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Research Article

Effect of CJX2, an Amlodipine Derivative, Combined With Verapamil on P-Glycoprotein Efflux Function In Vitro

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ABSTRACT In an effort to inhibit P-glycoprotein (P-gp) efflux function with greater activity and less side effects, the combined effect of CJX2 and verapamil (Ver) was evaluated isobolographically in fixed ratio combinations of 1:1, 1:2, 1:4, 1:8, and 1:10 in doxorubicin-resistant human myelogenous leukemia (K562/DOX) cells and rat brain microvessel endothelial cells (RBMEC). The results displayed that mixtures of both drugs at the fixed ratios of 1:1, 1:2, 1:4, 1:8, and 1:10 exerted synergistic interactions, indicating that when the two blockers that bind P-gp on separate membrane sites were combined, each contributes to the overall interaction with P-gp, leading to the greater effect than that seen by either agent alone. Drug Dev Res 70 : 445–449, 2009.

Key words: P-glycoprotein; CJX2; synergistic interaction

INTRODUCTION

A serious problem associated with cancer chemotherapy is the development of multidrug-resistant (MDR) tumor cells during the course of treatment [Krishna and Mayer, 2000]. The key mechanism of the acquired MDR phenotype in mammalian cells is an enhanced expression of a 170-kDa plasma membrane-associated glycoprotein, known as P-glycoprotein (P-gp), coded by the MDR1 gene, which acts as an active efflux pump for hydrophobic and cationic anticancer drugs [Gottesman and Pastan, 1993]. P-gp is widely expressed, not only in cancer drug-resistant cells, but also in liver, kidney, intestine, and brain where P-gp is expressed in the luminal membrane of the brain capillary endothelium and has an important role in limiting the distribution of various substances into the brain to prevent neurotoxicity [Asperen et al., 1997; Thiebaut et al., 1987]. It is likely that the expression of P-gp in the blood-brain barrier also leads to chemotherapy failure in brain cancer by limiting access to many effective anticancer drugs. Therefore, the strategy for improving clinical response during the treatment of cancer has been a search for more effective modulators. Another strategy has been to investigate the use of pairs of P-gp inhibitor in the hope of finding synergistic effects between them [Lehnert et al., 1991; Hu et al., 1990]. Previous reports [Ji et al., 2006; Ji and He, 2008] showed CJX2, an amlodipine derivative, to have potent in vitro effects in inhibiting

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P-gp function. Moreover, kinetic analysis showed that the binding sites of CJX2 and tetrandrine on P-gp were either identical or partially overlapping, while CJX2 and Ver can bind P-gp on separate sites in K562/DOX cells, indicating a possible cooperative interaction may exist between CJX2 and Ver on P-gp efflux function. To further assess this hypothesis, the effect of CJX2 in combination with Ver on P-gp function in K562/DOX cells and in RBMECs was evaluated.

MATERIALS AND METHODS

Materials

K562/DOX cells were purchased from the Shanghai Institutes for Biological Science, Chinese Academy of Sciences (Shanghai, China); CJX2 was obtained from the School of Traditional Chinese Pharmacy, China Pharmaceutical University (Nanjing, China); Rhodamine123 (Rh123) and Ver were purchased from Sigma (St. Louis, MO); and fetal calf serum (FCS), RPMI-1640 medium, and DMEM/F12(1:1) medium were purchased from Gibco (Grand Island, NY). All other chemicals used in the experiments were of reagent grade.

Cell Culture of K562/DOX Cells

Human doxorubicin-resistant myelogenous leukemia (K562/DOX) cells were grown in RPMI 1640 medium supplemented with 10% FCS at 37°C in a humidified atmosphere of 5% CO_2. K562/DOX cells were cultured in the presence of 0.5 μM doxorubicin and grown in drug-free medium 2 weeks before the experiments were carried out.

