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Reversal of P-glycoprotein-mediated multidrug resistance by a synthetic α-aminoxy peptidomimetic

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ABSTRACT

The lack of selectivity and adequate potency of currently known P-glycoprotein (P-gp) inhibitors obscured their further development for clinical use to circumvent P-gp-mediated multidrug resistance (MDR), which necessitates the investigation of novel ones with higher potency and better specificity. The present study investigated the reversal effect of a new synthetic α-aminoxy lysine-peptidomimetic (Lys-P) on P-gp-mediated MDR. Effects of Lys-P on cytotoxicity of P-gp substrate doxorubicin (Dox) and intracellular accumulation of another P-gp substrate rhodamine 123 were examined in HEK293 cells. Its interaction mechanism and effect on P-gp expression were further investigated using ATPase assay and Western blot in Caco-2 cells, respectively. Lys-P restored the cytotoxicity of Dox toward the resistant MDR1-transfected HEK293 and MCF-7 TX400 cells without affecting their corresponding parental cells. It also significantly increased intracellular accumulation (21-fold) of rhodamine 123 in HEK293 MDR1 cells. Further mechanistic studies demonstrated that in the Caco-2 cell monolayer model, Lys-P abolished the P-gp-mediated efflux of Dox due to uncompetitive inhibition of P-gp ATPase without altering P-gp expression. Our findings demonstrated that Lys-P can be used as a promising lead compound for further development into selective and efficient MDR reversing agents for combination use with P-gp substrate drugs in cancer chemotherapy.

1. Introduction

Multidrug resistance (MDR) of cancer cells to a wide spectrum of anti-cancer agents is one of the major obstacles to cancer chemotherapy. It is estimated that MDR tumors account for up to half of all cancer-related deaths (Leonard et al., 2003; Sheps and Ling, 2007). The anti-cancer drugs are actively pumped out of the cells by the ATP-binding cassette (ABC) transporters, thereby preventing their intracellular accumulation and subsequent interaction with the respective drug targets. Among all known ABC transporters, overexpression of P-glycoprotein (P-gp, encoded by the *ABCB1* (or *MDR-1*) gene) on the surface of cancer cells is one of the most common causes of MDR, leading to resistance to its substrate anti-cancer drugs such as doxorubicin (Dox) (Hait and Yang, 2005; Perez-Tomas, 2006). Given the clinical relevance of P-gp mediated MDR, considerable efforts have been made to develop P-gp inhibitors as a means of reversing MDR. A variety of agents with different chemical structures have been identified to be able to inhibit the function of P-gp and thus increase intracellular concentrations and efficacy of chemotherapeutic agents. Several inhibitory mechanisms have been proposed for these agents, including competitive or noncompetitive inhibition of P-gp ATPase (Ambudkar et al., 1999; Yu et al., 2009), down-regulation of P-gp expression (Donmez et al., 2011; Limtrakul et al., 2004), alteration of the conformation by changing cell membrane fluidity (Rege et al., 2002), sterically hindering substrate binding (Batrakova et al., 2004), and covalently modifying essential cysteines in the nucleotide binding domains of P-gp (Loo et al., 2004). Recently, some new inhibitors have been developed based on the mechanisms such as the inhibition of P-gp-mediated transport and/or down regulation of P-gp expression (Petropoulos et al., 2010; Zhang et al., 2010). However, to date, most of identified P-gp inhibitors are not specific or potent

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; Dox, doxorubicin; ER, efflux ratio; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; IS, internal standard; Lys-P, lysine-peptidomimetic; MDR, multidrug resistance; MRP1, multidrug resistance-associated protein 1; NEAA, non-essential amino acids; P-gp, P-glycoprotein; P_{app}, permeability coefficient; PBS, phosphate buffered saline; Rho 123, rhodamine 123; RLU, relative light unit; TEER, transepithelial electrical resistance.

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enough for reversing the P-gp mediated MDR. Therefore, the investigation of better P-gp inhibitors with improved specificity and potency is highly warranted.

Peptidomimetics are synthetic oligomeric molecules designed to mimic functions of natural peptides and proteins for clinical use (Goodman et al., 2007; Hill et al., 2001). Some glutathione peptidomimetics have been reported to modulate MDR *in vitro* by inhibiting multidrug resistance-associated protein 1 (Burg et al., 2002; O'Brien et al., 1999). Our research team has been working on the development of a variety of stable and beneficial peptidomimetics for their potential clinical use for many years (Li and Yang, 2006; Yang et al., 2001, 2003). The present studies reports the MDR reversal effect of a new synthetic peptidomimetic, lysine-peptidomimetic (Lys-P, Fig. 1), and its underlying mechanism of inhibiting P-gp-mediated MDR.

