DDR

DRUG DEVELOPMENT RESEARCH 66:278–285 (2006)

Research Article

CJX2, an Amlodipine Derivative, Reverses p-Glycoprotein-Mediated Multidrug-Resistance in Doxorubicin-Resistant Human Myelogenous Leukemia (K562/DOX) Cells

Bian-Sheng Ji,[1]* Ling He,[2] and Guo-Qing Liu[3]

[1]*Institute of Pharmacy, Henan University, Kaifeng, China*
[2]*Department of Pharmacology, China Pharmaceutical University, Nanjing, China*
[3]*Department of Pharmacology, China Pharmaceutical University, Nanjing, China*

Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT P-glycoprotein-mediated drug efflux can yield a multidrug-resistance (MDR) phenotype that is associated with a poor response to cancer chemotherapy. Development of safe and effective MDR-reversing agents is an important approach in the clinic. The aim of this study was to observe the effects of CJX2, an amlodipine derivative, on the inhibition of P-gp function and P-gp-mediated MDR in K562/DOX cells and parental K562 cells. Based on the flow cytometric technology, the uptake, accumulation, and efflux of rhodamine123 (Rh123) were detected in these cells by measuring Rh123-associated mean fluorescence intensity (MFI). The effects of CJX2 on the doxorubicin cytotoxicity were evaluated by assaying for MTT (3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide) reduction and the reversal fold (RF) values. The DNA content, percentage of apoptosis, and cell cycle analysis were monitored with flow cytometry. Intracellular accumulation of doxorubicin was also assessed by the determination of doxorubicin-associated MFI. Verapamil was employed as a comparative agent. Incubation of K562/DOX cells with CJX2 caused a marked increase in accumulation, uptake, and a notable decrease in efflux of Rh123. No such results were found in parental K562 cells. The inhibitory effect of the agent on P-gp function was reversible, but it persisted at least for 90 min after removal of 2.5 μM CJX2 from incubation medium. The doxorubicin-induced cytotoxicity, apoptosis, and cell cycle perturbations were significantly potentiated by CJX2. The intracellular accumulation of doxorubicin was enhanced in the presence of various concentrations of CJX2. The CJX2 exhibited potent effects in vitro in the reversal of P-gp-mediated

Abbreviations used: AIF, apoptosis-inducing factor; aSMase, acid sphingomyelinase; CysA, cyclosporin A; DOX, doxorubicin; IC_{50}, concentration resulting in 50% inhibition of cell growth; K562, cells human myelogenous leukemia cells; K562/DOX, cells doxorubicin-resistant human myelogenous leukemia cells; MDR, multidrug resistance; MFI mean, fluorescence intensity; MTT, 3-(4,5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide; nSMase, neutral sphingomyelinase; P-gp, p-glycoprotein; PI, propidium iodide; RF, reversal fold; Rh123, rhodamine123; SAPK/JNK, stress-activated protein kinase/c-JUN N-terminal kinase; SM, sphingomyelin; TOPOII, topoisomerase II; Ver, verapamil.

Grant sponsor: National 863 Program Foundation of China; Grant number: 2002AA2Z343E; Grant sponsor: Nature Science Foundation of Jiangsu Province, China; Grant number: BK 2004110.

*Correspondence to: Bian-Sheng Ji Department of Pharmacology, School of Pharmacy, Henan University, 357 Xi Men Street, Kaifeng, 475001, The People's Republic of China.
E-mail: jibiansheng@yahoo.com.cn

Received 15 October 2005; Accepted 1 January 2006

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ddr.20059

MDR, suggesting that the compound may become a candidate for an effective MDR-reversing agent in cancer chemotherapy. Drug Dev. Res. 66:278–285, 2006.

