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Cepharanthine is a potent reversal agent for MRP7(ABCC10)-mediated multidrug resistance

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ABSTRACT

Multidrug resistance protein 7 (MRP7; ABCC10) is an ABC transporter that confers resistance to anticancer agents such as the taxanes. We previously reported that several inhibitors of P-gp and MRP1 were able to inhibit the *in vitro* transport of $E_2$17βG by MRP7 in membrane vesicles transport assays. However, compounds that are able to reverse MRP7-mediated cellular resistance have not been identified. In this study, we examined the effects of cepharanthine (6′,12′-dimethoxy-2,2′-dimethyl-6,7-[methylenebis(oxy)]oxyacanthan), an herbal extract isolated from *Stephania cepharantha Hayata*, to reverse paclitaxel resistance in MRP7-transfected HEK293 cells. Cepharanthine, at 2 μM, completely reversed paclitaxel resistance in MRP7-transfected cells. In contrast, the effect of cepharanthine on the parental transfected cells was significantly less than that on the MRP7-transfected cells. In addition, cepharanthine significantly increased the accumulation of paclitaxel in MRP7-transfected cells almost to the level of control cells in the absence of cepharanthine. The efflux of paclitaxel from MRP7-transfected cells was also significantly inhibited by cepharanthine. The ability of cepharanthine to inhibit MRP7 was analyzed in membrane vesicle assays using $E_2$17βG, an established substrate of MRP7, as a probe. $E_2$17βG transport was competitively inhibited by cepharanthine with a K_i value of 4.86 μM. These findings indicate that cepharanthine reverses MRP7-mediated resistance to paclitaxel in a competitive manner.

1. Introduction

The ATP-binding cassette (ABC) transporters, ABCB1 (P-gp/MDR1), ABCC (such as multidrug resistance proteins, MRPs) subfamily and ABCG2 (BCRP/MXR) have been shown to be involved in mediating resistance to certain anticancer drugs [1]. The MRP subfamily of ABC transporters consists of nine members [2,3] and some of the functional properties of the

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Abbreviations: MDR, multidrug resistance; ABC, ATP-binding cassette; BCRP/ABCG2/MXR, breast cancer resistance protein; P-gp/MDR1/ABCB1, P-glycoprotein; MRP7/ABCC10, multidrug resistance protein 7; cepharanthine, 6′,12′-dimethoxy-2,2′-dimethyl-6,7-[methylenebis(oxy)oxyacan-than]; $E_2$17βG, 17-β-estradiol-(17-beta-D-glucuronide); VCR, vincristine; VBL, vinblastine; MTT, (3-(4,5-dimethylthiazol-2-yl)-3, 5-diphenylformazan.

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MRP family have been determined. MRP1 is able to confer resistance to anthracyclines, vinca alkaloids, epipodophyllotoxins, camptothecins and methotrexate, but not to taxanes, which are an important component of the P-gp profile [4,5]. The drug resistance profile of MRP2 is similar to that of MRP1 with respect to anthracyclines, vinca alkaloids, epipodophyllotoxins and camptothecins [6–8]. MRP3 only confers resistance to a few natural anticancer drugs such as etoposide, teniposide and vincristine [9–11]. It has been reported that MRP6 confers a low level of resistance to etoposide and teniposide as well as cisplatin [12]. The absence of a third membrane-spanning domain in the N-terminal of MRP4, MRP5, MRP8 and MRP9 suggests that these four transporters might have distinct properties and this has proved to be the case with respect to their drug resistance profiles. Indeed, MRP4, MRP5 and MRP8 do not confer resistance to most compounds that are natural products, but do confer resistance to some nucleobase or nucleoside analogs [13–16]. Neither substrate nor resistance profile of MRP9 has yet been found [17]. Multidrug resistance protein 7 (MRP7/ABCC10) is a member of MRP subfamily [18]. The topology of MRP7 is similar to those of MRP1, 2, 3 and 6, with two nucleotide-binding domains and three membrane-spanning domains. We previously reported that MRP7 transports conjugates such as the glucuronide conjugate $E_2$17βG [19], and its drug resistance profile includes both taxanes and vinca alkaloids [20]. A recent report indicated that vincristine-treated mouse and human salivary gland adenocarcinoma (SGA) cells overexpressed not only P-gp and MRP1 but also MRP7 [21]. Oguri et al. reported that MRP7 is expressed at a high level in paclitaxel resistant non-small cell lung cancer [22]. Tsuruo et al. first discovered that verapamil enhances cytotoxicity of VCR in a VCR-resistant cell line, which overexpressed P-gp [23]. These findings suggested that inhibiting drug efflux pumps could reverse drug resistance. However, in clinical trials, verapamil failed to show an improvement in the therapeutic outcome and toxic side effects were common [24]. PSC-833 (the second generation) induces pharmacokinetic interactions that limited the clearance and metabolism of anticancer drugs, and this increased plasma drug concentrations beyond acceptable levels of toxicity [25]. Currently, the third-generation of P-gp inhibitors is being studied for their clinical efficacy. Several inhibitors of MRP, such as MK571 and probenecid, have been identified and characterized *in vitro* [26], but clinical studies have not been conducted with MRP family member inhibitors. The discovery of potent and specific inhibitors of MRPs is of great interest, and may represent a strategy to circumvent clinical drug resistance.