2. Materials and methods

2.1. Materials

Lys-P was synthesized in our laboratory. The identity was characterized by UV, ^{1}H and ^{13}C NMR and MS analysis, and the purity was determined to be higher than 98.6% by HPLC–UV analysis. The detailed synthetic procedures and spectrometric data for its structural elucidation will be reported in a separate paper. Dox, HEPES, penicillin, pimozide, rhodamine 123 (Rho 123) and verapamil were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), non-essential amino acids (NEAA), phosphate buffered saline (PBS) and streptomycin were obtained from Gibco BRL (Carlsbad, CA, USA). A P-gp ATPase assay system was purchased from Promega (Madison, WI, USA). The primary monoclonal antibody of P-gp C219 was from Covance Research Products (Dedham, MA). Anti-mouse IgG horseradish peroxidase conjugate was obtained from Thermo Scientific (Rockford, USA). HPLC grade methanol was obtained from Merck (Darmstadt, Germany). All other compounds and reagents not listed were of analytical grade.

2.2. Cell lines and culture conditions

The cell lines with overexpression of P-gp and their parental cell lines were kindly provided by Dr Susan Bates (National Cancer Institute, USA). P-gp overexpressed MCF-7 TX400 cells were developed from their parental MCF-7 cells by stepwise selection in increasing concentrations of paclitaxel (Huff et al., 2006). Human embryonic kidney HEK293 cell lines transfected with either MDR1 (HEK293-MDR1) or the empty vector (HEK293-pcDNA3) were also used. The P-gp expression levels in P-gp overexpressing resistant cells and sensitive cells were verified by Western blot analysis (Supplementary Fig. 1). HEK293 cells were maintained in RPMI 1640 supplemented with 10% FBS, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. MCF-7 cells were maintained in DMEM supplemented with 10% FBS, 2 mmol/1 L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, USA) and they were cultured in DMEM supplemented with 10% FBS, 1% NEAA, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were incubated in a humidified incubator equilibrated with 5% CO_2 at 37 °C.

2.3. Cell cytotoxicity

The three-day cytotoxicity assays were performed as described by Vichai and Kirtikara (2006). MCF-7, MCF-7 TX400, HEK293-pcDNA3 or HEK293-MDR1 cells were seeded in 96-well plates (4000 or 5000 cells/well), respectively, and allowed to attach overnight. A range of different concentrations of Dox (10^{-4} to 10^{2} μM), with or without verapamil (50 μM) or Lys-P (25 μM), was added to MCF-7, MCF-7 TX400, HEK293-pcDNA3 or HEK293-MDR1 cells and incubated at 37 °C for the P-gp-mediated MDR reversal study. After 72 h incubation, the cellular proteins were fixed with 10% cold trichloroacetic acid, stained in 0.057% sulforhodamine B and incubated at room temperature for 30 min. The plate was then rinsed with 1% acetic acid to remove the unbound dye, and the remaining bound dye was solubilized in 10 mmol/l Tris base (pH 10.5) solution. Cell density was determined by measuring its absorbance at 510 nm. All samples were tested in quadruplicates. The IC_{50} values (concentrations that result in 50% inhibition of cell viability) of Dox in the presence of verapamil or Lys-P were determined by comparing average absorbance of the treated wells to that of the controls.

2.4. P-gp drug efflux assay by flow cytometry

Detection of intracellular accumulation of Rho 123 and its P-gp-mediated efflux in HEK293-MDR1 cells was performed using flow cytometric assay as described previously (Robey et al., 2001). The cells were trypsinized and incubated in 1 μg/ml Rho 123 in the absence or presence of Lys-P at 1, 2 and 5 μM in complete medium (phenol red-free RPMI 1640 with 10% FBS) at 37 °C in 5% CO_2 for 30 min, respectively. Cells were then washed and allowed to incubate in complete medium at 4 °C continuing with or without Lys-P for 1 h to generate the Lys-P/efflux or efflux fluorescence signals, respectively. Cells were then washed with cold PBS and placed on ice in the dark until analysis. Verapamil at 5 μM was also tested in parallel as a positive control P-gp inhibitor. Samples were analyzed on a BD LSRFortessa cell analyzer (BD Biosciences, MD, USA) equipped with a 488-nm argon laser. The fluorescence signal of intracellular Rho 123 was measured by a 530 nm band-pass filter. For all samples, 10,000 events were collected and debris was eliminated by gating on forward *vs* side scatter. The data were processed by FlowJo 5.7.2 software (Tree Star, Inc, Ashland, OR). The results were presented as fold change, which corresponded to the change in the intracellular Rho 123 accumulation (mean fluorescence intensity) in HEK293-MDR1 cells in the presence and absence of the inhibitor.

