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Lapatinib and erlotinib are potent reversal agents for MRP7 (ABCC10)-mediated multidrug resistance

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Abstract

In recent years, a number of TKIs (tyrosine kinase inhibitors) targeting epidermal growth factor receptor (EGFR) family have been synthesized and some have been approved for clinical treatment of cancer by the FDA. We recently reported a new pharmacological action of the 4-anilinoquinazoline derived EGFR TKIs, such as lapatinib (Tykerb®) and erlotinib (Tarceva®), which significantly affect the drug resistance patterns in cells expressing the multidrug resistance (MDR) phenotype. Previously, we showed that lapatinib and erlotinib could inhibit the drug efflux function of P-glycoprotein (P-gp, ABCB1) and ABCG2 transporters. In this study, we determined if these TKIs have the potential to reverse MDR due to the presence of the multidrug resistance protein 7 (MRP7, ABCC10). Our results showed that lapatinib and erlotinib dose-dependently enhanced the sensitivity of MRP7-transfected HEK293 cells to several established MRP7 substrates, specifically docetaxel, paclitaxel, vinblastine and vinorelbine, whereas there was no or a lesser effect on the control vector transfected HEK293 cells. [^{3}H]-paclitaxel accumulation and efflux studies demonstrated that lapatinib and erlotinib increased the intracellular accumulation of [^{3}H]-paclitaxel and inhibited the efflux of [^{3}H]-paclitaxel from MRP7 transfected cells but not in the control cell line. Lapatinib is a more potent inhibitor of MRP7 than erlotinib. In addition, the Western blot analysis revealed that both lapatinib and erlotinib did not significantly affect MRP7 expression. We conclude that the EGFR TKIs, lapatinib and erlotinib reverse MRP7-mediated MDR through inhibition of the drug efflux function, suggesting that an EGFR TKI based combinational therapy may be applicable for chemotherapeutic practice clinically.

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Keywords

tyrosine kinase inhibitor; EGFR; lapatinib; erlotinib; ABC transporters; MRP7/ABCC10; multidrug resistance

1. Introduction

The signaling pathways of tyrosine kinases (TKs) are involved in cancer cell proliferation, apoptosis, angiogenesis and metastasis [1,2]. Tyrosine kinase inhibitors (TKIs) are generally reversible competitors against ATP for binding to the intracellular catalytic domain of the TKs. Consequently, they inhibit autophosphorylation as well as downstream signaling processes, thereby representing a promising class of anticancer agents in the clinic [3,4]. The FDA has approved TKIs including imatinib, gefitinib, erlotinib and lapatinib for the treatment of various cancers in recent years [5,6]. Two predominant classes of TKIs have been developed and used in the clinic or clinical trials. These include BCR-ABL TKIs such as imatinib, nilotinib, dasatinib and bosutinib, and epidermal growth factor receptor (EGFR, HER1) TKIs such as gefitinib, erlotinib, lapatinib, caneritinib and AG1478. Recent studies have identified TKIs as modulators of ATP-binding cassette (ABC) transporter-mediated multidrug resistance (MDR) in cancer cells [7–9].

MDR is the development of resistance to a variety of anticancer drugs that are structurally and mechanistically unrelated, presenting a major obstacle to successful chemotherapy treatment [10]. Recently, a significant effort to elucidate the mechanism of MDR has been focused on the ABC transporters, and their abilities to extrude drugs from the cells [11–16]. These transporter proteins originate from one of the largest protein families which is divided into seven subfamilies (A–G) based on sequence similarities, including ABCB1, also called P-glycoprotein (P-gp) [11], and multidrug resistance proteins (MRPs, ABCCs) [14] and ABCG2 [15,16]. These ABC transporters are highly varied transporters which function to extrude a wide range of structurally and mechanistically different drugs from the cells. For example, drugs transported by P-gp include vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes [17]; drugs transported by MRP1 such as vinca alkaloids, anthracyclines, epipodophyllotoxins and some heavy metal anions [18]; drugs transported by ABCG2 include anthracyclines, mitoxantrone, antifolates, and flavopiridol [19]. Mechanistically, these ABC transporters are coupled to an ATP hydrolysis process, thereby utilizing energy to transport drugs outside of cells. Inhibition of ABC transporter-mediated drug efflux may re-sensitize MDR cancer cells to an effective MDR tumor treatment with chemotherapeutic agents. Currently, three generations of P-gp inhibitors and a number of MRP1 and ABCG2 inhibitors have been developed to enhance the effect of chemotherapeutic drugs on MDR cancer cells *in vitro* and *in vivo*[20–23]. Recently, we and others have reported that several TKIs are dual modulators of P-gp and ABCG2. For example, resistance to imatinib was related to P-gp overexpression and imatinib could reverse P-gp-mediated drug resistance. In addition, imatinib reverses ABCG2-mediated MDR [24,25]. Our recent data suggests that AG1478, an EGFR TKI, interacts with the substrate binding sites of P-gp and ABCG2 [26], we have also shown for the first time that lapatinib and erlotinib enhance the cytotoxic effects of multiple anti-cancer drugs by increasing the accumulation of P-gp and ABCG2 substrates due to their direct interaction at the substrate binding site[27,28]. Based on the amino acid sequence similarity, multidrug resistance protein 7 (MRP7/ABCC10) was recently characterized [29]. On the basis of amino acid sequence comparisons, the topology of MRP7 is similar to those of MRP1, 2, 3 and 6, with two nucleotide-binding domains and three membrane-spanning domains [29]. Phylogenetic analysis indicated that MRP7 is related to lipophilic anion pumps and is also involved in the regulation of ion channels. Previous *in vitro* studies on MRP7 transfected cell lines suggested that 17-β-estradiol-(17-beta-D-glucuronide), some taxanes and vinca alkaloids

are substrates of MRP7 [30,31]. Bessho Y et al recently reported that MRP7 confers resistance to vinorelbine in non-small cell lung cancer (NSCLC) cells [32]. The discovery of potent and specific inhibitors of MRP7 is of great interest, and may represent a strategy to overcome clinical drug resistance. It was hypothesized that since MRP7 shares some common substrates and functions with other members in the ABC family, modulators that overcome P-gp or ABCG2-linked MDR may also alleviate MRP7-mediated drug resistance. Indeed, we found that a P-gp inhibitor cepharanthine could also reverse MRP7-mediated resistance to paclitaxel [33]. In the present study, by using our previously established MRP7 transfected HEK293 cells, we conducted experiments to determine whether TKIs such as lapatinib and erlotinib could reverse MRP7-mediated MDR to elucidate their reversal mechanisms.