Cepharanthine, 6′,12′-dimethoxy-2,2′-dimethyl-6,7-[methylenebis(oxy)oxyacan-than], is an herbal extract isolated from *Stephania cepharantha* Hayata [27]. Cepharanthine was found to completely reverse the resistance of a multidrug-resistant subline, ChR-24, derived from human carcinoma cells, to vincristine, actinomycin D, and daunomycin, and partially overcome resistance to adriamycin [28]. As a substrate of P-gp, cepharanthine directly interacts with P-gp and competitively binds to P-gp thereby inhibiting its transport activity [29,30]. Cepharanthine, by binding to this drug. Therefore, cepharanthine may be useful in reversing the resistance to cancer agents in cells overexpressing P-gp.

Currently, information on compounds that reverse MRP7-mediated drug resistance is unavailable. In this study, we screened various chemical compounds using HEK293 cells ectopically transfected with MRP7. We found that cepharanthine potently reversed MRP7-mediated MDR and further investigated the reversal mechanisms of cepharanthine.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), bovine serum and penicillin/streptomycin were purchased from Hyclone (Logan, UT). Paclitaxel, VCR, VBL, etoposide (VP-16), cytarabine (AraC), colchicine, verapamil, probenecid, dimethyl sulfoxide (DMSO) and 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) were purchased from Sigma–Aldrich (St. Louis, MO). MK571 was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Cepharanthine (Fig. 1) was generously provided by Kakenshoyaku Co. (Tokyo, Japan) and 7-ethyl-10-hydroxy-20(S)-camptothecin (SN-38) were generously provided by Daiichi Sankyo Pharmaceutical Co. Ltd. and Yakult Pharmaceutical Co. (Japan). Sildenafil was a product of Toronto Research Chemicals (Toronto, ON, Canada). [^{3}H]paclitaxel (3.0 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). [^{3}H]-$E_2$17βG (40.5 Ci/mmol) were obtained from PerkinElmer Life Sciences (Boston, MA).

The monoclonal antibody against P-gp C219 was purchased from Signet Laboratories Inc. (Dedham, MA) and used at a dilution of 1:200. Another monoclonal (P7965) antibody against P-gp from Sigma–Aldrich (St. Louis, MO) was also used because this antibody specifically detects human MDR1/P-gp, but does not appear to recognize the human MDR3 product. The secondary antibody human anti-mouse IgG for the detection of P-gp and antibody complex was purchased from Sigma–Aldrich (St. Louis, MO). Polyclonal antibody against human MRP7 protein was used for Western blotting as described previously [19], and horseradish peroxidaselabeled goat anti-rabbit IgG from NEN (Boston, MA) was used as secondary antibody.

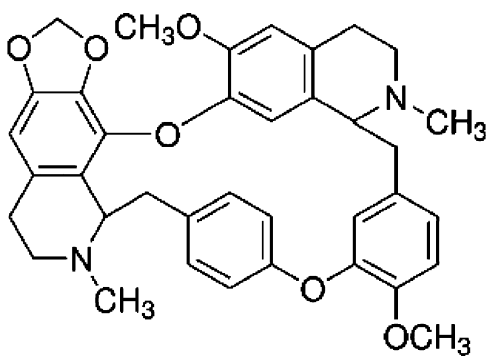

Fig. 1 – The chemical structure of cepharanthine.

2.2. Cell lines and cell culture

Previously described MRP7 expression vector and parental plasmid [19] were introduced into HEK293 cells by electroporation. Individual clones were selected in the medium containing G418 (1 mg/ml). One colony (HEK-pcDNA3.1) of empty vector transfectants and one colony (HEK-MRP7-2) of MRP7 cDNA transfectants in which MRP7 protein was detected by immunoblotting analysis were employed in the present study. Parental HEK293 cells without transfection and HEK-MRP7-C18 cells were also used to confirm the results. The parental drug-sensitivity human epidermoid carcinoma cell line KB-3-1 and its P-gp-overexpressing cell line KB-C2 were described previously [31]. All the cell lines were grown in DMEM supplemented with 10% bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin under standard culture condition (37 °C, 5% CO_2) in the incubator.

2.3. Preparation of membrane vesicles and cell lysates

The nitrogen cavitation method for the preparation of membrane vesicles from mammalian cells was reported previously [32]. Briefly, cells were separately grown in 24 × 24 cm dishes under standard culture condition (37 °C, 5% CO_2). Confluent monolayer cells were suspended into PBS containing 1% aprotinin (by scrapping or flushing using cold PBS). Cells were collected by centrifugation (4000 × *g* for 5 min), suspended in buffer A (10 mM Tris–HCl, pH 7.4, 0.25 M sucrose, 1 mM p-amindinophenylmethanesulfonylflouride, and 0.2 mM $CaCl_2$) and equilibrated at 4 °C for 15 min under a nitrogen pressure of 500 psi. EDTA was added to the suspension of lysed cells to a final concentration of 1 mM, and the suspension was then diluted 1:4 with buffer B (10 mM Tris–HCl, pH 7.4, 0.25 M sucrose, and 1 mM p-amindinophenylmethanesulfonylflouride) and centrifuged at 4000 × *g* for 10 min at 4 °C to remove nuclei and unlysed cells. The supernatant was then layered onto a sucrose cushion (10 mM Tris–HCl, pH 7.4, 35% sucrose and 1 mM EDTA) and centrifuged for 30 min at 16,000 × *g* at 4 °C. The interface was collected and centrifuged at 100,000 × *g* for 45 min at 4 °C. The pellet was resuspended in buffer B by repeated passage through a 25-gauge needle. To prepare the cell lysates, cells were harvested and rinsed twice with cold PBS. The cell extracts were prepared in RIPA buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for 30 min with occasional rocking and centrifuged at 12,000 × *g* at 4 °C for 15 min. The supernatant was the cell lysates. Protein concentrations in membrane vesicles and cell lysates were determined by the method of Bradford [33]. Both vesicles and cell lysates were stored at −80 °C.