2.5. P-gp-mediated efflux transport assay

Caco-2 cells were cultured according to the conditions described previously (Zhang et al., 2007). The cells (passages from 39 to 41) were plated onto six-well plate Transwell inserts (0.4 μm pore size, 4.67 cm^2 of growth area, Corning Costar Co., NY) at a density of 3×10^5 cells/well and cultured for 21 days prior to transport experiments. The integrity of the monolayer was monitored by measuring the transepithelial electrical resistance (TEER). Only data obtained using Caco-2 monolayer with TEER above 600 Ω cm^2 or 450 Ω cm^2 (after subtracting the background value of the Transwell) prior to or after the experiment were accepted in the transport study. The study was carried out in HBSS supplemented with 10 mM HEPES transport buffer solution (pH 7.4). The Caco-2 cell monolayer was rinsed twice and equilibrated with transport buffer in the absence or presence of 25 μM Lys-P at 37 °C for 15 min, respectively. Dox (20 μM) was loaded onto the apical (A) or basoleteral (B) side, respectively. Aliquots of individual sample (0.4 ml) were taken from the other side at different time points (0, 30, 45, 60, 90, 120 min) during the experiment. The same volume of blank buffer was added back after each sampling.

An aliquot of each sample (200 μl) was mixed with 30 μl internal standard (IS) pimozide (final concentration: 5 μM), and 50 μl

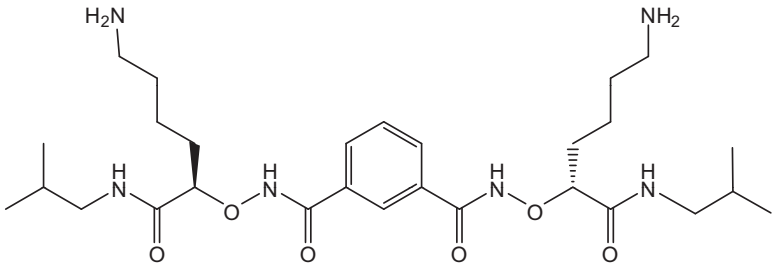

Fig. 1. Chemical structure of Lys-P.

of the resultant sample was analyzed by HPLC-UV using an Agilent 1100 series LC system coupled with a diode array detector. Separation of Dox and IS was achieved by using a Zorbax SB C_{18} column (150 mm × 4.6 mm i.d., 5 μm; Agilent, USA) with an Agilent C_{18} guard column (10 mm × 4.6 mm i.d., 5 μm) at 25 °C with a detecting wavelength at 233 nm. The mobile phase consisted of a mixture of methanol and 5 mM ammonium acetate buffer (47:53, v/v) with a flow rate of 0.90 ml/min. In addition, the recovery of Dox was determined to be higher than 95% by adding up its concentration measured in both apical and basolateral chamber after experiment.

2.6. P-gp ATPase assay

In this assay, the ATP level, which is inversely correlated to the activity of P-gp ATPase, was measured by luminescent signal. The assay was performed according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). Briefly, verapamil (control) or tested sample containing 0.25 mM verapamil and Lys-P (final concentration: 25 and 100 μM) was added to recombinant human P-gp membranes. After pre-incubation at 37 °C for 5 min, MgATP was added into the resultant samples to initiate the reaction, and followed by incubation at 37 °C for 40 min on a plate shaker. Subsequently, the samples were removed from heating source, mixed with ATP detection reagent on a plate shaker, and then incubated at room temperature for 20 min to allow luminescent signals to develop. The luminescence was measured by a Victor 1420 Multilabel Counter (EG&G Wallac, USA) as relative light unit (RLU). The ATPase activity was determined in the absence or presence of Lys-P at 25 or 100 μM. The data were processed by constructing a Lineweaver–Burk plot (or double reciprocal plot) to analyze the inhibitory kinetics of ATPase. The reciprocal of ATPase activity, which was defined as the change in luminescence (RLU) over 40 min, in the absence or presence of Lys-P, was plotted against the reciprocal of substrate concentrations (verapamil: 35, 50, 65, 80 and 95 μM) to construct linear regressions for each concentration of Lys-P used (*i.e.* 25 or 100 μM).