Key words: CJX2; doxorubicin; P-glycoprotein; multidrug resistance

INTRODUCTION

A serious problem associated with cancer chemotherapy is the development of multidrug-resistant (MDR) tumor cells during the course of treatment. An important mechanism of acquired MDR phenotype in mammalian cells is the enhanced expression of a 170-kDa plasma membrane associated glycoprotein, known as p-glycoprotein (P-gp) [Gottesman and Pastan, 1993]. P-gp is coded by the MDR1 gene and functions as an energy-dependent multidrug membrane transporter that rapidly extrudes a variety of hydrophobic anticancer drugs from target cancer cells and thereby prevents the drugs from exerting cytotoxic effects. Initial physiological and pharmacology studies with multidrug-resistant mutant cell lines correlated resistance as being attributable to reduced accumulation of drugs within the cell as a result of increased efflux or decreased influx [Gottesman and Pastan, 1993]. Because the efflux pump is an ATP-dependent transport system [Horio et al., 1991], agents that inhibit the ATP-dependent drug transport should inhibit the efflux of hydrophobic drugs from resistant cells and increase cellular accumulation. Chemotherapeutic agents such as doxorubicin can select for mutation leading to increased expression of P-gp and appearance of MDR in tissue culture models. Coadministration of P-gp inhibitor was found to suppress activation of MDR 1 gene expression and decrease resistance to doxorubicin [Chen et al., 1994; Page et al., 2000]. In light of these findings, it appears that the most effective way to use chemotherapeutic agents that are P-gp substrates will be in conjunction with a P-gp inhibitor at the time of tumor treatment. Accordingly, a variety of agents including verapamil (Ver), cyclosporin A (CysA), and others have been reported as agents for overcoming MDR [Twentyman, 1988]. However, Ver and CysA are used as an antiarrhythmic drug and an immunosuppressant, respectively. Therefore, side effects are expected to arise when these drugs are used as MDR-reversing agents with antitumor agents. Accordingly, the development of safe and effective MDR reversing agents without other pharmacological activities is eagerly required. Amlodipine, which belongs to dihydropyridines, is a calcium channel antagonist and is currently applied in the treatment of hypertension [Ohbayashi et al., 2003]. A previous report has shown that amlodipine could exhibit an inhibitory effect on P-gp-mediated transport of daunorubicin and digoxin [Katoh et al., 2000]. The aim of this study was to investigate the effects of CJX2, a derivative of amlodipine, on the P-gp function and P-gp-mediated MDR in human myelogenous leukemia (K562) cells and K562/DOX cells.

MATERIALS AND METHODS

Materials

K562 cells and K562/DOX cells were purchased from Shanghai Institutes for Biological Science, Chinese Academy of Sciences; CJX2 was obtained from the School of Traditional Chinese Pharmacy, China Pharmaceutical University; Doxorubicin (DOX) was obtained from Wanle Pharmaceutical Co Ltd, Shenzhen, China; Rhodamine123 (Rh123), 3-(4,5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), RNase, and verapamil were purchased from Sigma (St. Louis, MO); Fetal calf serum and RPMI-1640 medium were purchased from Gibco, Gaithersburg, MD). All other chemicals used in the experiments were commercial products of reagent grade.

Cell Culture

Human myelogenous leukemia K562 cells and their doxorubicin-resistant variant K562/DOX cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum 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 were grown in drug-free medium 2 weeks before the experiments.

Intracellular Rh123 Accumulation Assay

The K562 cells and K562/DOX cells at a density of 5×10^5/ml in exponential growth were used for the test. Cells were incubated in the presence or absence of various concentrations of CJX2 with medium containing 5 μM Rh123 at 37°C for 1 h. Verapamil was used as a positive control for an MDR-inhibitory agent. The intracellular mean fluorescence intensity (MFI) associated with Rh123 was measured with FACScan flow cytometry (Becton Dickinson, San Jose, CA). Excitation was performed by an argon ion laser operating at 488 nm and the emitted fluorescence was

collected through a 530-nm pass filter. Data analysis was performed using Cell Quest software.