2.4. Western blotting analyses

Membrane vesicles or cell lysates was separated by a 4–12% SDS-PAGE gel and transferred into nitrocellulose membranes using a wet transfer system, as described previously [20]. MRP7 was detected using a previously described polyclonal anti-MRP7 antibody at a dilution of 1:1000 and alkaline phosphatase-conjugates secondary antibody at a dilution of 1:2000 [19]. P-gp was detected using the monoclonal antibody C219 or P7965 at a dilution of 1:200 and HRP-conjugated secondary antibody at a dilution of 1:1000 [21]. Enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ) was used for visualization. Protein abundance was quantified by Scion Image Software (Scion Co., USA). Because the normally used control such as actin is not detectable in the samples prepared from the membrane vesicles, therefore, we used the Coomassie blue (CBB) stain to demonstrate approximately equal loading.

2.5. Analysis of drug sensitivity

Drug sensitivity was analyzed using a slightly modified MTT colorimetric assay as described previously [34]. HEK293, HEK-pcDNA3.1, HEK-MRP7-2 and HEK-MRP7-C18 cells were seeded in 96-well plates in triplicate at 5000 cells/well. After incubation in DMEM supplemented with 10% bovine serum at 37 °C for 24 h, the anticancer drugs being diluted to various concentrations were added and incubated continuously for 72 h. The potential inhibitors were added 1 h before the anticancer drugs were added. Growth assays were performed after 72 h of incubation in the presence of drug.

2.6. Drug accumulation and efflux

Intracellular paclitaxel accumulation and efflux were measured in HEK-pcDNA3.1 cells and HEK-MRP7-2 cells. The cells were trypsinized and two aliquots (2.5×10^6 cells) from each cell line were resuspended in the medium. To measure drug accumulation, cells were incubated at 37 °C with 0.1 μM [^{3}H]paclitaxel in DMEM medium with or without cepharanthine at 5 μM for 1 h at 37 °C after incubation with DMEM with or without cepharanthine for 1 h at 37 °C. The suspension cells were pelleted at 4 °C and washed twice with 10 ml ice-cold PBS. The cells were lysed in 1% SDS, and radioactivity was measured in a liquid scintillation counter. For the efflux study, cells were incubated with 0.1 μM [^{3}H]paclitaxel according to the method for the accumulation study. After being washed once with cold PBS, the cells were washed in fresh medium with or without 5 μM of cepharanthine at co-culture cell suspensions in the medium containing [^{3}H]paclitaxel at a concentration of 0.1 μM with or without cepharanthine at 37 °C. After 0, 10, 30, and 60 min, the aliquots of cells were removed and immediately added to the ice-cold PBS (10 ml) 4 °C and washed twice with 10 ml ice-cold PBS. The cell pellets were collected for radioactivity detection.

2.7. In vitro transport assays

Transport assays were performed using the rapid filtration method essentially as previously described [19]. Assays were carried out at 37 °C for 10 min in a total volume of 50 μl medium (membrane vesicles 10 μg, 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 4 mM ATP or 4 mM AMP, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, and 0.25 μM [^{3}H]$E_2$17βG). Reactions were stopped by the

addition of 3 ml of ice-cold stop solution (0.25 M sucrose, 100 mM NaCl, and 10 mM Tris–HCl, pH 7.4). For the rapid filtration step, samples were passed through 0.22 μm GVWP filters (Millipore Corporation, Billerica, MA) presoaked in the stop solution. The filters were washed three times with 3 ml of ice-cold stop solution and dried at room temperature. Radioactivity was measured by a liquid scintillation counter. For inhibition experiments, membrane vesicles were incubated with or without cepharanthine at 5 μM for 1 h on ice, and then transport reactions using various concentrations of [^{3}H]$E_2$17βG were carried out for 10 min at 37 °C in uptake medium containing 4 mM ATP.

2.8. Statistical analysis

Two-tailed student's *t*-test was used to compare the difference of the parameters between the two cell groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of MRP7 and P-gp in HEK-pcDNA3.1 and HEK-MRP7-2 cells

HEK293 cells transfected with an MRP7 expression vector were used as a cellular model in this study. As shown in Fig. 2A, MRP7 was detected in membrane vesicles prepared from HEK-MRP7-2 cells but not in membranes derived from parental-vector transfected HEK-pcDNA3.1 cells. P-gp expression was also examined. P-gp was undetectable in HEK293, HEK-pcDNA3.1 and HEK-MRP7-2 cells when we used P7965 antibody (Fig. 2B). Surprisingly, endogenous P-gp was detected in both HEK-pcDNA3.1 and HEK-MRP7-2 cells, but the expression level in HEK-pcDNA3.1 was unexpectedly about 2-fold higher than in HEK-MRP7-2 cells when we used P-gp antibody C219 antibody (data not shown). Additional studies need to be done to confirm whether the detectable bands are P-gp/MDR1 or MDR3 since C219 cross reacts with both human MDR1 and MDR3.