2.7. Western blot analysis for P-gp protein expression

Caco-2 cells were seeded at a density of 2×10^6 cells/dish on 60-mm petri dishes and treated with Lys-P at 100 μM in DMEM on day 18 and 20, respectively. On day 21, after exposure to Lys-P for 24 and 72 h, respectively, the cells were washed twice and scraped in lysis buffer containing protease inhibitors. The mixture was then centrifuged at 15,000 × *g* at 4 °C for 15 min. The supernatant containing the cell membrane proteins was collected and the protein concentration was measured by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. An aliquot (60 μg) of the extracted membrane proteins was used and separated on a 7.5% SDS–polyacrylamide gel for 2 h. The gel was then transferred onto a nitrocellulose membrane. The membrane was blocked by incubating with 5% non-fat milk in washing buffer for 1 h at room temperature, and then incubated overnight with the primary monoclonal antibody P-gp C219 (1:1000). The membrane was incubated with anti-mouse IgG horseradish peroxidase conjugate (1:3000) for 1 h and reacted with chemiluminescence detection reagent (Immun Star Western C Kit, Bio-Rad) for additional 5 min. The protein was visualized by exposing the membrane to a ChemDoc XRS detection system (Bio-Rad, Milan, Italy). P-gp protein for each sample was normalized to β-actin.

2.8. Data analysis

The permeability coefficient (P_{app}) values were calculated using the equation described by Artursson and Karlsson (1991):

$$P_{app} = \frac{dC/dt \times V}{AC_0}$$

where dC/dt is the change of concentration of Dox in the period of incubation time, V is the volume of the receiver chamber, A is the area of the membrane in the insert and C_0 represents initial concentration of Dox in the donor chamber at 0 min. Efflux ratio (ER) was calculated according to the following equation:

$$\mathrm{ER} = \frac{P_{app}(\mathrm{B\ to\ A})}{P_{app}(\mathrm{A\ to\ B})}$$

All the data are expressed as the mean ± SD from at least three independent experiments. One-way ANOVA followed by Dunnett post test was used for multiple comparisons, whereas the two-tailed unpaired *t*-test was used for two-group comparison. A *P* value less than 0.05 was considered significant.

3. Results

3.1. Reversal of Dox MDR in HEK293-MDR1 and MCF-7 TX400 cells

Firstly, cytotoxicity of Lys-P toward HEK293-MDR1, HEK293-pcDNA3, MCF-7 TX400 and MCF-7 cells was tested, and the highest concentration (25 μM) tested did not show toxicity to all the cells (Supplementary Fig. 2). Subsequently, the effect of Lys-P at 25 μM on the cytotoxicity of Dox toward both pairs of cell lines was investigated. The representative concentration–response curves of Dox in both pairs of cell lines in the presence of Lys-P are presented in Fig. 2, and the IC_{50} values are summarized in Table 1. HEK293-MDR1 exhibited an 8-fold higher IC_{50} value than its control HEK293-pcDNA3 (108 ± 2.33 nM in HEK293-MDR1 cells *vs* 13.1 ± 2.38 nM in HEK293-pcDNA3 cells, $P < 0.001$). MCF-7 TX400 also exhibited a 14-fold higher IC_{50} value than its parental MCF-7 (2730 ± 226 nM in MCF-7 TX400 *vs* 201 ± 65.5 in MCF-7 cells, $P < 0.001$). While no significant effect was observed in HEK293-pcDNA3 or MCF-7, Lys-P at 25 μM circumvented Dox MDR in HEK293-MDR1 and MCF-7 TX400 cells and almost returned the IC_{50} values to that in the sensitive control cells. Moreover, it was noted that a similar MDR reversing

Table 1
Effect of Lys-P on the sensitivity of HEK293 and MCF-7 cells to Dox.

Compound	$IC_{50} \pm SD$ (nM)[a]			
	HEK293-pcDNA3	HEK293-MDR1	MCF-7	MCF-7 TX400
Dox	13.1 ± 2.38	108 ± 2.33###	201 ± 65.5	2730 ± 226###
Dox + verapamil (50 μM)	10.5 ± 2.66	13.8 ± 1.94***	242 ± 54.1	372 ± 55.3***
Dox + Lys-P (25 μM)	9.01 ± 1.45	8.94 ± 2.52***	188 ± 43.9	288 ± 39.8***

Data represent the mean values of quadruplicate determinations ± SD ($n = 3$).
[a] IC_{50}: concentration of Dox that reduced cell viability by 50%.
*** $P < 0.001$, comparing with Dox group on the same cell line.
$P < 0.001$, comparing with the corresponding treatment group on HEK293-pcDNA3 or MCF-7 cell line.

