, it remains as one of the least characterized protein level of MRP7 in HEK293-MRP7 cells remained unaltered were examined by Western blot analysis. We found that the effect of PD173074 on MRP7 expression, HEK293-MRP7 cells were treated with PD173074 and the levels of MRP7 expression following paclitaxel treatment, 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.

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. 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. The 4-anilinoquinazoline-derived EGFR TKIs, such as lapatinib (Tykerb®) and erlotinib (Tarceva®), have been shown to inhibit the ABCG2-mediated drug resistance. 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. FGFR signaling has been related to neangiogenesis, induction of SCLC cell proliferation, and resistance to cytotoxic drugs. When used in vivo, PD173074 was shown to inhibit FGF-driven neangiogenesis, while being exempt of general toxicity. Recently, our group reported that PD173074 could significantly reverse P-gp (ABCB1)-mediated MDR. 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 [3H]-paclitaxel in MRP7-overexpressing cells. The efflux data suggested that increased intracellular accumulation of [3H]-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.

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