Isolation and Culture of RBMEC

RBMECs were isolated as previously described with minor modifications [Abbott et al., 1992; He and Ji, 2008]. Cortex was obtained from the brain of 10 Sprague-Dawley rats (d5) purchased from the animal center of Henan province and placed in ice-cold phosphate-buffered saline (PBS). After removal of the surface vessels and meninges, cortical gray matter was minced and incubated at 37°C for 25 min in Hank's balanced salt solution containing 0.05% trypsin. Then, the samples were filtered with 150-μm nylon mesh. After centrifugation at 800 *g* for 5 min, the pellet was resuspended in PBS containing 20% bovine serum albumin (BSA) and centrifuged at 2000 *g* at 37°C for 5 min. Fat, cell debris, and myelin floating on BSA were removed and the pellet containing microvessels was resuspended and incubated at 37°C for 30 min in PBS containing 0.1% collagenase II. Microvessels were finally collected by centrifugation at 800 *g* for 5 min; the pellet was then washed twice with PBS and cultured in DMEM/F12 (1:1) medium supplemented with 20% FBS at 37°C in 5% CO_2 humidified atmosphere.

Rh123 Cellular Accumulation Assay in K562/DOX Cells

K562/DOX cells at a density of 5×10^4/ml in exponential growth were used. Cells were incubated in the presence or absence of various concentrations of the agents with medium containing 5 μM Rh123 at 37°C for 60 min. The intracellular mean fluorescence intensity (MFI) associated with Rh123 was measured with FACScan flow cytometry (Becton Dickinson). Excitation was performed by an argon ion laser operating at 488 nm; the emitted fluorescence was collected through a 530-nm pass filter. Data analysis was performed using Cell Quest software. Inhibition of Rh123 accumulation (%) was calculated from the obtained fluorescence value (F) obtained according to the following formula:

$$\text{Inhibition of Rh123 accumulation (\%)} = (F_{drug} - F_{control})/F_{control} \times 100\%.$$

Rh123 Cellular Accumulation Assay in RBMEC

RBMECs were seeded at a density of 5×10^4/ml in 24-well plates. After reaching confluence, cell monolayers were exposed to 5 μmol/L Rh123 in serum-free DMEM/F12 medium containing various concentrations of the agents at 37°C for 90 min. After incubation, the medium was removed, and monolayers were washed three times with ice-cold PBS and then dissolved in 1% TritonX-100. Fluorescence of Rh123 was determined using fluorescence spectrophotometry; concentration of Rh123 was measured from the fluorescence value by the Rh123 standard curve. The amount of Rh123 in cell samples was normalized with the amount of protein in each sample as described previously [Fontaine et al., 1996; Sarver et al., 2002].

Inhibition of Rh123 accumulation (%) was calculated from obtained fluorescence value (F) obtained according to the following formula:

$$\text{Inhibition of Rh123 accumulation (\%)} = (F_{drug} - F_{control})/F_{control} \times 100\%.$$

Data Analysis and Statistics

Data from the experiments testing the role of single CJX2 and Ver on the P-gp efflux function were presented as the mean ± SD and analyzed using analysis of variance (ANOVA), followed by the *q*-test. Statistical significance between the theoretical additive value and the experimentally derived value was evaluated by using Student's *t*-test, $P \ll 0.05$ was considered to indicate a significant synergistic

interaction between the two agents according to isobolographic analysis [Tallarida, 2000, 2001].

RESULTS

Effect of Single CJX2 and Ver on Cellular Rh123 Accumulation in K562/DOX Cells and RBMEC

K562/Dox cells and RBMECs were used to assess the effect of CJX2 and Ver on the accumulation of Rh123 by determining intracellular Rh123-associated MFI and Fluorescence, respectively. As shown in Figures 1 and 2, the amount of intracellular Rh123 was increased in a concentration-dependent manner, the EC_{50} values (μM) for CJX2 were 1.42 ± 0.23 and 0.85 ± 0.03 in K562/DOX cells and RBMEC, respectively, and for Ver were 2.24 ± 0.42 and 1.42 ± 0.12. CJX2 was more potent than Ver in reversing P-gp efflux function, consistent with previous reports [Ji et al., 2006].

Effect of Combination of CJX2 and Ver on P-gp Efflux Function in K562/DOX Cells and RBMEC

Isobolographic analysis of the inhibitory effect offered by mixtures of CJX2 and Ver, at 5 fixed drug–dose ratio combinations revealed that all the fixed ratios tested were synergistic (Tables 1 and 2). The experimentally derived $EC_{50\ mix}$ values for the fixed ratios of 1:1,1:2, 1:4, 1:8, and 1:10 were significantly lower than the theoretically calculated $EC_{50\ add}$, and thus, indicated synergistic interactions between CJX2 and Ver (Tables 1 and 2).