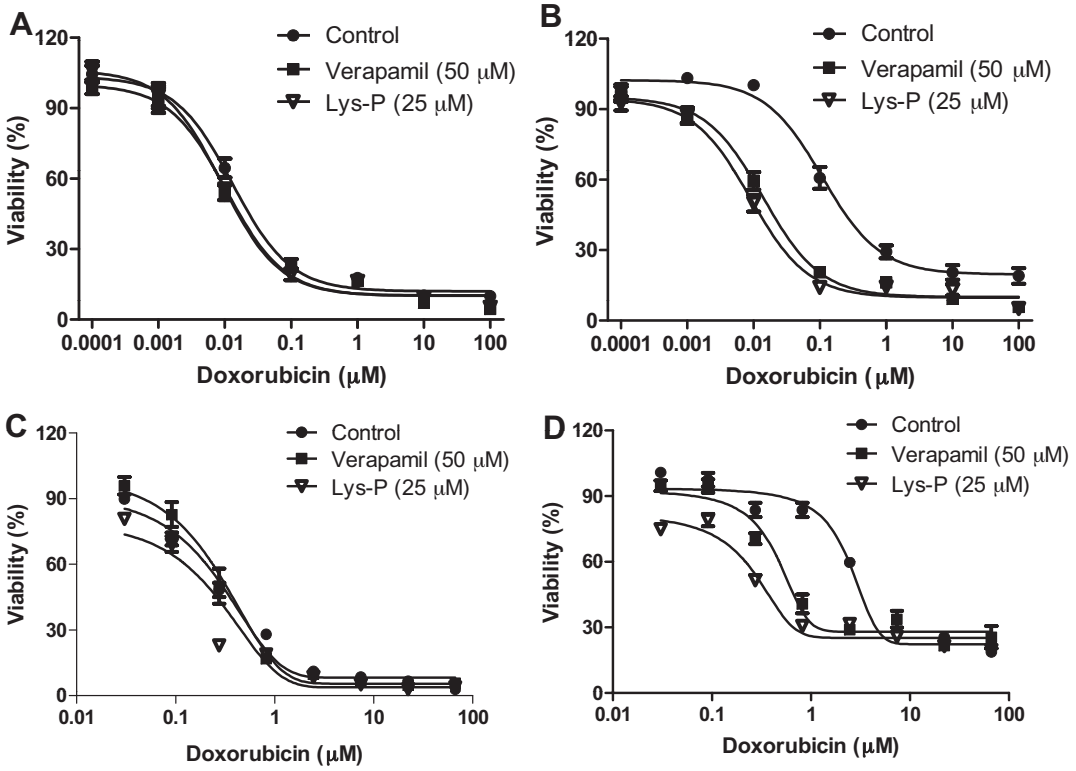

Fig. 2. Cytotoxicity of Dox in the absence (control) or presence of Lys-P in HEK293-pcDNA3 (A), HEK293-MDR1 (B), MCF-7 (C) and MCF-7 TX400 (D) cells ($n = 4$).

effect was achieved in the presence of verapamil at the concentration double than that of Lys-P, indicating that Lys-P was much more potent than verapamil in reversing Dox MDR.

3.2. Increase in intracellular accumulation of Rho 123 in HEK293-MDR1 cells

To investigate whether the MDR reversal effect of Lys-P was due to increasing the intracellular concentration of P-gp substrate, the effect of Lys-P on the intracellular accumulation of P-gp substrate Rho 123 in the P-gp overexpressing HEK293-MDR1 cells was evaluated using flow cytometry analysis. As shown in Fig. 3, the intracellular accumulation of Rho 123 after a 1-h efflux was significantly increased by Lys-P in a concentration-dependent manner. In the presence of Lys-P at 1, 2 and 5 μM, the intracellular fluorescence signals of Rho 123 were increased by 3-, 7- and 21-fold, respectively. Moreover, at the highest concentration (5 μM) tested Lys-P was more potent than verapamil in enhancing intracellular accumulation of Rho 123.