Rh123 Uptake and Efflux Assay

In the uptake study, K562 cells and k562/DOX cells were incubated with medium containing 5 μM Rh123 in the presence or absence of 10 μM CJX2 and verapamil at 37°C for 10, 25, 45, 60, and 90 min, respectively. The rate constant (K) was obtained from fitting the data to $Ft = Fss(1\text{-}e^{-kt})$, where Ft is the Rh123-associated MFI at time t, Fss is the MFI at time 90. In the efflux study, K562/DOX cells were first incubated with medium containing 5 μM Rh123 at 37°C for 90 min, washed three times with Rh123-free medium, and then incubated in the presence or absence of 10 μM CJX2 and verapamil at 37°C for 5, 10, 25, 30, 60, and 90 min, respectively. The rate constant (K) was obtained from fitting data to $Ft = F_0e^{-kt}$, where Ft is the Rh123-associated MFI at time t. The MFI was measured as described for the accumulation assay. Graphs were plotted of cell-associated MFI against time.

Persistence of Activity

K562/DOX cells were incubated with medium containing 5 μM Rh123 in the presence or absence of 2.5 μM CJX2 and verpamil at 37°C for 90 min, and washed three times with rhodamine-free and drug-free medium. At subsequent time points as indicated, the ability of the cells to accumulate Rh123 was assessed. Graphs were plotted of cell-associated MFI against time, where T_0 represents the end of the modulator incubation phase.

Cytotoxicity Assay

The ability of CJX2 to potentiate doxorubicin cytotoxicity was evaluated in K562/DOX cells and K562 cells by the conversion of MTT to a purple formazan precipitate as previously described [Morten et al., 1998]. Cells were seeded into 96-well plates at 5×10^4/well. Various concentrations of doxorubicin and CJX2 were subsequently added and incubated for 48 h. IC_{50} values for doxorubicin (concentration resulting in 50% inhibition of cell growth) were calculated from plotted results using untreated cells as 100%. The reversal fold (RF) values, as potency of reversal, were obtained from fitting the data to $RF = IC_{50}$ of cytotoxic drug alone/IC_{50} of cytotoxic drug in the presence of a modulator.

Intrinsic Cytotoxicity Assay

K562 cells and K562/DOX cells were seeded into 96 well plates at 5×10^4/well, 50 μM CJX2 was added, cells were incubated for 48 h, and the intrinsic cytotoxicity of CJX2 was determined as previously described.

Flow Cytometric Apoptosis Assay and Cell Cycle Analysis

The K562/DOX cells at a density of 5×10^5/ml in exponential growth were exposed to 10 μM doxorubicin in the presence or absence of various concentrations of CJX2 at 37°C in a humidified atmosphere of 5%CO_2 for 24 h, then the cells were collected, fixed with 70% ethanol (−20°C), and stored at −20°C for 24 h. The internuleosomally fragmented DNA were removed from apoptotic cells by incubation in 0.2M citrate-phosphate buffer (pH 7.8) containing 0.2 mg/ml RNase for 30 min, and then 100 μg/ml propidium iodide was added. The remaining DNA content was measured using FACScan flow cytometry (Becton Dickinson) [Gong et al., 1994]. Data acquisition and analysis were controlled by Modifit software.

Intracellular Doxorubicin Accumulation

The K562/DOX cells at a density of 5×10^5/ml in exponential growth were exposed to 10 μM doxorubicin in the presence or absence of various concentrations of CJX2 for 1 h. The intracellular doxorubicin-associated MFI was measured with FACScan flow cytometry. Excitation was performed by an argon ion laser operating at 488 nm and the emitted fluorescence was collected through a 615-nm pass fitter. Data analysis was performed using Cell Quest software.

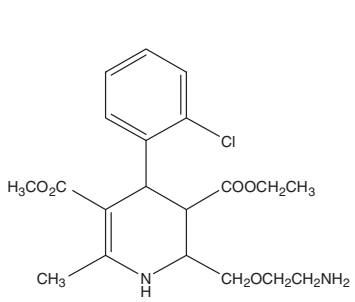
Data Analysis

All data were presented as mean ± SD and analyzed using Student's *t*-test or analysis of variance (ANOVA) followed by *q* test.

