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Oxaliplatin up-regulated the function and expression of P-glycoprotein/MDR1 in porcine kidney epithelial LLC-PK$_1$ cells

Noriaki Kitada[1], Kohji Takara[1,*], Hisato Kishi[1], Toshiyuki Sakaeda[2], Noriaki Ohnishi[1], Teruyoshi Yokoyama[1]

[1]Department of Hospital Pharmacy, Faculty of Pharmaceutical Sciences, Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan, Tel: +81-75-595-4628, Fax: +81-75-595-4752, E-mail: takara@mb.kyoto-phu.ac.jp (*corresponding author), [2]Department of Hospital Pharmacy, School of Medicine, Kobe University, Kobe, Japan.

ABSTRACT

The purpose of this study was to clarify the effects of oxaliplatin on the function and expression of the multidrug efflux transporter P-glycoprotein/MDR1 in a porcine kidney epithelial cell line, LLC-PK$_1$, as a renal tubular epithelial model. LLC-PK$_1$ cells were pretreated with or without oxaliplatin for 48 h, and the growth inhibitory effects of a MDR1 substrate, paclitaxel, and the transport of a MDR1 substrate, Rhodamine123, were assessed. The level of MDR1 mRNA and protein was also examined in the cells treated with or without oxaliplatin for 48 h using RT-PCR and immunoblotting. In the present study, the pretreatment with oxaliplatin tended to suppress the growth inhibitory effects of paclitaxel in LLC-PK$_1$ cells, presumably by accelerating the functions of MDR1. In addition, the uptake of Rhodamine123 was reduced significantly by pretreatment with oxaliplatin, and the efflux of Rhodamine123 from LLC-PK$_1$ cells was enhanced significantly. These accelerated functions were supported by the suppression of Rhodamine123's transport by a representative MDR1 substrate/inhibitor, ciclosporin, at 10 μM. The exposure to oxaliplatin for 48 h resulted in an increase in the expression of MDR1 in LLC-PK$_1$ cells. These findings were similar to those obtained with cisplatin, a nephrotoxic drug. In conclusion, the present findings suggested that transient exposure for 48 h to oxaliplatin caused the up-regulation of MDR1 function and expression in LLC-PK$_1$ cells, as was the case for cisplatin.

Key words: oxaliplatin, LLC-PK$_1$, MDR1, P-glycoprotein, up-regulation

INTRODUCTION

Oxaliplatin, an antitumor platinum complex first synthesized by Kidani et al. (1980), shows a stronger antitumor activity against various colon cancer cell lines than its analogues, cisplatin and carboplatin (Armand et al., 2000).

Oxaliplatin has been used for the treatment of metastatic colorectal cancer in combination with fluoropyrimidines in Europe, the USA and Asia, and its clinical efficacy has been confirmed (de Gramont et al., 2000; Fakih 2004). In 2005, oxaliplatin was also approved for use in Japan, where over four thousands patients have been treated with it. Oxaliplatin has a characteristic side effect, i.e., sensory neurotoxicity, however, it is less nephrotoxic than cisplatin (Cassidy and Misset 2002).

Multidrug resistance (MDR) is one of the major causes of failure in cancer chemotherapy (Takara et al., 2006a). The mechanism involved is considered to be an acceleration of efflux, and P-glycoprotein/MDR1, belonging to the ATP-Binding Cassette (ABC) superfamily, has especially attracted attention (Bradshaw and Arceci 1998). MDR1 has been shown to act as an efflux pump to remove anticancer drugs, resulting in a decrease in their cellular concentration to below the lethal level. In addition, MDR1 was clarified to be expressed in the apical membrane of normal tissues including the liver, kidneys, intestine, and brain (Borst et al., 1993; Fojo et al., 1987; Kusuhara et al., 1998; Thiebaut et al., 1987). Recently, MDR1 was also demonstrated to participate in the control of apoptosis (Huang et al., 2001; Johnstone et al., 2000; Sakaeda et al., 2002; Smyth et al., 1998). Thus, MDR1 is considered to be a regulatory factor for maintaining homeostasis, being involved in protection against various types of toxins and stress.

Previously, we have examined whether MDR1-dependent protection was activated under cytotoxic conditions induced by cisplatin, a nephrotoxic anticancer drug that is not a substrate for MDR1 (Takara et al., 2003a). The study demonstrated that transient exposure to cisplatin caused up-regulation of the function and expression of MDR1 in porcine kidney epithelial LLC-PK_1 cells as a model of renal tubular cells. This suggests that changes in the function and expression of MDR1 are one of the factors protecting against the nephrotoxicity of cisplatin.