2. Material and Methods

2.1 Materials

Lapatinib and erlotinib were purchased from ChemieTeck Inc. (Indianapolis, IN). [^{3}H]-paclitaxel (3.0 Ci/mmol) was purchased from Moravek Biochemicals. (Brea, CA). The monoclonal mouse antibody against P-gp (P7965), the polyclonal goat antibody against MRP7 (C-19), the secondary horseradish peroxidase-labeled anti-goat or anti-mouse IgG, docetaxel, paclitaxel, vinblastine, vinorelbine and cisplatin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). A polyclonal antibody against human ABCC1 (MRP1) [34] was kindly provided by Dr. Shin-ichi Akiyama (Kagoshima Univ., Japan). A monoclonal antibody BXP-34 (against ABCG2) was acquired from Signet Laboratories Inc (Dedham, MA). Cepharanthine was generously provided by Kakenshoyaku Co. (Tokyo, Japan).

2.2 Cell lines

We used MRP7 expression vector, parental plasmid and MRP7 transfected cell lines previously described by Chen et al. [30]. The parental drug-sensitive human epidermoid carcinoma cell line KB-3-1 and its corresponding resistant KB-C2 cell line were kindly provided by Drs. Michael M. Gottesman (NCI, NIH, Bethesda) and Shin-ichi Akiyama (Kagoshima Univ., Japan), respectively. The P-gp-overexpressing KB-C2 cells were established from KB-3-1 cells by exposing them to increasing concentrations of colchicine up to 2 μg/ml, in a gradual manner [35]. All the cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator containing 5% CO_2 at 37°C.

2.3 Cell cytotoxicity by MTT assay

Drug sensitivity was analyzed using an MTT colorimetric assay [27]. HEK293-pcDNA3.1 and HEK293-MRP7-2 cells were seeded into 96-well plate in triplicate at 5,000 cells/well. After incubation in DMEM supplemented with 10% bovine serum at 37°C for 24 h, three different concentrations of lapatinib and erlotinib (0.625, 1.25, 2.5 μM) were added 1 h prior to the addition of the anticancer drugs. After 72 h of incubation, 20 μl of MTT solution (4 mg/ml) was added to each well. The plate was further incubated for 4 h, the medium discarded, and 100 μl of dimethylsulfoxide (DMSO) was added into each well to dissolve the formazan crystals. The absorbance was determined at 570 nm by an OPSYS microplate Reader from DYNEX Technologies, Inc. (Chantilly, VA). The concentrations required to inhibit growth by 50% (IC_{50}) were calculated from survival curves. The degree of resistance was calculated by dividing the IC_{50} of the MDR cells by that of the parental sensitive cells.

2.4 [^{3}H]-paclitaxel accumulation and efflux

The parental HEK293-pcDNA3.1 and HEK-MRP7-2 transfected cells were seeded in two T75 flasks and incubated with DMEM supplemented with 10% bovine serum at 37°C. After the

cells reached 90% confluency, the cells were trypsinized and two aliquots (48×10^6 cells) from each cell line were suspended in the medium, pre-incubated with or without lapatinib/erlotinib (2.5 μM) at 37°C for 1 h. Subsequently, cells were suspended in the medium containing 0.1 μM [^{3}H]-paclitaxel with or without lapatinib/erlotinib at 37°C for 1 h. The cells were washed with PBS for three times, and then suspended in fresh medium with or without lapatinib/erlotinib at 37°C. Aliquots (1×10^6 cells) were collected at various time points (0, 30, 60, 120 min), followed by placed in scintillation fluid to measure the radioactivity by a Packard TRI-CARB 1900CA liquid scintillation counter (Packard Instrument Inc., Downers Grove, IL).

2.5 Preparation of cell lysates

Cells in T-25 flask treated with lapatinib or erlotinib for different time periods (0, 36, 72 h), then were harvested and rinsed twice with cold PBS. The cell extracts were prepared by incubating the cells with the Radioimmunoprecipitation assay (RIPA) buffer [1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μM p-aminophenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM aprotinin] for 30 min on ice with occasional rocking, followed by centrifugation at 12,000 rpm at 4°C for 15 min. The supernatant containing total cell lysates were collected and stored at −80°C until future experiments. The protein concentration was determined by bicinchoninic Acid (BCA™)-based protein assay (Thermo Scientific, Rockford, IL).

2.6 Immunoblotting

Equal amounts of total cell lysates (40 μg) were resolved by 4–12% sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto PVDF membrane, then immersed in blocking solution (5% skim milk in TBST) to block nonspecific binding for 1 h at room temperature. The membrane was then immunoblotted overnight with primary antibodies (polyclonal anti-MRP7, monoclonal anti-P-gp or monoclonal anti-ABCG2 at 1:500 dilution, or polyclonal anti-MRP1 at 1: 3,000 dilution) at 4° C. The following day, the membrane was washed with TBST buffer (0.3% Tris, 0.8% NaCl, 0.02% KCl, 0.05% Tween 20) for three times and followed by 3 h incubation with horseradish peroxide (HRP)-conjugated secondary anti-goat IgG for MRP7, anti-mouse IgG for P-gp and ABCG2 (1:1,000) or anti-rabbit IgG for MRP1 (1:1,000) for 3 h, respectively. Proteins were detected by enhanced chemoluminescence detection system (Amersham, NJ). β-Actin was used to confirm equal loading in each lane in the samples prepared from cell lysates. We also used 10 and 20 μg protein to detect MRP7 in the treatment of lapatinib/erlotinib experiments in order to avoid that overloading of the protein may mask the differences in the expression.

2.7 Statistical analysis

Unless otherwise indicated, all experiments were repeated at least three times and the differences were determined by the two-tailed Student's t-test. When statistical differences between more than 2 groups were analyzed, one-way ANOVA followed by Tukey's multiple comparison test was performed, as indicated. Results are presented as means ± standard deviations (SD). The statistical significance was determined to be $P < 0.05$.

3. Results

3.1 Expression of ABC transporters in MRP7-transfected HEK293 cells

Since MRP7 and P-gp share some common substrates and some EGFR TKIs were proved to be effective in reversing P-gp-mediated drug resistance, we decided to determine if MRP7 and P-gp were present in HEK293-pcDNA3.1 and HEK-MRP7-2 cells using immunoblotting analysis. We found that the MRP7 protein, with a molecular weight of 171 kD, was expressed in HEK-MRP7-2 cells but not in HEK293-pcDNA3.1 cells (Fig. 1A). P-gp, with a molecular

weight of 170 kD, was highly expressed in the positive control KB-C2 cell line, but was undetectable in the negative control KB-3-1 cells as well as in both HEK293-pcDNA3.1 and HEK-MRP7-2 cell lines (Fig. 1B). To determine whether other ABC transporters are expressed and/or contribute to MDR in the cell lines used for this study, we also did Western blot analyses for MRP1 and ABCG2 expression in HEK293-pcDNA3.1 and HEK-MRP7-2 cells. We confirmed that in the MRP7 transfected HEK293 cells used in this study, only MRP7 protein is overexpressed and the levels of other ABC transporters such as MRP1 and ABCG2 are not up-regulated compared with the empty vector transfected HEK293/pcDNA3.1 cells (data not shown).