3.2. Effect of reversing agents on the sensitivity of MRP7-transfected HEK293 cells to paclitaxel

In this study, MRP7-transfected HEK293 cells were resistant to paclitaxel, VCR, and VBL compared with their empty vector transfected cells. In addition, HEK-MRP7-2 cells conferred low levels of resistance to VP-16, AraC and colchicine but did not confer significant resistance to SN-38 and cepharanthine (supplemental Fig. 1). These findings are in accord with our previous observations using other MRP7-transfected HEK293 cell lines (supplemental Fig. 1) [20,35]. Subsequently, experiments were conducted to determine if cepharanthine, verapamil and other potential reversal agents alone produced cytotoxicity. Cepharanthine, alone at concentrations of up to 2 μM (supplemental Fig. 1), and other reversal agents up to 5 μM (data not shown), did not exert a significant toxic effect. Resistance to paclitaxel was used to analyze the effects of several classes of reversal agents. The first reversal compound examined was cepharanthine. The structure of cepharanthine

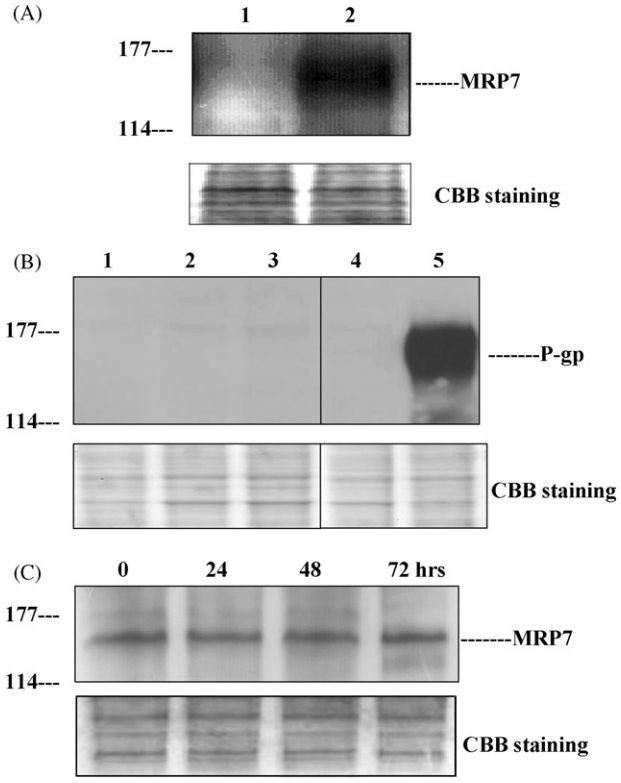

Fig. 2 – Immunoblot detection of MRP7 and P-gp. The expression of MRP7 (A) and P-gp (B) in membrane vesicles prepared from HEK-pcDNA3.1, HEK-MRP7-2 and/or HEK293 cells. Membrane vesicles were prepared from non-transfected parental HEK293 cells (lane 1 in B), HEK293 cells transfected with empty vector (HEK-pcDNA3.1, lane 1 in A and lane 2 in B) or MRP7 (HEK-MRP7-2, lane 2 in A and lane 3 in B) as well as KB-3-1 (parental cell line, lane 4 in B) and KB-C2 (P-gp overexpression drug selected cell line, lane 5 in B) as described in Section 2. Equal amount (15 μg protein/lane) of membrane vesicles were used for each sample, subjected to SDS-PAGE gel and transfer to nitrocellulose membranes. Membranes were subsequently probed with antibodies against MRP7 or P-gp (P7965) as outlined in Section 2. Effect of cepharanthine on the expression of MRP7 in HEK-MRP7-2 cells (C). Cell lysates were prepared from HEK293 cells transfected with MRP7 expression vector (HEK-MRP7-2) incubated without cepharanthine (lane 1) or with cepharanthine (2 μM) for 24 h (lane 2), 48 h (lane 3), and 72 h (lane 4). Proteins (15 μg/lane) were resolved by 4–12% SDS-PAGE gel, electrotransferred to nitrocellulose membranes, and incubated with polyclonal MRP7 antibody. The bands of MRP7 were indicated. The bottom panel is a section of an identical gel stained with CBB to demonstrate approximately equal loading. Results from a representative experiment are shown.

is shown in Fig. 1. Compared to control HEK-pcDNA3.1 cells, HEK-MRP7-2 cells exhibited 6.9-fold resistance to paclitaxel (Table 1), with IC_{50} values of 81.3 ± 19.7 nM and 11.7 ± 1.9 nM for HEK-MRP7-2 and parental-vector transfected cells, respec-

Table 1 – Effect of reversal agents on the sensitivity of HEK-MRP7-2 and control HEK-pcDNA3.1 cells to paclitaxel. To examine the effects of cepharanthine, verapamil, MK571, probenecid or sildenafil on paclitaxel sensitivity, cells were pre-incubated with or without cepharanthine, verapamil, MK571, probenecid or sildenafil for 1 h and then incubated with various concentrations of paclitaxel. Data represent the means of triplicate determinations (±SD) from at least three separate experiments.