Herein, the effects of oxaliplatin on the function and expression of MDR1 were examined in a porcine kidney epithelial cell line, LLC-PK_1, as a model of renal tubular cells. In addition, the findings obtained were compared with those for cisplatin.

MATERIALS AND METHODS

Chemicals

Oxaliplatin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Paclitaxel and ciclosporin (CsA) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). WST-1 and 1-methoxy PMS were purchased from Dojindo Laboratories (Kumamoto, Japan). Rhodamine123 was obtained from Molecular Probes, Inc. (Eugene, OR, USA).

Cells and cell culture

The porcine kidney epithelial LLC-PK_1

cells (220-232 passages) were maintained in a culture medium consisting of Medium 199 (Invitrogen Corp., GrandIland, NY, USA) supplemented with 10% fetal bovine serum (Lot No. 99H2314, Sigma-Aldrich) without antibiotics. LLC-PK_1 cells (1.0×10^6) were seeded, and grown in a humidified atmosphere of 5% CO_2-95 % air at 37°C, being passaged every 3 or 4 days with 0.02% EDTA-0.05% trypsin solution (Invitrogen).

Growth inhibition assay

The cytotoxicity of paclitaxel, a substrate for MDR1, was evaluated in LLC-PK_1 cells pretreated with oxaliplatin by using a WST-1 colorimetric assay (Takara et al., 2002a, 2005). LLC-PK_1 cells were pretreated with or without 0.1 or 1 μM oxaliplatin for 48 h, and then the cells (5,000 cells/well) were re-seeded into 96-well plates (Corning Inc., NY, USA) in 100 μL of culture medium without paclitaxel on Day 0, and 24 h later the culture medium was exchanged for one containing paclitaxel (on Day1). After 3 days at 37°C, the WST-1 colorimetric assay was performed (on Day 4). The culture medium was exchanged for one containing WST-1 reagent solution, and 3h later, the absorbance was determined at 450 nm with a reference wavelength of 620 nm using a SpectraFluor microplate reader (Tecan Seetrasse, Switzerland). The 50% growth inhibitory concentration (IC_{50}) of paclitaxel in LLC-PK_1 cells was calculated according to the sigmoid inhibitory effect model; $E=E_{max} \times [1 - C^{\gamma}/(C^{\gamma}+IC_{50}^{\gamma})]$, by means of a nonlinear least-squares method (Solver, Microsoft Excel 2001). E and E_{max} represent the surviving fraction (% of control) and its maximum, respectively, and C and γ represent the drug concentration in the medium (nM) and the sigmoidicity factor, respectively.

Transport of Rhodamine123

The uptake of Rhodamine123, a substrate for MDR1, was determined as described previously (Takara et al., 2002b, 2003a, 2003b). LLC-PK_1 cells (5.0×10^4 cells/well) were seeded into 24-well plates, and incubated for 48 h in a humidified atmosphere of 5% CO_2-95% air at 37°C. Then, the culture medium was exchanged for a fresh culture medium containing 0.1, 1 or 10 μM oxaliplatin, and further incubated for 48 h in a humidified atmosphere of 5% CO_2-95% air at 37°C. After pretreatment with oxaliplatin, cells were washed twice with a warmed Hanks' balanced salt solution (HBSS), and the uptake experiments were started by addition of fresh HBSS containing 10 μM Rhodamine123 with or without 10 μM ciclosporin (CsA), which is a representative substrate/inhibitor for MDR1. CsA was used in order to clarify the contribution of MDR1 to the transport of Rhodamine123. The reaction was stopped by aspiration of HBSS from the wells, followed by washing three times with ice-cold phosphate buffered saline (PBS).

In the efflux experiments, LLC-PK_1 cells were cultured in the same manner as

described for the uptake experiments. Cells were washed twice with warmed HBSS and incubated in fresh HBSS containing 10 μM Rhodamine123 with or without 10 μM CsA for 1 h (Loading time). After loading, HBSS was removed immediately from the wells and cells were washed twice with ice-cold HBSS. Efflux experiments were started immediately by addition of warmed HBSS excluding Rhodamine123 with or without 10 μM CsA, and further incubated for 1 h at 37°C. The reaction was stopped by aspiration of HBSS from the wells, followed by washing three times with ice-cold PBS.