3.2 The effect of lapatinib and erlotinib on drug sensitivity of MRP7-transfected HEK293 cells

Consistent with our previous findings [33], the colorimetric sensitivity assay revealed that HEK-MRP7-2 cells, compared to HEK293-pcDNA3.1 cells, exhibited a significant resistance to various MRP7 substrates such as docetaxel (12-fold), paclitaxel (8.6-fold), vinblastine (5.4-fold), and vinorelbine (3.6-fold), but showed no significant sensitivity difference to cisplatin (0.9-fold), (Table 1, Fig. 2).

We tested lapatinib and erlotinib in the combination with the above mentioned MRP7 substrates to determine if they would significantly reverse MRP7-mediated MDR. To avoid toxicity, the highest concentration of lapatinib and erlotinib used in the reversal experiments was 2.5 μM, a concentration that caused <10% growth inhibition in all the cell lines (data not shown). Lapatinib and erlotinib at 0.625, 1.25 and 2.5 μM, dose-dependently decreased the IC_{50} values of docetaxel, paclitaxel, vinblastine and vinorelbine of HEK-MRP7-2 cells (Table 1). In addition, lapatinib, at 2.5 μM, significantly sensitized the parental HEK293-pcDNA3.1 cells; however, this effect was significantly lower than measured in HEK-MRP7-2 cells. In contrast, lapatinib and erlotinib did not significantly reverse the resistance of cells to cisplatin, a non-MRP7 substrate (Table 1, Fig. 2). Previously, we showed that cepharanthine could reverse MRP7-mediated resistance to paclitaxel in a competitive manner, hence to compare lapatinib and erlotinib we used cepharanthine as a positive control in the present experiment (Table 1). The effect of cepharanthine was comparable to the effect of lapatinib and erlotinib (Table 1).

3.3 The effects of lapatinib and erlotinib on the intracellular accumulation of [^{3}H]-paclitaxel

The effects of lapatinib and erlotinib on the accumulation of [^{3}H]-paclitaxel in HEK293-pcDNA3.1 and HEK-MRP7-2 cells were examined. Our data showed that the intracellular concentration of [^{3}H]-paclitaxel in HEK-MRP7-2 cells was significantly lower (41.91%) than that in HEK293-pcDNA3.1 cells (Fig. 3, $P < 0.05$). After the cells were incubated with either lapatinib or erlotinib at 2.5 μM for 1 h, intracellular [^{3}H]-paclitaxel accumulation was significantly enhanced in HEK-MRP7-2 cells by 2.82-fold and 1.96-fold, respectively ($P<0.05$). However, in the control HEK293-pcDNA3.1 cells, neither lapatinib nor erlotinib significantly altered the intracellular [^{3}H]-paclitaxel accumulation. In consistent with our previous findings, cepharanthine produced a 2.80-fold increase in the intracellular accumulation of [^{3}H]-paclitaxel in HEK-MRP7-2 cells, which was consistent with our previous findings [33].

3.4 The effects of lapatinib and erlotinib on the efflux of [^{3}H]-paclitaxel

To ascertain whether the increase in the intracellular [^{3}H]-paclitaxel accumulation caused by lapatinib and erlotinib was due to an inhibition of [^{3}H]-paclitaxel efflux, we conducted a time course study to determine [^{3}H]-paclitaxel efflux in the presence of lapatinib or erlotinib. Our results indicated that HEK-MRP7-2 cells extruded a significantly higher percentage of intracellular accumulated [^{3}H]-paclitaxel than HEK293-pcDNA3.1 cells (Fig. 4, $P < 0.05$). When we incubated cells with lapatinib or erlotinib at 2.5 μM, they significantly blocked the intracellular [^{3}H]-paclitaxel efflux at different time periods (0, 30, 60, 120 min) from HEK-

MRP7-2 cells, but not in the parental HEK293-pcDNA3.1 cells. The accumulation of [^{3}H]-paclitaxel at 0 min was set as 100%, at 30, 60, 120 min, the percentages were 60.60%, 30.71%, 24.53%, respectively, of the accumulated [^{3}H]-paclitaxel that remained in HEK-MRP7-2 cells in the absence of lapatinib or erlotinib. When HEK-MRP7-2 cells were incubated with lapatinib, the percentages at 30, 60 and 120 min were increased to 83.15%, 74.64%, 57.26% respectively (Fig. 4A, $P<0.05$ for the same time point comparison). Erlotinib increased the percentage of [^{3}H]-paclitaxel accumulation at 30, 60, and 120 min to 76.09%, 58.64% and 40.47%, respectively (Fig. 4B, $P<0.05$ for the same time point comparison). Lapatinib was more potent than erlotinib, which is consistent with the results in colorimetric growth assay and [^{3}H]-paclitaxel accumulation experiments. As expected, cepharanthine, the positive control, could also effectively block MRP7 function and significantly inhibited the efflux of [^{3}H]-paclitaxel from HEK-MRP7-2 cells (data not shown).

3.5 The effects of lapatinib and erlotinib on the expression of MRP7 in HEK-MRP7-2 cells

The reversal of MRP7-mediated MDR can be achieved either by inhibiting the function of MRP7 or by decreasing MRP7 expression. To evaluate the effect of lapatinib or erlotinib on MRP7 expression, HEK-MRP7-2 cells were treated with lapatinib or erlotinib and MRP7 expression levels were examined by Western blotting analysis. We found that the protein level of MRP7 in HEK-MRP7-2 cells was not altered after treatment with lapatinib or erlotinib at 2.5 μM for 0, 36 and 72 h (Fig. 5), no matter in three (10, 20 and 40 μg) different protein loading. This suggests that the regulatory mechanisms of lapatinib and erlotinib are not due to MRP7 expression levels.

4. Discussion

Lapatinib is a reversible, small molecule inhibitor of EGFR (HER1) and HER2 receptor TKs that has been approved by the FDA for the treatment of HER2-positive metastatic breast cancers [36]. Recently, we showed for the first time that lapatinib could reverse P-gp- and ABCG2-mediated MDR by directly inhibiting their transport function [28]. Furthermore, our *in vivo* experiments indicated that lapatinib significantly enhanced the sensitivity of paclitaxel on P-gp mediated MDR cancer xenograft model in nude mice [28]. Moreover, lapatinib increased topotecan accumulation and cytotoxicity by inhibiting P-gp and ABCG2 in both *in vitro* and *in vivo* studies [37]. Erlotinib, another selective small molecule inhibitor of the EGFR tyrosine kinases, is used clinically for the treatment of chemotherapy resistance in advanced NSCLC patients as well as advanced pancreatic cancer in combination with gemcitabine [38]. Previously, we reported that erlotinib significantly reversed MDR mediated by both P-gp and ABCG2 transporters [27]. Furthermore, we observed that erlotinib dose-dependently inhibits ABCG2-mediated transport of methotrexate and 17-β-estradiol-(17-beta-D-glucuronide) in membrane vesicles [27].