Treatment	HEK-pcDNA3.1			HEK-MRP7-2		
	IC_{50} (nM)	FR[a]	DMF[b]	IC_{50} (nM)	FR	DMF
Paclitaxel	11.7 ± 1.9	1.0		81.3 ± 19.7	6.9	
+Cepharanthine (2 μM)	4.7 ± 0.74	0.40	2.5[c]	6.3 ± 1.0	0.53	13[c]
+Verapamil (5 μM)	2.0 ± 0.32	0.17	5.8[c]	6.9 ± 1.4	0.59	11[c]
+MK571 (5 μM)	10.1 ± 2.2	0.86	1.2	63.9 ± 9.9	5.5	1.3
+Probenecid (5 μM)	5.52 ± 0.82	0.45	2.1[c]	22.5 ± 5.4	2.1	3.6[c]
+Sildenafil (5 μM)	2.23 ± 0.10	0.19	5.3[c]	10.34 ± 0.91	0.88	7.9[c]

[a] FR: Fold-resistance was determined by dividing the IC_{50} values of paclitaxel in HEK-MRP7-2 cells by the IC_{50} of paclitaxel in HEK293-pcDNA3.1 cells in the absence of reversal agent; or the IC_{50} of paclitaxel in HEK293-pcDNA3.1 cells in the presence of reversal agent divided by the IC_{50} of paclitaxel in HEK293-pcDNA3.1 cells in the absence of reversal agent.
[b] DMF: Dose-modifying factor was the ratio of IC_{50} without reversal agent and IC_{50} with reversal agents.
[c] Values obtained for drug combined with the reversing agents, are significantly different from those obtained for drug alone ($P < 0.05$).

tively. In the presence of 2 μM cepharanthine, resistance to paclitaxel in HEK-MRP7-2 cells was completely reversed (~13-fold reversal, Table 1), with IC_{50} values of 6.3 ± 1.0 nM and 4.7 ± 0.74 nM for HEK-MRP7-2 and control cells, respectively, in the presence of cepharanthine. As shown in Table 1, cepharanthine also had an appreciable effect on parental-vector transfected cells (~2.5-fold). However, this effect was modest by comparison with the MRP7-transfected cells. A representative survival curve of cells incubated with paclitaxel in the presence or absence of cepharanthine is presented in Fig. 3A. Verapamil also reversed paclitaxel resistance in MRP7-transfected cells. However, verapamil also had a sizable effect on the parental-vector transfected cells, such that the MRP7-transfected cells were only sensitized 2-fold more than the parental-vector transfected cells (Fig. 3B). MK571, a leukotriene D4 (LTD) receptor antagonist that is a potent inhibitor of MRP1 [36], had only a modest effect on HEK-MRP7-2 (Table 1 and Fig. 3C). Probenecid, an inhibitor of organic ion transporters, partially reversed (3.6-fold) the resistance of HEK-MRP7-2 cells to paclitaxel. However, this compound had a similar effect on the parental-vector transfected cells (Table 1 and Fig. 3D). Finally, we tested sildenafil, a phosphodiesterase 5 and 6 inhibitor which has been reported to be a potent inhibitor of MRP4 [15] and MRP5 [37]. Sildenafil at 5 μM completely reversed (~7.9-fold) the paclitaxel resistance in HEK-MRP7-2 cells, but it also significantly enhanced the paclitaxel sensitivity (~5.3-fold) in parental HEK-pcDNA3.1 cells (Table 1 and Fig. 3E). These results suggest that cepharanthine might be the single best reversal agent for MRP7-mediated drug resistance. Non-transfectant HEK293 cells and HEK-MRP7-C18 cells were also used for the reversal study of cepharanthine and the results were similar to those using HEK-pcDNA3.1 and HEK-MRP7-2 cells (data not shown).

3.3. *Effect of cepharanthine on cellular accumulation and efflux of [^{3}H]paclitaxel*

To determine the mechanism by which cepharanthine circumvents MRP7-mediated resistance, its effect on the accumulation of paclitaxel in MRP7-transfected cells was examined. As shown in Fig. 4A, the intracellular concentration of paclitaxel in HEK-MRP7-2 cells was approximately 55% of that in control cells. Cepharanthine at 5 μM enhanced the accumulation of paclitaxel in control cells by only 1.1-fold, while in HEK-MRP7-2 cells cepharanthine increased accumulation by about 2-fold. Notably, in the presence of cepharanthine, paclitaxel accumulation in MRP7-transfected cells was restored to the levels in the parental-vector transfected cells in the absence of the reversal agent (Fig. 4A). The accumulation of paclitaxel in the presence of cepharanthine in HEK-pcDNA3.1 cells is inconsistent with our MTT data in the present of cepharanthine in HEK-pcDNA3.1 cells. One explaination for this is that we only incubated cells with paclitaxel for 1 h in the accumulation study but we incubated the cells with drugs for 72 h in the MTT assay.

In another series of experiments, we determined the effect of cepharanthine on paclitaxel efflux. Following 1 h of drug accumulation under energy depletion conditions, a time course of intracellular drug levels was determined (Fig. 4B). As expected, HEK-MRP7-2 cells released a high percentage of intracellular paclitaxel compared to control cells, and the amount of extruded paclitaxel increased with time. After 60 min of co-incubation in the presence of 5 μM cepharanthine, 43% of the accumulated paclitaxel was released from HEK-MRP7-2 cells, whereas only 12% of the accumulated paclitaxel was released from parental-vector transfected cells. Thus, cepharanthine markedly inhibited the paclitaxel efflux from HEK-MRP7-2 cells such that efflux from this cell line was comparable to that of the control cells (Fig. 4B).