After finishing the transport experiments, cells were lysed with 0.3 M NaOH, and neutralized with an equal volume of 0.3 M HCl. Aliquots (200 μL) of cell lysate were transferred into 96-well black plates, and the fluorescence intensity of Rhodamine123 was measured using the SpectraFluor (Tecan) with an excitation wavelength of 485 nm and emission wavelength of 535 nm. Protein content was determined by the method of Lowry (Lowry et al., 1951), and bovine serum albumin was used as the standard.

RT-PCR analysis of MDR1 mRNA

LLC-PK_1 cells (1.0×10^6) were seeded on plastic culture dishes, and incubated for 48 h. The culture medium was exchanged for that containing oxaliplatin at the indicated concentrations, and further incubated at 37°C for 48 h. Total RNA was isolated using a GeneElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich) and aliquots (0.75 μg) of RNA were used for reverse transcription and cDNA-PCR, using an RNA PCR kit (AMV) version 2.1 (TakaraBio Inc., Shiga, Japan). The PCR oligonucleotide primers for amplification of MDR1 and β-actin (BA) cDNA were synthesized by Proligo Japan K.K. (Kyoto, Japan) and their sequences were shown in a previous report (Takara et al., 2003a, 2006b).

PCR amplification was initiated by one cycle at 94°C for 2 min followed by 35 (MDR1) or 25 (BA) sequential cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, and extension at 72°C for 45 sec in a thermal cycler (BioRad Laboratories, Inc., CA, USA). PCR products were separated on Tris-acetate-EDTA 3% agarose gels containing ethidium bromide. Densitometric analysis was performed on a Macintosh computer using NIH Image ver.1.63 (National Institutes of Health, Bethesda, MD, USA), and the ratios of band intensity (MDR1/BA) were calculated.

Western blot analysis

Cells were cultured and treated with the protocol mentioned above. After the treatment, cells were harvested and lysed with CelLytic™-M (Sigma-Aldrich) and centrifuged at 10,000 rpm (9,100 g) at 4°C for 15 min. The supernatant was collected, and the total protein content was determined according to the Bradford method (Bradford 1976) using bovine γ-globulin as the standard.

Table 1: IC_{50} values for paclitaxel in LLC-PK_1 cells pretreated with or without oxaliplatin for 48 h

Treatment	IC_{50} (nM)	RR[a)]
Control	2.33 ± 0.31	-
+ 0.1 μM oxaliplatin	3.52 ± 1.12	1.51
+ 1 μM oxaliplatin	4.96 ± 0.50	2.13

Cells were pretreated with or without oxaliplatin (0.1 and 1 μM) for 48 h at 37°C, and then the cytotoxic effect of paclitaxel for 72 h was evaluated by a WST-1 assay. Each IC_{50} value represents the mean ± SEM for four independent experiments. [a)] Relative resistance; the IC_{50} value in the pretreated cells was divided by that in the control.

An aliquot of protein (20 or 10 μg) was loaded in each lane to detect the expression of MDR1 or BA, respectively, electrophoresed on a 7.5% SDS-polyacrylamide gel by the method of Laemmli (Laemmli 1970), and transferred to a polyvinylidene difluoride (PVDF) membrane Immun-Blot™ (pore size 0.2 μm, Bio-Rad). For immunoblotting, the membranes were blocked with 5% skim milk (Wako) in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 137 mM NaCl and

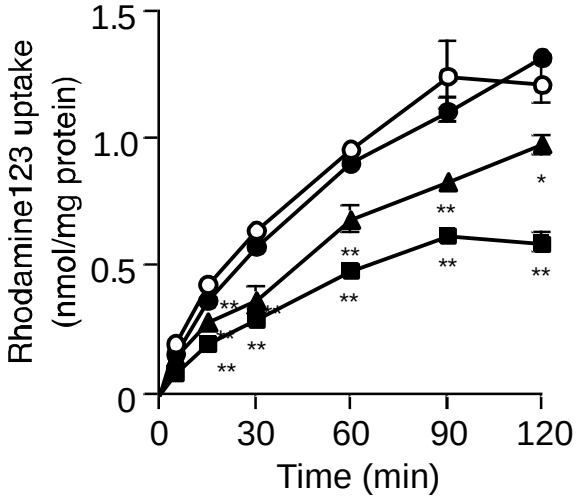

Figure 1: Uptake of Rhodamine123 in LLC-PK_1 cells pretreated with oxaliplatin for 48 h. Cells were pretreated with (closed symbol) or without (open circle) oxaliplatin at 0.1 (●), 1 (▲), or 10 μM (■) for 48 h at 37°C, and then the accumulation of Rhodamine123 (10 μM) was evaluated for the periods indicated at 37°C. Each point represents the mean ± SEM for three independent experiments. * and ** p<0.05 and 0.01 significantly different from the un-treated groups at the corresponding time points, respectively. Each point in the pre-treated cells at 5 min was also significantly different (p<0.01) from the control.