In the present study, we tested both lapatinib and erlotinib to determine if they could also reverse MRP7-mediated drug resistance. The transfected HEK293-pcDNA3.1 and HEK-MRP7-2 cell lines used in our experiments have been used in a previous study from our laboratory [33]. We used Western blot analysis to detect the expression of MRP7. We could not detect P-gp (Fig. 1B), MRP1 and ABCG2 (data not shown) expression in the two HEK293 transfectants. These findings indicate that the effects of the erlotinib and lapatinib are due to their interaction with the MRP7 protein.

We found that the drug resistance profile of established MRP7 substrates such as docetaxel, paclitaxel and vinblastine in HEK-MRP7-2 cells was consistent with our previous report [31] (Table 1). In addition, we found that HEK-MRP7-2 exhibited a lower level of resistance to vinorelbine (3.6-fold) (Table 1;Fig. 2B, 2G) and this was in accordance with a recent report that MRP7 is associated with vinorelbine resistance in non-small cell lung cancer [32]. As

some EGFR TKIs such as lapatinib and erlotinib are competitive inhibitors of P-gp and ABCG2, we designed our present study to determine if these EGFR TKIs also have the ability to reverse MRP7-mediated drug resistance. We used non-toxic concentrations of lapatinib and erlotinib (0.625, 1.25, 2.5 μM) in the present study. In human pharmacokinetic studies, the highest peak plasma lapatinib and erlotinib levels were ~3 μM [39] suggesting that the *in vitro* concentrations used in our experiments are similar to those obtained in plasma after therapeutic treatment. Thus, the concentrations used in the present study are clinically relevant and it is possible to achieve a reversal effect in tumor xenograft model study.

The reversal effect of MRP7-mediated MDR by lapatinib is more potent than that of erlotinib. One possible explanation is due to the dual selectivity of lapatinib on the TKs of both EGFR and HER2 receptors, whereas erlotinib is selective only for EGFR [40]. Lapatinib at 2.5 μM also caused a relatively small reduction of the IC_{50} of all four drugs we tested in HEK293-pcDNA3.1 cells whereas erlotinib at 2.5 μM only decreased the IC_{50} of vinorelbine in HEK293-pcDNA3.1 cells. This may be due to the endogenous expression of drug transporters in HEK293-pcDNA3.1 cells. In addition, neither lapatinib nor erlotinib could significantly alter the sensitivity of HEK293-pcDNA3.1 and HEK-MRP7-2 cells to cisplatin, a compound that is not an MRP7 substrate.

Since the MTT assay results can not be used as a direct evidence of MRP7-mediated drug transport, we determined the effect of lapatinib and erlotinib on the accumulation and efflux of [^{3}H]-paclitaxel, a known chemotherapeutic substrate of MRP7 transporter, in HEK293-pcDNA3.1 and HEK-MRP7-2 cells [31]. In our experiments, both lapatinib and erlotinib at 2.5 μM significantly increased the intracellular concentration of [^{3}H]-paclitaxel, and decreased the intracellular [^{3}H]-paclitaxel efflux from the HEK-MRP7-2 cells but not in parental HEK293-pcDNA3.1 cells. The reversal effect of lapatinib was similar to a known MRP7 inhibitor cepharanthine [33]. This suggests that lapatinib and erlotinib modulate MRP7-mediated MDR by increasing intracellular drug accumulation through inhibiting drug efflux function of MRP7.

Our Western blotting analysis suggests that neither lapatinib nor erlotinib at 2.5 μM significantly affects the expression of MRP7 in HEK-MRP7-2 cells after 36 h and 72 h of incubation (Fig. 5). It is most likely that regulatory mechanisms of MRP7-mediated MDR reversal is due to the functional interaction of lapatinib and/or erlotinib with MRP7 and is not related to changes in the expression level of MRP7. Further experiments are needed to deduce the interactions between EGFR TKI and MRP7. It is possible that lapatinib and erlotinib may be substrates of multiple ABC transporters such as P-gp, ABCG2 and MRP7.

Currently, pre-clinical research and clinical trials are investigating the combination of EGFR TKIs with other anticancer drugs to improve the therapeutic outcome of cancer patients. Therefore, the interaction of EGFR TKIs with MRP7, P-gp and/or ABCG2 should be addressed when exploring the combinational use of EGFR TKIs with cytotoxic anticancer drugs that are substrates of MRP7, P-gp and ABCG2. Recently, several clinical studies have discovered that first-line therapy with lapatinib and paclitaxel, which is a substrate of MRP7 and P-gp, could significantly improve the clinical outcome in HER-2-positive patients [41,42]. TKIs may affect the pharmacokinetics of anticancer drugs, potentially resulting in increased responses but also potentially increasing adverse effects. This is especially true in the case of normal tissues and cancer tissues expressing a high level of P-gp and ABCG2, where the concentration and distribution of anticancer drugs might be altered. In fact, it has reported that when gefitinib, another TKI, was combined with camptothecins derivatives in mice, gefitinib was found to enhance the oral absorption of camptothecins, decrease the clearance of topotecan, and increase the oral bioavailability of irinotecan [43]. Our group previously found that lapatinib significantly enhanced the therapeutic effect of paclitaxel in a tumor xenograft in mice that

overexpressed P-gp [28]. It is possible that these findings including those in this paper, could assist in the development of a new therapeutic regimen.

In conclusion, our findings indicate for the first time that the EGFR TKIs lapatinib and erlotinib are able to effectively reverse MRP7-mediated MDR. The mechanism of MDR modulation by lapatinib and erlotinib is associated with an increase in intracellular drug accumulation by inhibiting drug efflux from MDR cells. These results suggest that lapatinib and erlotinib could be used to augment the clinical response to conventional chemotherapeutic agents that are substrates of MRP7. Therefore, the use of lapatinib and erlotinib with drugs that are MRP7 substrates warrants further study.