3.4. *Cepharanthine does not alter the expression of MRP7*

The reversal of MRP7-mediated resistance by cepharanthine could occur either by it (1) decreasing MRP7 expression; (2) exerting its own toxicity or (3) inhibiting MRP7 activity. To determine the effect of cepharanthine on the expression of MRP7, HEK-MRP7-2 cells were incubated with 2 μM of cepharanthine for 24, 48 or 72 h. As shown in Fig. 2C, cepharanthine treatment did not significantly alter the expression of MRP7 compared to control cells. Experiments

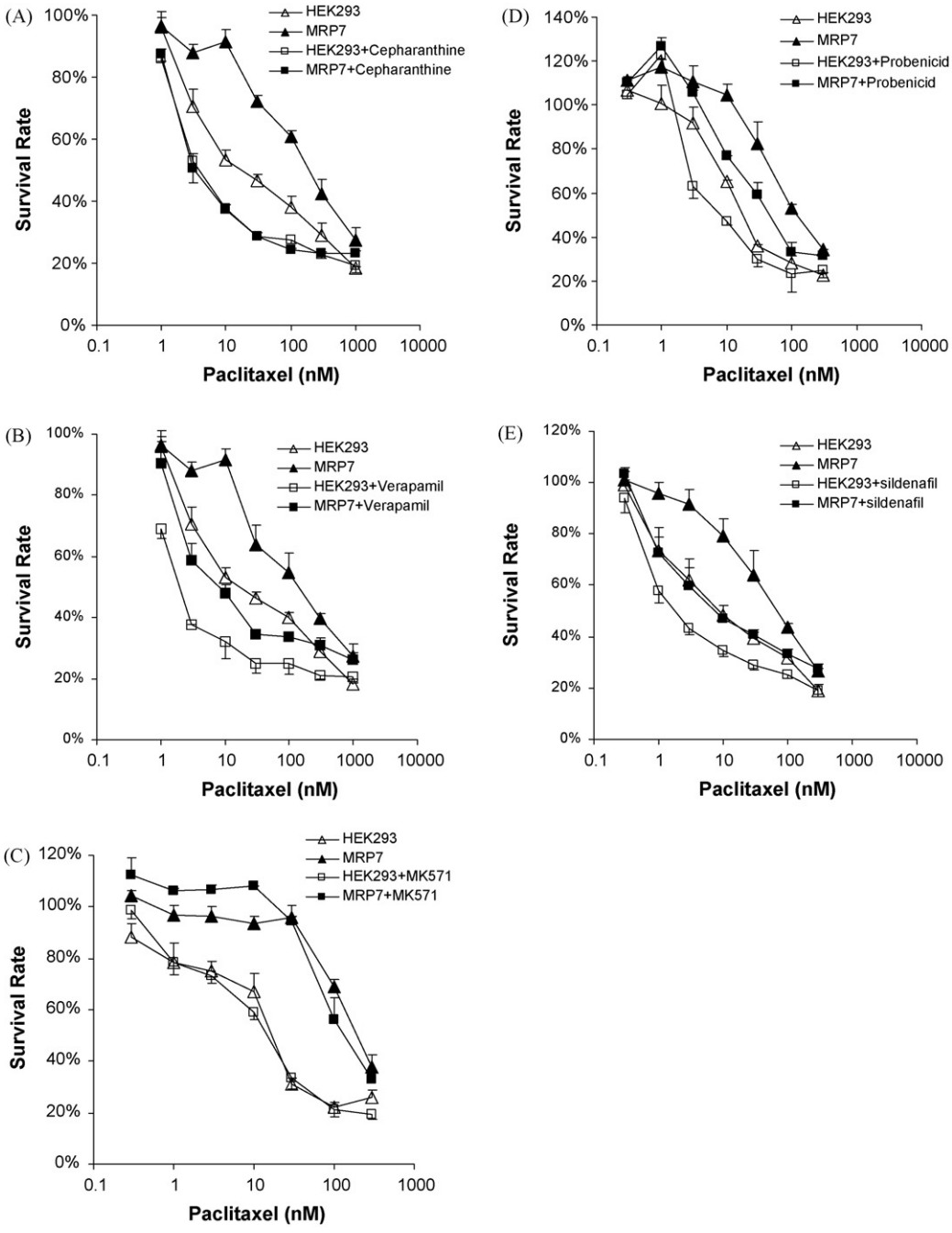

Fig. 3 – Effect of reversing agents on the sensitivity of MRP7-transfected HEK293 cells to paclitaxel. Two cell lines, HEK-pcDNA3.1 and HEK-MRP7-2, were represented as HEK293 and MRP7, respectively. After seeding and culturing cells for 24 h, the reversal agents were added into HEK-pcDNA3.1 cells (□) or HEK-MRP7-2 cells (■), or the equal volumes PBS were added into HEK-pcDNA3.1 cells (△) or HEK-MRP7-2 cells (▲). Paclitaxel was then added after 1 h. Cepharanthine at 2 μM while other compounds were used at 5 μM in the corresponding experiments which were nontoxic for cell growth. Representative experiments are shown.

were also conducted to determine if cepharanthine alone had a cytotoxic effect on HEK293 cells. Cepharanthine, at concentrations of up to 2 μM, did not produce significant toxicity (Supplemental Fig. 1H). In combination, these findings suggest that reversal by this agent is likely attributable to direct inhibition on the activity of the pump.