DISCUSSION

Considerable effort has been expended to find ways to more effectively block the efflux action of P-glycoprotein, the main mechanism for the multidrug resistance in cancer therapy. Verapamil (Ver) and Cyclosporin A (CsA) have been reported as agents for

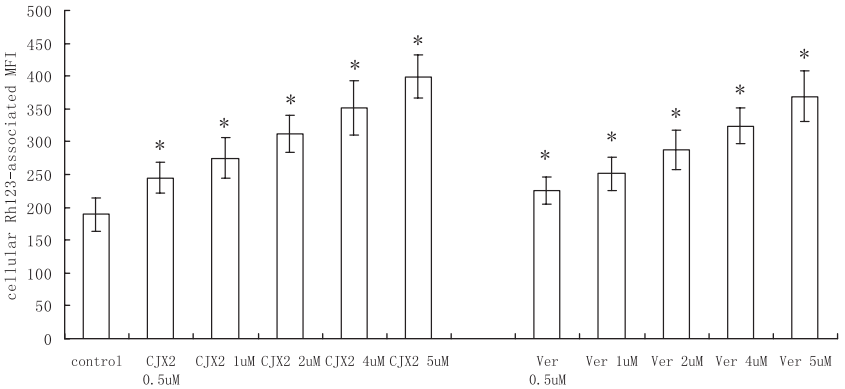

Fig. 1. Effect of CJX2 and verapamil alone on cellular Rh123 accumulation in K562/DOX cells. *Significantly different from the control at $P<0.05$ (ANOVA followed by a *q*-test). Each value represents the mean ± SD from four experiments and was measured as described in Materials and Methods.

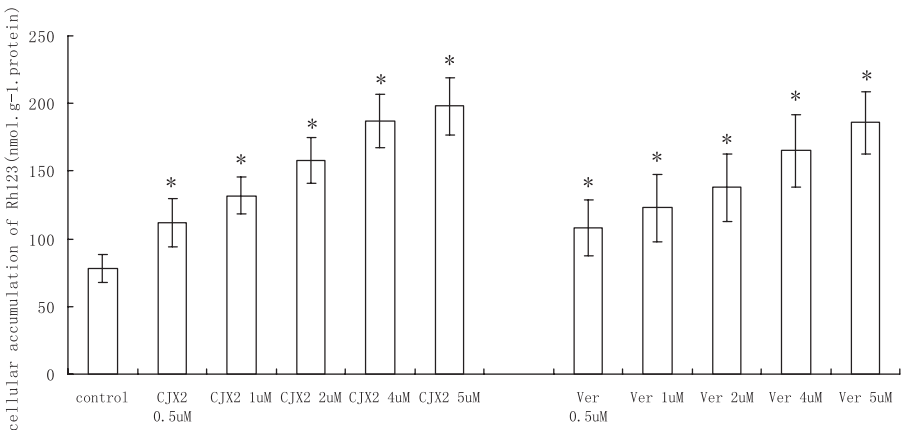

Fig. 2. Effect of CJX2 and verapamil alone on the Rh123 cellular accumulation in RBMEC. Significantly different from the control at *$P<0.05$ (ANOVA followed by *q*-test). Each value represents the mean ± SD from four experiments and was measured as described in Materials and Methods.