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Abbreviations

TKI	tyrosine kinase inhibitor
EGFR	epidermal growth factor receptor
MDR	multidrug resistance
ABC	ATP-binding cassette
P-gp (ABCB1)	P-glycoprotein
ABCG2	also called BCRP (breast cancer resistance protein)/MXR (mitoxantrone resistance protein)
MRP7 (ABCC10)	multidrug resistance protein 7
HER	human epidermal receptor

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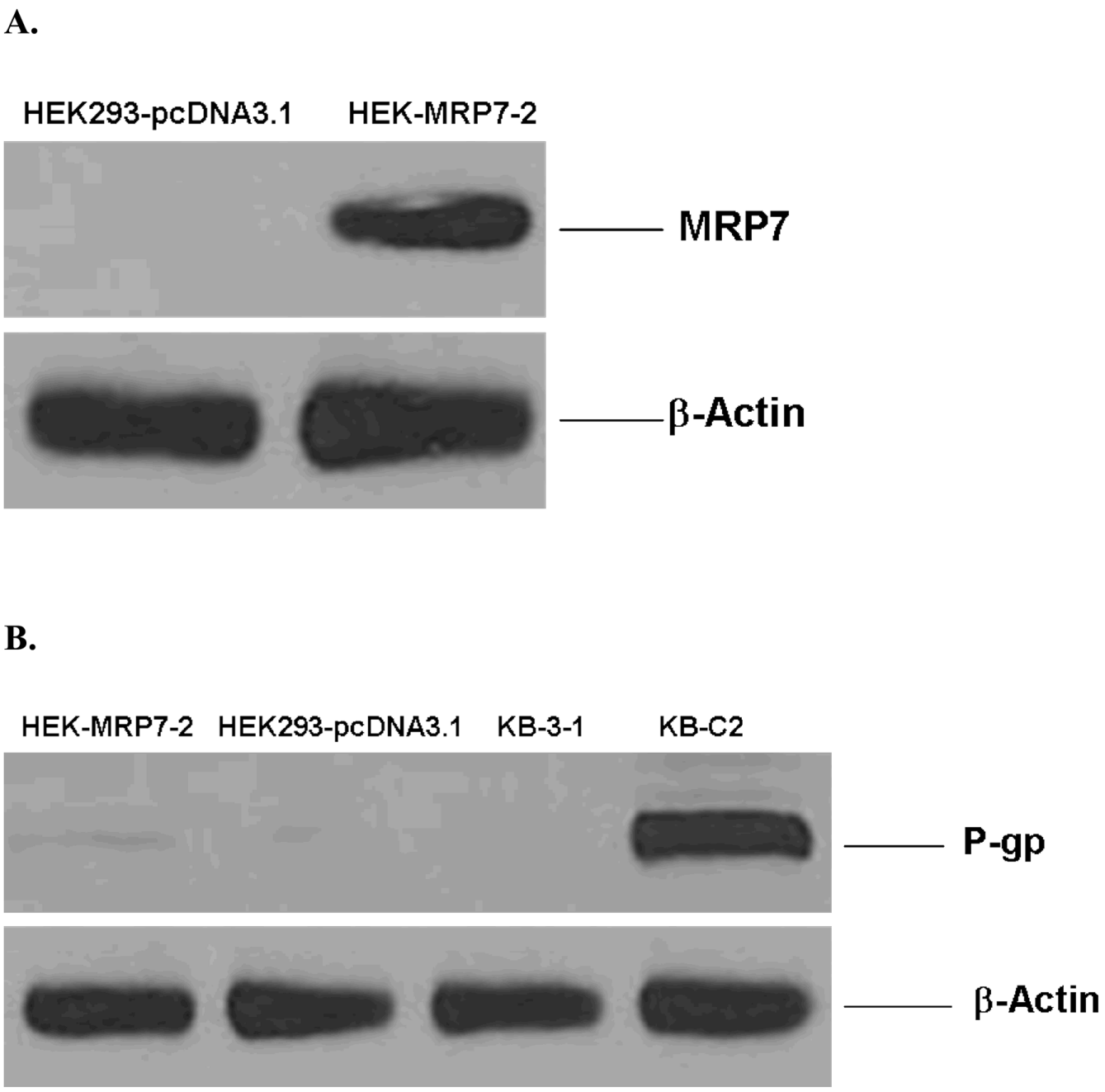
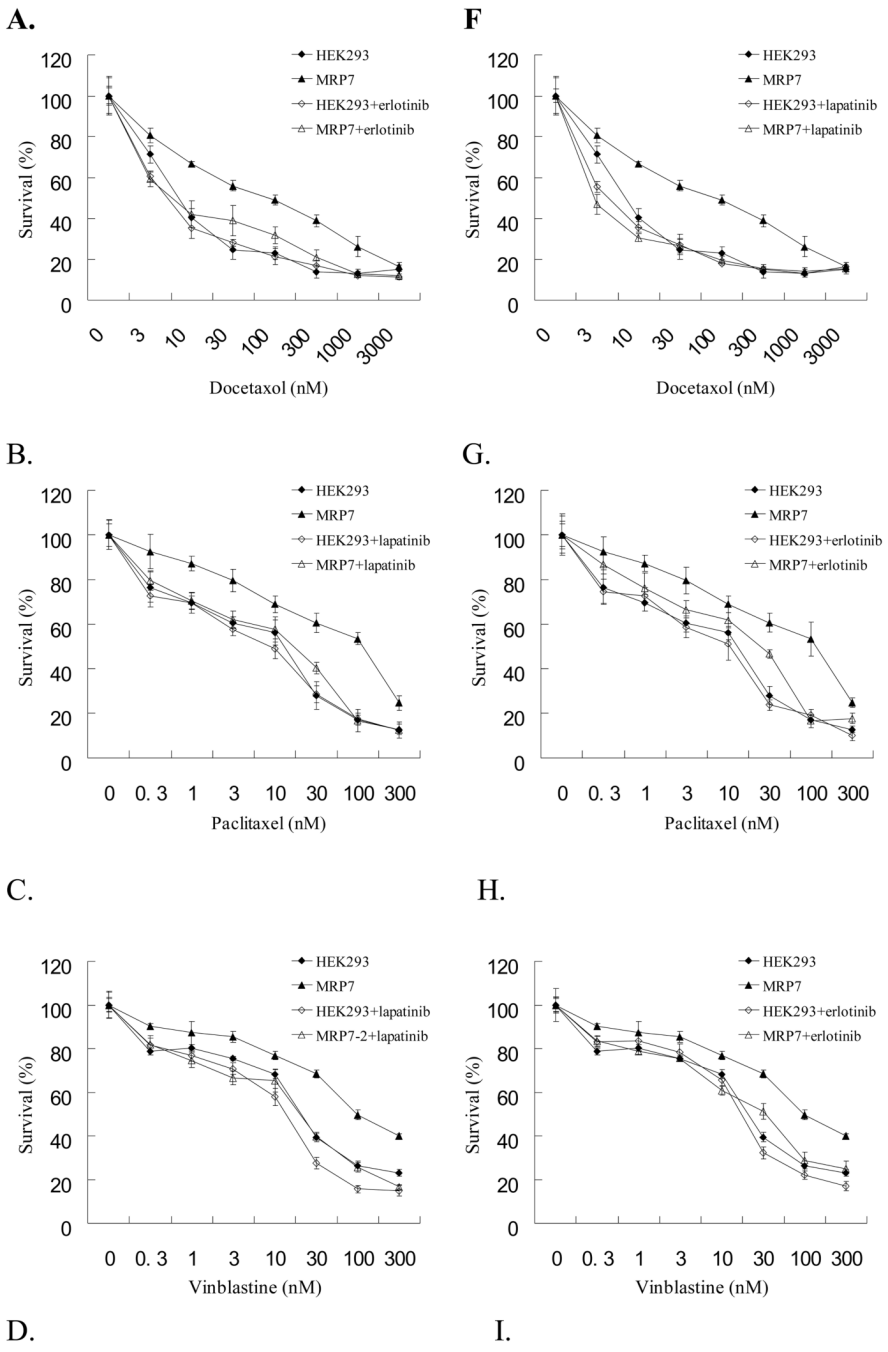