3.3. Inhibition of P-gp-mediated transport across Caco-2 cell monolayer

Since both Dox and Rho 123 are P-gp substrates, the enhanced cytotoxicity of Dox and the reduced efflux of Rho 123 by Lys-P were

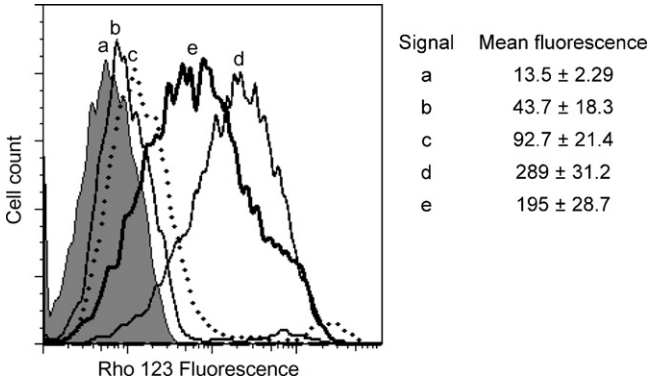

Fig. 3. Effect of Lys-P on the intracellular concentration of Rho 123. The fluorescence of Rho 123 in the absence (a) or presence of Lys-P at 1 μM (b), 2 μM (c) and 5 μM (d), or verapamil (positive control) at 5 μM (e) was measured by flow cytometry.

Table 2
P_{app} and ER values of Dox in the absence or presence of Lys-P.

Compound	P_{app} ($\times 10^{-6}$ cm/s)		Efflux ratio
	A to B	B to A	
Dox	0.513 ± 0.036	3.76 ± 0.461	7.29 ± 1.04
Dox + Lys-P (25 μM)	1.51 ± 0.020***	2.14 ± 0.118**	1.42 ± 0.08***

Data are presented as mean values ± SD ($n = 3$).
** $P < 0.01$, comparing with Dox group.
*** $P < 0.001$, comparing with Dox group.

very likely due to the inhibition of P-gp. The inhibitory effect of Lys-P on the P-gp-mediated efflux transport of Dox was then investigated *via* the inhibition of P-gp expressed in the apical membrane of Caco-2 cell monolayer (Giacomini et al., 2010). The ER value, the ratio of $P_{app\ (B\ to\ A)}/P_{app\ (A\ to\ B)}$, was determined for evaluating efflux transport. The P-gp substrate Dox exhibited an ER value of about 7 (Table 2), suggesting that it was predominantly transported outside the cell from apical membrane by a P-gp-mediated efflux process. In the presence of Lys-P at 25 μM, the efflux transport of Dox was abolished and the ER value returned to 1, demonstrating that Lys-P, at the same concentration for reversing Dox MDR in cytotoxicity study (Table 1), was able to completely inhibit the P-gp-mediated efflux of Dox in the Caco-2 cell model.

3.4. Inhibition of P-gp ATPase

To further understand the mechanism for P-gp inhibition by Lys-P, the P-gp ATPase assay was performed. In this assay, an increase in luminescence signal was used as the read-out to indicate the decrease of ATP consumption by P-gp ATPase upon exposure to verapamil, which acted as a substrate of P-gp to stimulate the activity of ATPase. Decrease in the consumption of ATP corresponded to the reduction of activity of ATPase and thus the inhibition of P-gp. Lys-P significantly inhibited the activity of P-gp ATPase by the evidence of concentration-dependent increase in luminescence signals in the presence of Lys-P (Fig. 4A). Further mechanistic study using a Lineweaver–Burk plot showed a parallel profile among different concentrations of Lys-P (Fig. 4B), indicating that the inhibitory mechanism of P-gp ATPase by Lys-P was uncompetitive (Zhang and Wong, 2005).

3.5. Effect on P-gp expression

Effect of Lys-P on P-gp protein expression was determined in Caco-2 cells by Western blot analysis. The results demonstrated that Lys-P at 100 μM, which exhibited significant inhibition of ATPase activity, did not affect the expression of P-gp in Caco-2 cells after incubation for 24 or 72 h (Fig. 5), proving that the circumvention of P-gp-mediated MDR by Lys-P was not due to the alteration of P-gp expression.