TABLE 1. Isobolographic Analysis of Combined Effect of CJX2 and Verapamil on P-gp Efflux Function in K562/DOX Cells*

FR	CJX2	+Ver	= $EC_{50\ mix}$ (μM)	N_{mix}	$EC_{50\ add}$ (μM)	= CJX2	+Ver	N_{add}
1:1	1.12	2.01	3.33±0.55*	12	3.66±0.45	1.42	2.24	12
1:2	0.87	3.35	4.22±0.41*	12	5.9±0.62	1.42	4.48	12
1:4	0.65	5.21	5.86±0.75*	12	10.38±0.54	1.42	8.96	12
1:8	0.42	6.02	6.44±0.74*	12	19.34±0.96	1.42	17.92	12
1:10	0.36	6.87	7.23±0.13*	12	23.82±1.78	1.42	22.4	12

FR, fixed drug-dose ratio combination; N_{add}, total number of samples calculated for the additive mixture of the agents examined; N_{mix}, total number of samples used for the experimental mixture.
*Results are presented as median effective doses (EC_{50}±SD) for drug mixtures, either experimentally determined ($EC_{50\ mix}$) or theoretically calculated ($EC_{50\ add}$). *$P<0.05$ vs. the respective $EC_{50\ add}$.

TABLE 2. Isobolographic Analysis of Combined Effect of CJX2 and Ver on P-gp Efflux Function in RBMEC*

FR	CJX2	+Ver	= $EC_{50\ mix}$ (μM)	N_{mix}	$EC_{50\ add}$ (μM)	= CJX2	+Ver	N_{add}
1:1	0.74	1.22	1.96±0.35*	12	2.27±0.35	0.85	1.42	12
1:2	0.53	2.08	2.61±0.40*	12	3.69±0.24	0.85	2.84	12
1:4	0.42	2.85	3.27±0.38*	12	6.53±0.75	0.85	5.68	12
1:8	0.34	4.19	4.53±0.55*	12	12.21±0.3	0.85	11.36	12
1:10	0.29	4.88	5.17±0.76*	12	15.05±1.4	0.85	14.2	12

FR, fixed drug-dose ratio combination; N_{add}, total number of samples calculated for the additive mixture of the agents examined; N_{mix}, total number of samples used for the experimental mixture.
*Results are presented as median effective doses (EC_{50}±SD) for drug mixtures, either experimentally determined ($EC_{50\ mix}$) or theoretically calculated ($EC_{50\ add}$). *$P<0.05$ vs. the respective $EC_{50\ add}$.

overcoming MDR [Twentyman, 1988]. However, Ver and CsA are used as antiarrhythmic drug and immunosuppressant, respectively, leading to the possibility of side effects when these drugs are used as MDR-reversing agents with antitumor agents. Although the first P-gp inhibitor was found more than 20 years ago, to date no effect P-gp modulator is used in cancer therapy. It is difficult to find potent, specific, and safe agents able to inhibit the efflux activity of P-gp [Van Zuylen et al., 2000]. It has therefore been proposed that combinations of P-gp inhibitors, each at a concentration below its toxic level, may overcome the problems mentioned above [Ayesh et al., 1996].

P-gp has two binding sites—one for transport and another for modulation—and it is possible that more than three sites may exist for drug interactions on P-gp [Martin et al., 2000]. Our previous report showed that CJX2 inhibited Ver-stimulated P-gp ATPase activity in a noncompetitive manner (unchanged K_m and reduced V_{max}); a similar result was observed in the effects of CsA on the CJX2-stimulated P-gp ATPase activity. In contrast, CsA inhibited Ver-stimulated P-gp ATPase activity in a competitive manner with unchanged V_{max} and increased K_m [Ji and He, 2008], indicating a possible synergistic interaction on P-gp function between CJX2, Ver, or CsA. The present study revealed that with CJX2 and Ver at five fixed dose ratios, the experimentally derived $EC_{50\ mix}$ values for the fixed ratios of 1:1,1:2, 1:4, 1:8, and 1:10 were significantly lower than the theoretically calculated $EC_{50\ add}$, respectively, suggesting a synergistic inhibitory effect on the P-gp efflux function in the K562/DOX cells and RBMECs with no predominant action being found for each agent. This approach may provide a valuable way to inhibit P-gp function in tumor cells and in the BBB with lower doses of individual agents leading to new, better P-gp modulators. It might be possible to design a modulator consisting of two or more modulator molecules joined together that would be expected to bind two or more sites to which single modulator molecules bind, potentiating its affinity and reversing activity.

In summary, the present report suggests that when the two inhibitors that bind P-gp at separate sites are combined, each contributes to the overall interaction with P-gp, leading to synergy in modulating P-gp function. Additional studies are ongoing to explore this possibility.

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