0.1% Tween 20) at 37°C for 1 h. The blots were incubated with anti-MDR1 monoclonal antibody C219 (1:200, Zymed Laboratories Inc., South San Francisco, CA, USA) or anti-BA monoclonal antibody (1:5,000, Sigma-Aldrich) for 2 h, then with horseradish peroxidase (HRP)-linked whole sheep antibody to mouse IgG (NA931, Amersham Biosciences) as a secondary antibody for 1 h, and washed five times with PBS-T. Except where stated specifically, all washing and incubation steps were performed at ambient temperature. MDR1 and BA were detected with the HRP chemiluminescent reaction (Immobilon^{TM} Western Chemiluminescent HRP Substrate, Millipore Corp., Billerica, MA, USA) according to the manufacturer's instructions. Blots were then exposed to a Polaroid film using an ECL Mini-Camera (Amersham Biosciences).

Statistical analysis

Comparisons between two groups were performed with the unpaired Student *t*-test (transport experiments). In the case of more than three groups, the one-way analysis of variance (ANOVA) followed by the Dunnett test (transport experiments and mRNA expression) was used. A *p* value of less than 0.05 (two-tailed) was considered significant.

RESULTS

Growth inhibitory effects of paclitaxel in oxaliplatin-pretreated cells

Table 1 shows the 50% growth inhibitory concentration (IC_{50}) for paclitaxel, a MDR1 substrate, in LLC-PK_1 cells pretreated with or without oxaliplatin for 48 h.

Table 2: Effects of ciclosporin (CsA) on the cellular accumulation of Rhodamine123 for 2 h in LLC-PK_1 cells pretreated with or without oxaliplatin

	Cellular accumulation (nmol/mg protein/2 h)		Fold increase[a)]
	Absence of CsA	Presence of CsA	
LLC-PK_1	1.22 ± 0.07	2.82 ± 0.31*	2.32
+ 0.1 μM oxaliplatin	1.33 ± 0.02	2.92 ± 0.12**	2.20
+ 1 μM oxaliplatin	0.98 ± 0.03	2.71 ± 0.15**	2.77
+ 10 μM oxaliplatin	0.59 ± 0.03	1.18 ± 0.01**	1.99

Cells were pretreated with or without oxaliplatin (0.1, 1, or 10 μM) for 48 h at 37°C, and then the accumulation of Rhodamine123 (10 μM) for 2 h was evaluated in the absence or presence of 10 μM ciclosporin (CsA). Each value represents the mean ± SEM for three independent experiments. * and ** $p<0.05$ and 0.01 significantly different from the respective group in the absence of CsA, respectively. [a)] The cellular accumulation of Rhodamine123 in the presence of CsA was divided by the respective value in the absence of CsA.

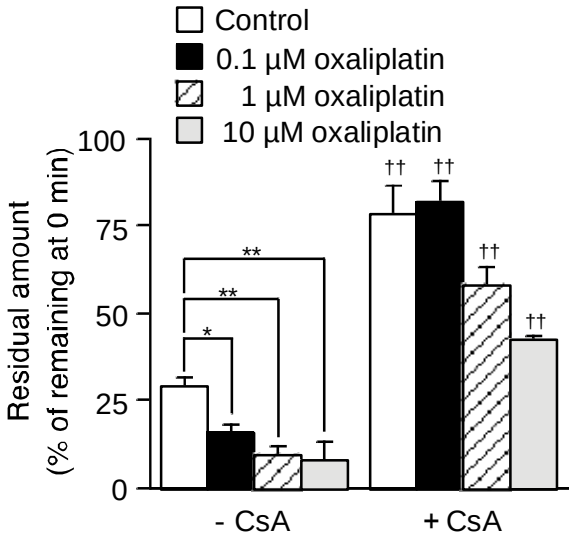

Figure 2: Rhodamine123 efflux for 1 h in LLC-PK_1 cells pretreated with or without oxaliplatin for 48 h and their alterations by ciclosporin (CsA). Cells were pretreated with or without oxaliplatin (0.1, 1 and 10 μM) for 48 h at 37°C, and then the efflux of Rhodamine123 was evaluated for 1 h in the absence or presence of 10 μM ciclosporin (CsA). Each bar represents the mean ± SEM of three independent experiments. * and ** $p<0.05$ and 0.01 significantly different from the control in the absence of CsA, respectively. †† $p<0.01$ significantly different from the respective group in the absence of CsA.