Fig 1. Immunoblot detection of MRP7 and P-gp in MRP7-transfected cells

Immunoblot detection of MRP7 was done in cell lysates prepared from HEK293 cells transfected with empty vector (HEK293-pcDNA3.1) and MRP7 vector (HEK-MRP7-2) cells (Fig. 1A). For the presence of P-gp, we used KB-3-1 and KB-C2 cells as negative and positive controls, respectively (Fig. 1B). β-Actin was used as an internal control for equal loading.



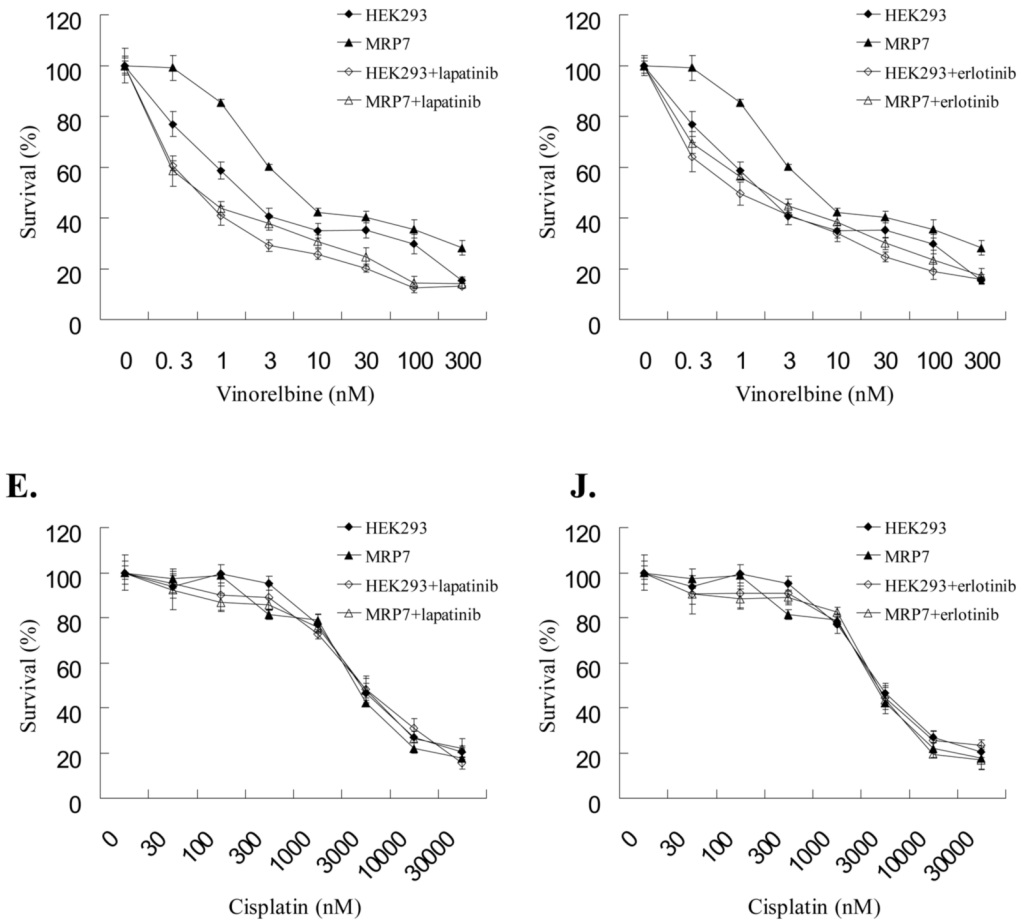


Fig 2. The effects of lapatinib and erlotinib on the sensitivity of HEK293-pcDNA3.1 and HEK-MRP7-2 cells to docetaxel, paclitaxel, vinblastine, vinorelbine and cisplatin

HEK293-pcDNA3.1 and HEK-MRP7-2 cells were seeded and cultured for 24 h, pre-treated with or without inhibitors at different concentration (0.625, 1.25, 2.5 μM) for 1 h, then added various concentrations of docetaxel (A and F), paclitaxel (B and G), vinblastine (C and H), vinorelbine (D and I) or cisplatin (E and J), and further incubated for 72 h. The absorbance was determined at 570 nm by an OPSYS microplate Reader. The above representative figures show the effects of lapatinib (A–E) and erlotinib (F–J) at 2.5 μM on the various drugs sensitivity of HEK293-pcDNA3.1 and HEK-MRP7-2 cells.