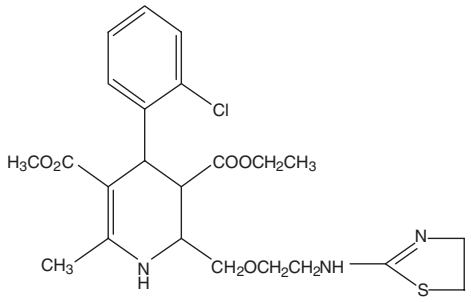
RESULTS

Effect on the Uptake and Intracellular Accumulation of Rh123

We used K562/DOX cells and the parental K562 cells to study the effects of CJX2 on P-gp function by determining intracellular Rh123-associated MFI. As shown in Figure 1, in the uptake study, after K562 cells were incubated with Rh123, the MFI increased in a time-dependent manner over a 90-min period. In contrast, untreated K562/DOX cells did not accumulate the substrate over this same time period. After the same cells were incubated in the presence of CJX2, it accumulated Rh123 in a manner similar to that of untreated K562 cells. The rate constant (K) for the uptake in CJX2-treated K562/DOX cells was comparable to that in control and that in verapamil-treated K562/DOX cells (Table 1). While CJX2 enhanced Rh123 accumulation in a concentration-dependent manner, no such increase in MFI was observed in CJX2-treated K562 cells (Fig. 2).



amlodipine



CJX2

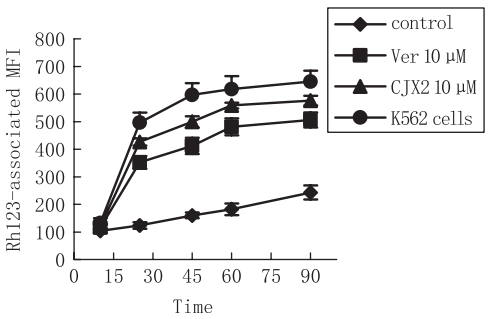


Fig. 1. Effect of CJX2 on the uptake of Rh123 in K562 cells and K562/DOX cells. K562 cells and K562/DOX cells were incubated with medium containing 5 μM Rh123 in the presence or absence of 10 μM CJX2 at 37°C for 10, 25, 45, 60, and 90 min, respectively. The Rh123-associated MFI was measured as described in Materials and Methods. Each point represents the mean ± SD from four experiments.

TABLE 1. Influence of CJX2 on the Rate Constant (K) for the Uptake and Efflux of Rh123 in K562/DOX Cells

Group	K_{uptake} (min^{-1})	K_{efflux} (min^{-1})
Control	0.032 ± 0.0012	0.0244 ± 0.0017
CJX2 (10 μM)	0.053 ± 0.0014**	0.0089 ± 0.00045**
Verapamil (10 μM)	0.048 ± 0.0034*	0.0111 ± 0.0015*

Each value represents the mean ± SD from four experiments and was measured as described in Materials and Methods.
Significantly different from control at $^{*}P<0.05$ (analysis of variance [ANOVA] followed by a *q* test).
Significantly different from verapamil at $\Delta^{**}P<0.05$ (analysis of variance [ANOVA] followed by a *q* test).

Inhibition of Rh123 Efflux and Persistence of Modulatory Activity

Figures 3 and 4 illustrated clearly that 10 μM CJX2 inhibited the efflux of Rh123 from K562/DOX cells and that the inhibitory effect remained even after 2.5 μM CJX2 was removed from the medium for 120 min, suggesting that the inhibitory effect of the agent on P-gp function was reversible and the CJX2 had a longer duration of action compared with verapamil. The rate constant (K) for the efflux in CJX2-treated K562/DOX cells was less than that in control and in verapamil-treated K562/DOX cells (Table 1).

Effect on Doxorubicin Cytotoxicity

The activity of CJX2 to reverse resistance of K562/DOX cells to doxorubicin is shown in Table 2. CJX2 gave a significant reversal of resistance to doxorubicin at a concentration as low as 1 μM. The RF of 10 μM CJX2 23.02 was comparable to that of verapamil 14.45. No such activity was found in K562 cells.