The IC_{50} value for paclitaxel in LLC-PK_1 cells increased in a concentration-dependent manner on pretreatment with oxaliplatin for 48 h, resulting in decrease in the sensitivity to the MDR1 substrate.

Transport activity of MDR1 using Rhodamine123

The uptake of Rhodamine123 was examined in LLC-PK_1 cells pretreated with or without oxaliplatin for 48 h (Fig. 1). The uptake was reduced significantly by pretreatment with oxaliplatin in a concentration-dependent manner. Also, the cellular accumulation for 2 h was significantly increased by the addition of 10 μM ciclosporin (CsA), a representative substrate/inhibitor for MDR1, and recovered to the level found in LLC- PK_1 cells, the effect in the cells pre-treated with 10 μM oxaliplatin was low (Table 2).

The residual cellular amounts of Rhodamine123 at 1 h decreased significantly dependent on the concentration of oxaliplatin (Fig. 2). The effluxes were significantly restored with the addition of 10 μM CsA, although it was low in the case of 10 μM oxaliplatin.

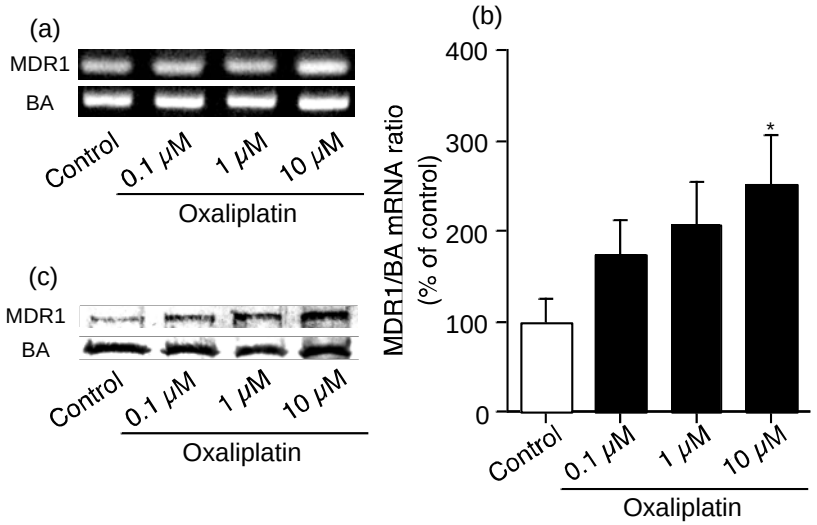

Figure 3: Effects of oxaliplatin on MDR1 expression in LLC-PK_1 cells. Cells were treated with or without the indicated concentrations of oxaliplatin for 48 h at 37°C (a) Total RNA was extracted from cells, and MDR1 and BA mRNA expression was evaluated by RT-PCR. A representative electrophoretogram is presented, and BA represents an internal standard gene. (b) Data are presented as a percentage of the MDR1/BA mRNA level in LLC-PK_1 cells. Each bar represents the mean ± SEM for four independent experiments. * p<0.05 significantly different from the control. (c) Total protein was extracted from cells, and electrophoresed on a 7.5% SDS-polyacrylamide gel. For immunoblotting, the blots were incubated with anti-MDR1 (C219) or anti-βactin (BA) antibodies. A representative immunoblot is presented, and BA was used as the reference protein.

Expression analysis of MDR1

Effects of oxaliplatin on MDR1 expression in LLC-PK_1 cells were examined using RT-PCR and immunoblotting methods (Fig. 3). The level of MDR1 mRNA was significantly increased by treatment with oxaliplatin in a concentration dependent manner (Figs. 3a and 3b). The level of MDR1 mRNA in LLC-PK_1 cells treated with 10 μM oxaliplatin was ca. 2.5-fold higher than that in the control. Immunoblotting also indicated that MDR1 expression was about 5-fold higher in LLC-PK_1 cells treated with 10 μM oxaliplatin than in the control (Fig. 3c).