3.5. *Inhibition of [^{3}H]$E_2$17βG transport by cepharanthine*

Previously, we reported that [^{3}H]$E_2$17βG is a substrate of MRP7 [19]. To gain further insight into how cepharanthine inhibits MRP7, its ability to inhibit [^{3}H]$E_2$17βG uptake into membrane vesicles prepared from HEK-MRP7-2 cells was determined. As

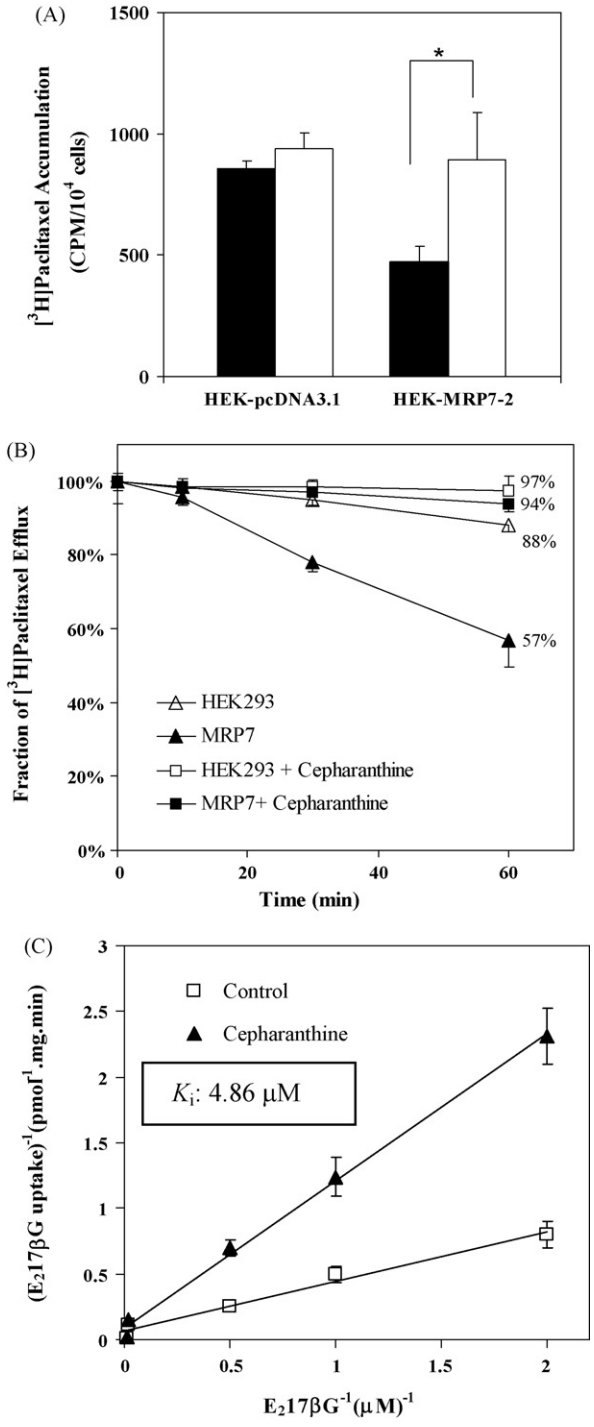

Fig. 4 – Effects of cepharanthine on accumulation, efflux and $E_2$17βG uptake of paclitaxel in HEK-pcDNA3.1 and/or HEK-MRP7-2 cells. (A) The accumulations of paclitaxel in HEK-pcDNA3.1 and HEK-MRP7-2 cells were measured after the incubation with 0.1 μM [^{3}H]paclitaxel for1 h without (■) or with (□) 5 μM cepharanthine in HEK-pcDNA3.1 and HEK-MRP7-2 cells. Columns represent means (±SD) of triplicate determinations. *Statistical calculations of $P < 0.05$ in the two groups. (B) The percentages of the released paclitaxel were plotted as a function of time. After 1 h incubation of [^{3}H]paclitaxel in HEK-pcDNA3.1 without (△) or with (□) cepharanthine, and in HEK-MRP7-2 cells without (▲) or with (■) cepharanthine, the cells were washed and re-incubated in the paclitaxel free medium. At time point at 0 min, 10 min, 30 min and 60 min, the cells were collected for the measurements of radioactivities. The values at 0 min were set as 100% for the comparison with values measured from other time points. Points represented means (±SD) of two experiments each with triplicate samples. (C) The rates of ATP-dependent uptake of [^{3}H]$E_2$17βG into membrane vesicles (10 μg) prepared from HEK-MRP7-2 cells were measured at 37 °C at various substrate concentrations in the absence (□) or presence of 5 μM cepharanthine (▲). K_i value was determined from the double reciprocal plots. Values shown (means ± SD) are rates measured in the presence of ATP minus rates measured in the presence of AMP for duplicate determinations. Representative experiments are shown.

shown in Fig. 4C, cepharanthine was a competitive inhibitor of [^{3}H]$E_2$17βG uptake, with a K_i value of 4.86 μM.