Intrinsic Cytotoxicity

Although CJX2 reversed P-gp-mediated MDR at the concentration of 1 μM, the compound was noncytotoxic by itself at a concentration up to 50 μM in K562/DOX cells and parental K562 cells (data not shown).

Effect on Doxorubicin-Induced Apoptosis and Cell Cycle Perturbations

As shown in Figure 5, the exposure of K562/DOX cells to 10 μM doxorubicin in the presence of various concentrations of CJX2 was associated with an enhancement of internucleosomal DNA fragment. The CJX2 elevated the doxorubicin-induced apoptosis in a concentration-dependent manner. The reduction of cells in the G1 phase and the accumulation of cells in the G2/M phase were also observed after the cells were exposed to 10 μM doxorubicin in the presence of CJX2.

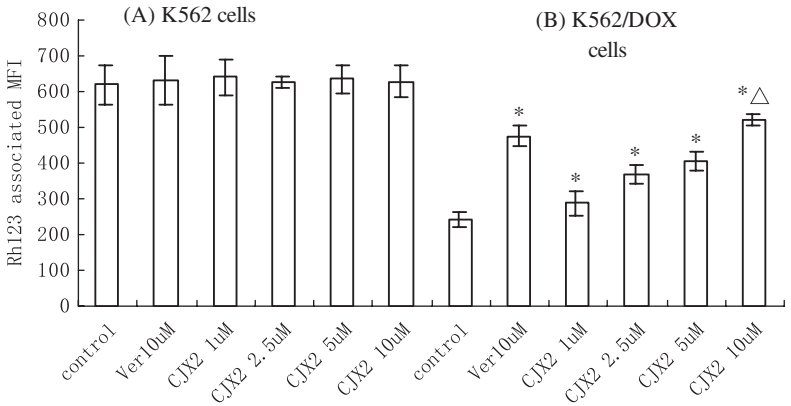


Fig. 2. Effect of CJX2 on the intracellular accumulation of Rh123 in K562 cells and K562/DOX cells. The K562 cells and K562/DOX cells were incubated with medium containing 5 μM Rh123 in the presence or absence of various concentrations of CJX2 and 10 μM verapamil at 37°C for 1 h. Each bar represents the mean ± SD from four experiments. Significant differences from the control were determined by using analysis of variance (ANOVA) followed by q test ($^*P<0.05$); Significant differences from the verapamil were determined by using analysis of variance (ANOVA) followed by q test ($\Delta P<0.05$).

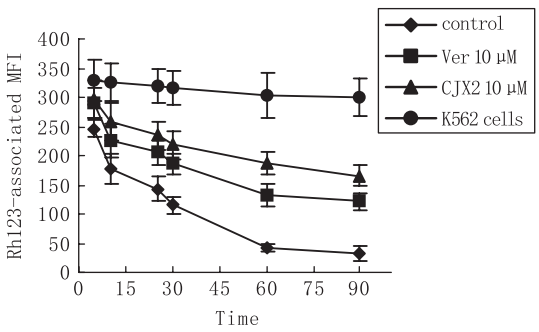


Fig. 3. Effect of CJX2 on the efflux of Rh123 from the K562/DOX cells. Cells were first incubated with medium containing 5 μM Rh123 at 37°C for 90 min, washed three times with Rh123-free medium, and then incubated in the presence or absence of 10 μM CJX2 and verapamil at 37°C for 5, 10, 25, 30, 60, and 90 min, respectively. Each point represents the mean ± SD from four experiments.