DISCUSSION

Pretreatment with oxaliplatin for 48 h tended to suppress the growth inhibitory effects of paclitaxel, a substrate of MDR1, in LLC-PK_1 cells, presumably by accelerating the functions of MDR1 (Table 1). This phenomenon was also obtained in a previous study on cisplatin (Takara et al., 2003a). Therefore, the transport function and expression of

MDR1 was examined. The uptake of Rhodamine123, another substrate of MDR1, was significantly and concentration-dependently reduced (Fig. 1), and the efflux of Rhodamine123 from LLC-PK_1 cells was significantly enhanced by pretreatment with oxaliplatin for 48 h (Fig. 2). These experiments suggest an acceleration of the transport function of MDR1 on pretreatment with oxaliplatin. In addition, these accelerated functions were supported by the suppression of Rhodamine123's transport by a representative MDR1 substrate/inhibitor, ciclosporin (CsA) (Table 2 and Fig. 2). As with cisplatin, exposure to oxaliplatin for 48 h resulted in an increase in the expression of MDR1 as detected using RT-PCR and immunoblotting (Fig. 3). Collectively, the present findings suggested that exposure for 48 h to oxaliplatin caused the up-regulation of MDR1 function and expression in LLC-PK_1 cells, being similar to the previous report concerning cisplatin, a nephrotoxic anticancer drug (Demeule et al., 1999; Huang et al., 2001; Takara et al., 2003a). Although oxaliplatin is less nephrotoxic than cisplatin, some reports have described renal dysfunction after the administration of oxaliplatin. Pinotti et al. documented a case of acute tubular necrosis due to oxaliplatin (Pinotti and Martinelli 2002). In addition, Labaye et al. reported that the spectrum of side effects from oxaliplatin included reversible acute renal failure with tubular necrosis (Labaye et al., 2005). Therefore, the up-regulation of MDR1 expression was considered to provide protection against the cytotoxic effects of oxaliplatin. Regrettably, the relationship between the change in the expression of MDR1 and nephrotoxic intensity could not be entirely explained.

Recently, MDR1 has attracted attention as the regulatory factor for maintaining homeostasis and the induction of apoptosis (Huang et al., 2001; Johnstone et al., 2000; Sakaeda et al., 2002; Smyth et al., 1998). Huang et al. assessed cisplatin-induced nephrotoxicity using a microarray, and demonstrated that cisplatin-treatment for 7 days induced the expression of MDR1 and proapoptotic proteins, i.e., annexin V, Bax, and Gadd153 (Huang et al., 2001). In addition, MDR1 was demonstrated to confer long-term resistance to caspase-dependent apoptotic stimuli (Johnstone et al., 2000). On the other hand, the expression of MDR1 was also demonstrated to be up-regulated by apoptotic stimuli, resulting in the suppression of apoptotic signaling presumably *via* the mitochondrial pathway (Sakaeda et al., 2002). Therefore, MDR1 is considered to have the ability to control and maintain normal conditions, and thus the present findings may represent one of the protective mechanisms *via* the apoptotic pathway against cell injury by anticancer drugs. In fact, it was reported that oxaliplatin apparently reduced the expression of apoptosis-inhibitory factors, and phosphorylation of Bcl-2 and Bcl-xl as early as 24 h after treatment, although it did not change the expression of Bax (Fujie et al., 2005).

In clinical practice, oxaliplatin has been frequently used in combination with 5-fluorouracil and levofolinate according to the FOLFOX protocol (de Gramont et al., 2000; Fakih 2004). A standard clinical dose of oxaliplatin in the FOLFOX protocol is 85-100 mg/m^2 delivered by infusion, and the mean maximum plasma concentration after dosing at 85 mg/m^2 over 2 h ranged from 0.587 to 1.563 μg/mL as free platinum (ca. 3 to 8 μM as platinum) (Kho et al., 2006). The concentration of oxaliplatin used in the present study was 10 μM at maximum. This was not very different from an achievable plasma concentration in patients receiving standard dosages of oxaliplatin, and thus the present findings may apply to the clinical situations.

In conclusion, the present findings suggested that transient exposure to oxaliplatin up-regulated the function and expression of MDR1 in the porcine kidney epithelial cell line LLC-PK$_1$. The results were comparable to those for cisplatin, although there was a difference in nephrotoxic intensity. The present study will provide valuable information for developing a more effective and safer cancer chemotherapy with oxaliplatin.

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