4. Discussion

The results of this study indicated that cepharanthine completely reversed the resistance to paclitaxel in HEK293 cells transfected with the ABC transporter gene *MRP7*. Cepharanthine significantly increased paclitaxel accumulation by inhibiting the MRP7-mediated efflux of paclitaxel. Furthermore, cepharanthine competitively inhibits (K_i = 4.86 μM) $E_2$17βG, a substarte for MRP7. The cepharanthine-induced reversal of paclitaxel resistance is most likely due to its inhibition of the activity of the MRP7 transporter as (1) the expression levels of MRP1 and ABCG2 in HEK-MRP7-2 cells are similar to those of the control HEK-pcDNA3.1 cells (data not shown), and (2) the expressional level of P-gp in HEK-pcDNA3.1 and HEK-MRP7-2 cells is not detected (Fig. 2B). Furthermore, cepharanthine does not significantly alter expression of MRP7 protein levels (Fig. 2C) and had a significantly weaker effect in empty vector transfected cells (Fig. 3A).

Cepharanthine and verapamil have been reported to reverse drug resistance by inhibiting the P-gp-mediated MDR [23,29]. These two P-gp inhibitors were tested in the current study to determine if they could reverse MRP7-mediated paclitaxel resistance. Both compounds significantly reversed paclitaxel resistance in MRP7 overexpressing HEK-MRP7-2 cells. Cepharanthine and verapamil strongly sensitized the MRP7-transfected HEK293 cells to paclitaxel. However, these P-gp inhibitors showed some effects on HEK-pcDNA3.1 cells, but to a lesser degree for cepharanthine (2.5-fold) and to a greater extent for verapamil (5.8-fold). In most of our Western blotting analyses, P-gp was undetectable in parental HEK293, HEK-pcDNA3.1 and HEK-MRP7-2 cells (Fig. 2B). However, we had one preparation of membrane vesicles show that P-gp is expressed in HEK-pcDNA3.1 cells at a relative higher level than in HEK-MRP7-2 cells using C219 antibody (data not shown). This result is neither happened when we switched to use P7965 monoclonal antibody (Fig. 2B) nor appeared in a

separated membrane vesicle preparation (data not shown). Further experiments are needed to confirm whether that membrane preparation was contaminated with proteins expressing low levels of P-gp, or that detected bands may be MDR3 but not MDR1/P-gp since C219 cross reacts with human MDR3. Since paclitaxel can also be transported by P-gp, compounds that inhibit P-gp can decrease the intracellular efflux of paclitaxel. However, the impact of these inhibitors on HEK-MRP7-2 cells would not be significant due to no or very low level of expression of P-gp in MRP7-tranfected cells.

In this study, we also found that sildenafil, an inhibitor of PDE5 and PDE6, was more effective than MK571, a leukotriene receptor antagonist, in reversing paclitaxel resistance in MRP7-transfected cells (Table 1). The findings with sildenafil are congruant with our previous report of $E_2$17βG in HEK-MRP7-2 vesicles [19].

Cepharanthine has been used in clinical trials to treat leukopenia induced by chemotherapy or radiotherapy in cancer patients. In P-gp negative K562 cells, co-treatment of cepharanthine with ADM and VCR resulted in the enhancement of cytotoxicity the anticancer drugs and the apoptosis induced by them [38]. This is due to a cepharanthine-induced alteration in the distribution of ADM from the cytoplasmic vesicles to nucleoplasm in K562 cells. The alteration was caused by cepharanthine via inhibition of the acidification of cytoplasmic organelles [39]. Similar to our findings, it has been reported that in human salivary gland adenocarcinoma (SGA) cells overexpressing MRP7, the incubation of cells with $E_2$17βG reversed resistance to vincristine [21]. Furthermore, in vincristine-resistant SGA cells, the intracellular accumulation of doxorubicin was increased and drug-cross-resistance to docetaxel decreased by $E_2$17βG [21]. It should be noted that in SGA cells, MRP7, P-gp and MRP1 were detected in the cells after incubation with vincristine [21]. MRP7, P-gp and MRP1 are drug efflux pumps responsible for transporting a variety of anticancer drugs outside the cells. When they are simultaneously present in tumor cells, each contributes to the decrease in intracellular drug concentrations, thereby producing insufficient intracellular levels for cytotoxicity.

Cepharanthine has been reported to be an inhibitor of the P-gp efflux pump [29,30] and a reversal agent that partially reverses drug resistance in the human glioma cells expressing MRP1 [40]. These findings suggest that cepharanthine might be effective in restoring the sensitivity of tumors to certain anticancer drugs by inhibiting the drug efflux activity of P-gp, MRP1, and/or MRP7. Oguri reported that the expression of MRP7 is induced by paclitaxel and MRP7 confers paclitaxel resistance by enhancing the efflux for paclitaxel [22]. The results from Oguri and our group utilized different approaches but the results are congruent.

In conclusion, our results tentatively suggest that *in vitro*, cepharanthine reverses paclitaxel resistance by acting in part as a competitive inhibitor of the efflux transporter MRP7. It is possible that cepharanthine could be used in clinically to produce chemosensitization although this remains to be verified. The determination of the effect of 0cepharanthine in a tumor xenograft model should be useful in determining its potential to reverse paclitaxel resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.12.005.

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