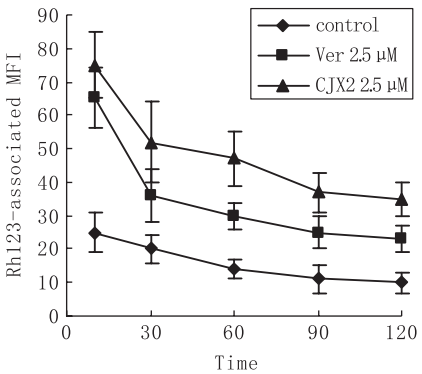


Fig. 4. Persistence of modulatory activity. K562/DOX cells were incubated with medium containing 5 μM Rh123 in the presence or absence of 2.5 μM CJX2 and verpamil at 37°C for 90 min, washed with rhodamine-free and drug-free medium for three times. At subsequent time points as indicated, the Rh123-associated MFI was measured. Each point represents the mean ± SD from four experiments.

Effect on Intracellular Accumulation of Doxorubicin

The ability of CJX2 to inhibit the P-gp function was also evaluated by determining the intracellular doxorubicin-associated MFI in K562/DOX cells. As shown in Figure 6, the MFI was increased in the presence of various concentrations of CJX2, which can explain the enhanced effects of CJX2 on doxorubicin cytotoxicity in K562/DOX cells.

DISCUSSION

Flow cytometric technology has proven to be invaluable for the discovery and characterization of agents capable of altering the MDR phenotype. The doxorubicin-resistant human myelogenous leukemia (K562/DOX) cells that were reported overexpressing p-glycoprotein by the Western blotting method are extremely useful in studying the MDR phenotype and the effects of P-gp inhibitor [Tatu et al., 1997; Ikegawa et al., 2000, 2002]. In the present report, based on the flow cytometric technology, we investigated the effects of CJX2 on the P-gp function and the reversal of P-gp-mediated MDR in doxorubicin-resistant human myelogenous leukemia (K562/DOX) cells.

The efflux of fluorescent dye Rh123 is known to be P-gp-dependent and consequently has been used extensively to determine efflux rate from drug-resistant cell lines expressing P-gp [Green et al., 2001]. After K562 cells and K562/DOX cells were incubated with Rh123, the intracellular Rh123-associated MFI in K562/DOX cells was lower than that in parental K562 cells and notably increased by CJX2 and verapamil, but was not affected in K562 cells. The uptake of Rh123 in

TABLE 2. Effect of CJX2 on Doxorubicin Cytotoxicity in K562 Cells and K562/DOX Cells

Group	IC_{50} (μM) (K562 Cells)	IC_{50} (μM) (K562/DOX cells)	RF (K562/DOX cells)
DOX	0.51±0.10	19.8±0.25	
DOX + CJX2 (1 μM)	0.50±0.07	9.90±0.34	2.0
(2.5 μM)	0.51±0.06	5.69±0.93	3.47
(5 μM)	0.52±0.09	3.40±0.59	5.82
(10μM)	0.50±0.09	0.86±0.09Δ*	23.02Δ*
DOX + Verapamil (10 μM)	0.51±0.06	1.37±0.10	14.45

Each value represents the mean ± SD from four experiments and was measured as described in Materials and Methods.
Significantly different from doxorubicin plus verapamil at Δ: $P<0.05$ (analysis of variance [ANOVA] followed by a q test). * refers to the IC_{50} and RF for the DOX+10μM CJX2 were significantly different from doxorubicin plus verapamil.