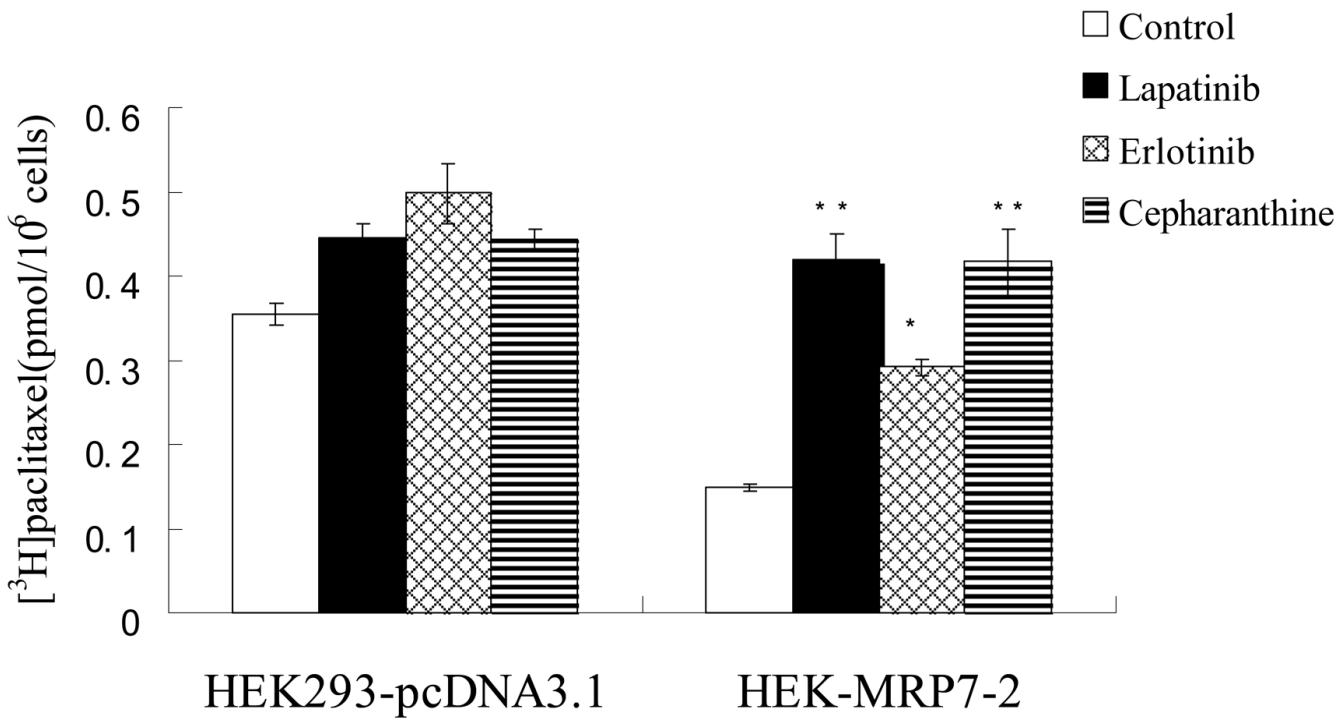


Fig 3. The effects of lapatinib or erlotinib on the accumulation of [^{3}H]-paclitaxel in HEK293-pcDNA3.1 and HEK-MRP7-2 cells

Cells were pre-treated with or without 2.5 μM lapatinib or erlotinib, and then incubated with 0.1 μM [^{3}H]-paclitaxel for 1 h. After that, cells were collected and the intracellular levels of [^{3}H]-paclitaxel were determined by scintillation counting. Cepharanthine was used as positive control. All experiments were performed in triplicate of three separated experiments. Data are presented as means ± SD. *$P < 0.05$, **$P < 0.01$.

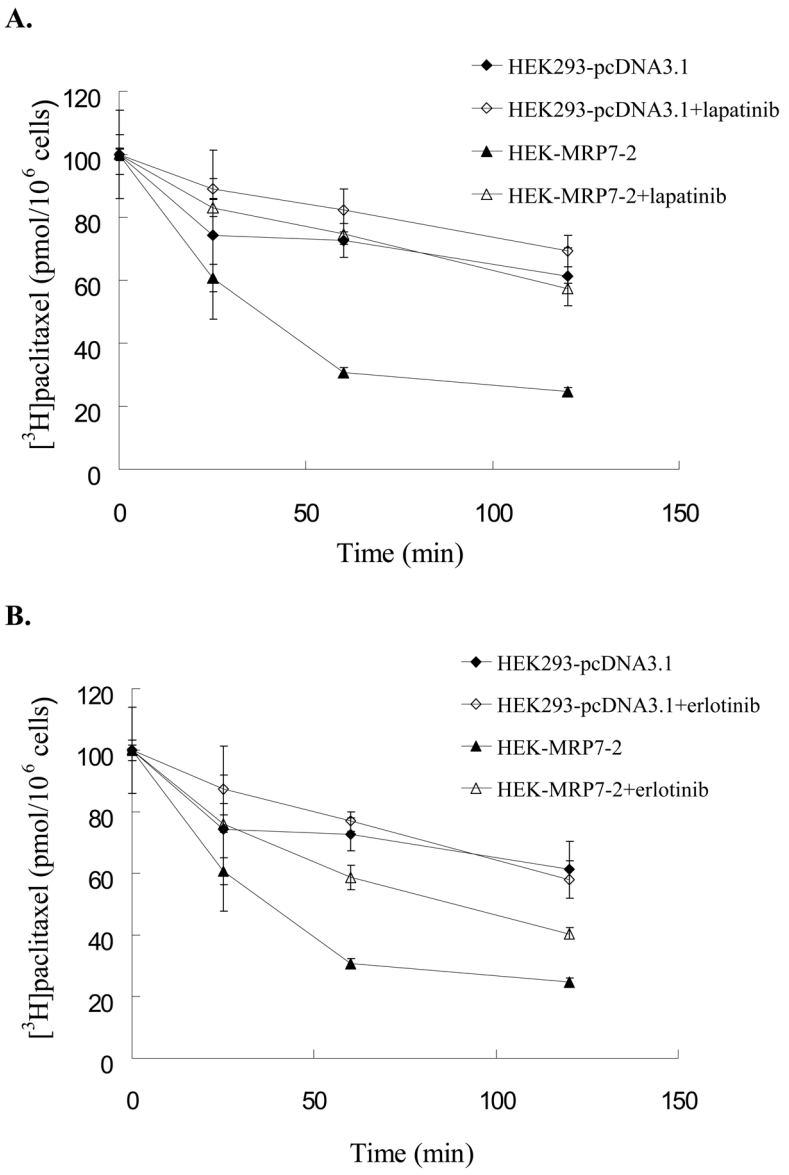


Fig 4. The effects of lapatinib or erlotinib on the efflux of [^{3}H]-paclitaxel in HEK293-pcDNA3.1 and HEK-MRP7-2 cells

Cells were pre-treated with or without lapatinib **(A)** or erlotinib **(B)** at 2.5 μM for 1 h at 37°C, and further incubated with 0.1 μM [^{3}H]-paclitaxel at 37°C for 1 h. Cells were then incubated in the 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, 120 min). All experiments were performed in triplicate of three separated experiments. Data are presented as means ± SD.