4. Discussion

The efficacy of chemotherapeutic drugs is greatly limited by the emergence of MDR *via* different mechanisms, among them, overexpression of P-gp is the most commonly observed in MDR cancer cells, causing the efflux of its substrate chemotherapeutic drugs out of the cells and leading to MDR. The P-gp substrate anticancer drug doxorubicin (Dox) is frequently used in combination chemotherapy. However, its efficacy is usually compromised by the up-regulation of P-gp in cancer cells (Gillet et al., 2007). In the present study, a new synthetic peptidomimetic Lys-P was demonstrated to significantly reverse Dox MDR (Fig. 2 and Table 1), through inhibition of P-gp-mediated drug efflux (Fig. 3), in a P-gp overexpressing HEK293-MDR1 cells. Similar reversal effect of Lys-P was observed in P-gp overexpressed MCF-7 TX400 cells. Importantly, when compared with verapamil, a potent P-gp competitive inhibitor commonly used in the P-gp inhibitory study (Baumert and Hilgeroth, 2009; Giacomini et al., 2010), Lys-P was found to exhibit a significantly higher effect in circumventing Dox MDR and was more potent in inhibiting the P-gp-mediated drug efflux in HEK293-MDR cells.

Our more in-depth mechanistic studies demonstrated that the reversal effect of Lys-P was due to its inhibition of P-gp transporter evidenced by abolishing the efflux of Dox. While, Lys-P itself was not a P-gp substrate with an efflux ratio close to 1 (data not shown), which might be an advantage because its transport would not be affected by other P-gp substrates or inhibitors when used concurrently with other drugs. Furthermore, the inhibitory mechanism of Lys-P on ATPase was found to be uncompetitive, which is different from most of the reported P-gp inhibitors in that competitive inhibition, such as verapamil (Ambudkar et al., 1999), and non-competitive inhibition, such as indomethacin (Yu et al., 2009). The uncompetitive inhibition means that the inhibitor does not directly compete with the substrate during the inhibition process, but binds to the ATPase–substrate complex to perform its inhibitory function. However, whether and how Lys-P binds to such a complex

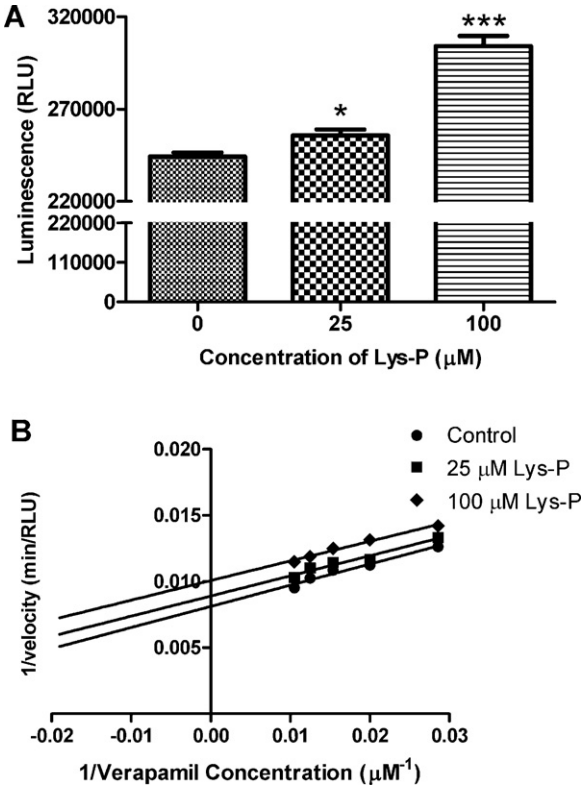

Fig. 4. Inhibition of Lys-P on P-gp ATPase activity. Inhibitory effect on verapamil-stimulated ATPase by Lys-P (A) and Lineweaver–Burk plot for uncompetitive inhibition (B). *$P < 0.05$, ***$P < 0.001$ compared with control ($n = 4$).