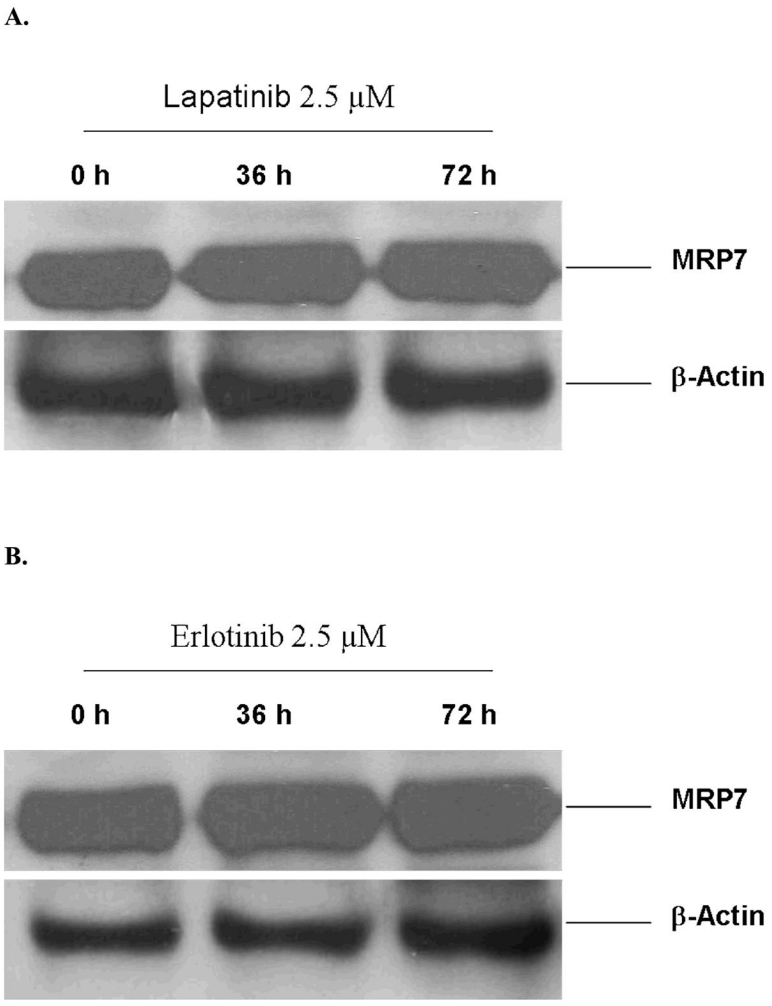


Fig 5. Immunoblot detection of MRP7 in HEK-MRP7-2 cells following incubation with lapatinib or erlotinib

Cell lysates were prepared from HEK-MRP7-2 cells incubated with 2.5 μM lapatinib (A) or erlotinib (B) for different time periods (0, 36, 72 h), immunoblot detection of MRP7 was done using polyclonal anti-MRP7 antibody, β-Actin was used as an internal control for equal loading. Equal amounts (40 μg protein) of total cell lysates were used for each sample. Each figure is a representative example of three replications.

Table 1

The effects of lapatinib and erlotinib on the sensitivity of HEK293-pcDNA3.1 and HEK-MRP7-2 cells to various therapeutic agents including docetaxel, paclitaxel, vinblastine, vinorelbine and cisplatin

Compounds	IC_{50} ± SD[a] (nM)	
	HEK293-pcDNA3.1	HEK-MRP7-2
Docetaxel	9.4 ± 0.15 (1.0)[b]	102.8 ± 1.58 (11.97)
+lapatinib 0.625 μM	9.1 ± 0.26 (1.07)	24.8 ± 0.76 (3.0)
+lapatinib 1.25 μM	5.3 ± 0.38 (0.71)	13.6 ± 0.24 (1.5)
+lapatinib 2.5 μM	3.5 ± 0.26 (0.47)	5.0 ± 0.09 (0.56)
+erlotinib 0.625 μM	9.7 ± 0.16 (1.04)	72.9 ± 1.93 (8.19)
+erlotinib 1.25 μM	7.6 ± 0.2 (0.82)	64.8 ± 2.31 (7.41)
+erlotinib 2.5 μM	9.4 ± 0.27 (0.96)	24.1 ± 1.28 (2.33)
+cepharanthine 2.5 μM	4.7 ± 0.25 (0.61)	6.7 ± 0.34 (0.86)
Paclitaxel	14.30 ± 2.36 (1.0)[b]	123.59 ± 34.67 (8.64)
+lapatinib 0.625 μM	12.56 ± 1.77 (0.88)	81.31 ± 7.88 (5.69)
+lapatinib 1.25 μM	10.71 ± 1.58 (0.75)	34.52 ± 6.12 (2.41)
+lapatinib 2.5 μM	9.08 ± 1.21 (0.63)	19.88 ± 1.95 (1.39)
+erlotinib 0.625 μM	12.92 ± 1.20 (0.90)	100.81 ± 20.93 (7.05)
+erlotinib 1.25 μM	11.95 ± 1.67 (0.84)	48.69 ± 15.66 (3.40)
+erlotinib 2.5 μM	10.85 ± 1.16 (0.76)	25.55 ± 4.89 (1.79)
+cepharanthine 2.5 μM	9.96 ± 1.29 (0.70)	24.71 ± 5.65 (1.73)
Vinblastine	22.78 ± 1.62 (1.0)[b]	124.09 ± 2.46 (5.37)
+lapatinib 0.625 μM	23.18 ± 2.63 (1.01)	40.83 ± 2.23 (1.77)
+lapatinib 1.25 μM	22.72 ± 4.15 (0.98)	35.63 ± 0.96 (1.54)
+lapatinib 2.5 μM	15.98 ± 2.7 (0.69)	22.03 ± 10.86 (0.97)
+erlotinib 0.625 μM	23.76 ± 0.41 (1.04)	86.4 ± 28.48 (3.79)
+erlotinib 1.25 μM	20.58 ± 1.12 (0.9)	71.77 ± 15.95 (3.14)
+erlotinib 2.5 μM	19.69 ± 1.78 (0.87)	33.91 ± 16.79 (1.5)
+cepharanthine 2.5 μM	17.75 ± 0.1 (0.78)	30.35 ± 8.38 (1.38)
Vinorelbine	1.98 ± 0.21 (1.0)[b]	6.97 ± 0.56 (3.58)
+lapatinib 0.625 μM	1.63 ± 0.62 (0.83)	2.92 ± 1.5 (1.54)
+lapatinib 1.25 μM	0.94 ± 0.3 (0.48)	1.02 ± 0.51 (0.52)
+lapatinib 2.5 μM	0.77 ± 0.45 (0.39)	0.76± 0.4 (0.39)
+erlotinib 0.625 μM	2.73 ± 0.99 (1.38)	0.66 ± 1.28 (3.36)
+erlotinib 1.25 μM	1.85 ± 0.07 (0.94)	5.17 ± 2.06 (2.67)
+erlotinib 2.5 μM	1.1 ± 0.28 (0.55)	1.75 ± 0.71 (0.87)
+cepharanthine 2.5 μM	1.36 ± 0.51 (0.68)	2.0 ± 1.06 (0.98)
Cisplatin	2216 ± 81.29 (1.0)[b]	1901 ± 180.05 (0.87)
+lapatinib 2.5 μM	2201 ± 29.78 (0.99)	2138 ± 58.64 (0.97)
+erlotinib 2.5 μM	2119 ± 243.96 (0.95)	2083 ± 202.31 (0.94)
+cepharanthine 2.5 μM	2226 ± 106.96 (1.0)	2388 ± 348.06 (1.07)

[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 docetaxel, paclitaxel, vinblastine, vinorelbine and cisplatin in HEK-MRP7-2 cells in the absence or presence of reversal agent, or HEK293-pcDNA3.1 cells with reversal agents, by the IC_{50} of docetaxel, paclitaxel, vinblastine, vinorelbine and cisplatin in HEK293-pcDNA3.1 cells without reversal agents.