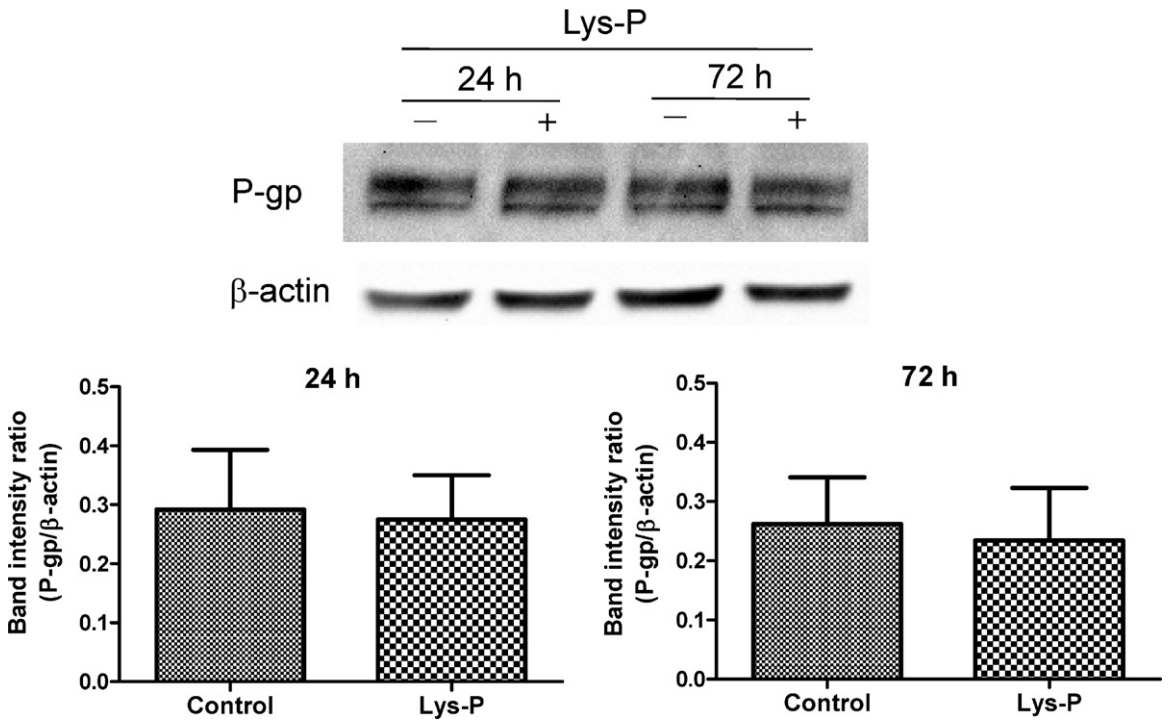

Fig. 5. Effect of Lys-P (100 μM) on the protein expression of P-gp in Caco-2 cells. A representative image from three independent Western blot analysis is shown.

are still unknown and requires further investigation. Nevertheless, the inhibitory mechanism by Lys-P may provide an undiscovered new direction for identifying and developing better P-gp inhibitors with the mechanism different from the currently well-investigated ones. Other compounds can also be modified from Lys-P, the representative peptidomimetic, and tested for their MDR reversal effect in future study. Furthermore, the *in vivo* reversal effects of MDR by Lys-P and whether Lys-P may cause pharmacokinetic interaction with chemotherapeutical drugs warrant further studies.

Decrease in protein expression of P-gp can help to circumvent P-gp-mediated MDR, and has been found to be a common mechanism for various known P-gp inhibitors. It was reported that verapamil significantly reduced P-gp expression in MCF-7/Dox cells (Donmez et al., 2011). However, normal expression of P-gp is generally important as a body defense mechanism. For instance, the inhibition of toxins absorbed through the small intestine, facilitation of drugs and their metabolites clearance from the liver to biliary excretion, and prevention of xenobiotics penetration into the brain (Matsson et al., 2009; Zhang et al., 2006). Therefore, a better way to reverse the function of P-gp is to reversibly inhibit its ATPase activity without significantly affecting the P-gp expression. In contrast to verapamil, our findings demonstrated that Lys-P at the concentration significantly higher (4-fold) than that completely reversed Dox MDR had no significant effect on the P-gp expression in Caco-2 cells after 72 h incubation. Therefore, the lack of effect on P-gp expression of our new P-gp inhibitor Lys-P may be beneficial and further *in vivo* safety and efficacy studies of Lys-P are warranted.

In addition, our preliminary study using MRP1-overexpressing MCF-7 VP cells and BCRP-overexpressing MCF-7 FLV1000 cells demonstrated that Lys-P could also inhibit MRP1 but not BCRP transporter, indicating that Lys-P may have a potential to circumvent MRP1, another major MDR transporter, mediated MDR. The detailed mechanism of the inhibitory effect of Lys-P on MRP1 transporter and the potential reversal of MRP1-mediated MDR by Lys-P is currently under investigation, and the detailed results will be reported later.

5. Conclusions

Our studies showed Lys-P a potent synthetic peptidomimetic exhibiting P-gp-mediated MDR reversal effect with a new mechanism *via* uncompetitive inhibition of P-gp ATPase activity, without significant alteration of P-gp expression. Therefore, Lys-P as a promising lead compound and its structurally related peptidomimetics have a potential for further investigation and development into better agents to circumvent P-gp-mediated MDR in cancer chemotherapy.

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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.ijpharm.2011.12.046.

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