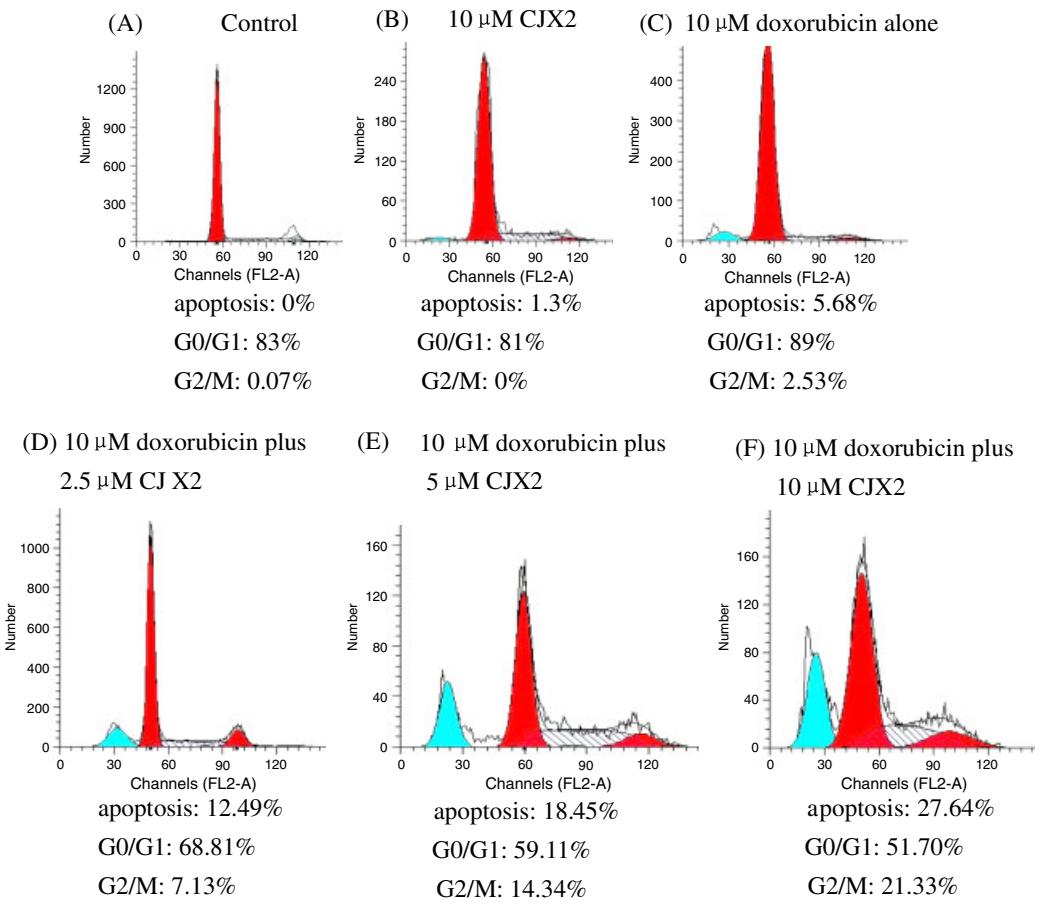


Fig. 5. Effect of CJX2 on doxorubicin-induced apoptosis and cell cycle perturbations in K562/DOX cells

the uptake time-course is the result of passive inward diffusion and active efflux. The rate constant for the uptake and efflux of Rh123 in CJX2-treated K562/DOX cells was determined and compared with that in the verapamil-treated K562/DOX cells. It was shown clearly that the rate constant for the uptake of Rh123 in K562/DOX cells co-incubated with CJX2 was greater than that with verapamil. In contrast, in the Rh123 efflux assay, there was a decreased efflux rate, and the rate constant for the efflux of Rh123 in K562/DOX cells co-incubated with CJX2 was reduced significantly. These results indicated that the CJX2 could inhibit P-gp-mediated transport of Rh123 and the inhibitory effect of CJX2 on P-gp function was more potent than that of verapamil. The persistence of activity assay showed that the CJX2 had a longer duration of action compared with verapamil. The compound inhibited P-gp function in K562/DOX cells in excess of 120 min after a short

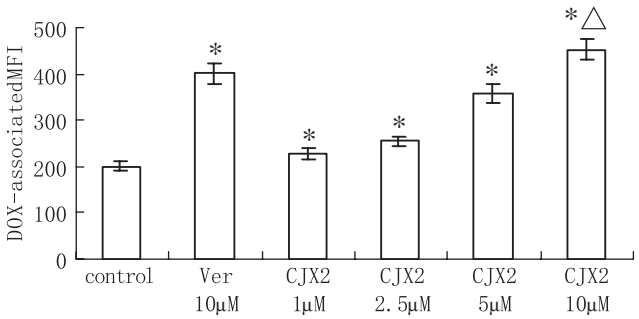


Fig. 6. Effect of CJX2 on the intracellular accumulation of doxorubicin in K562/DOX cells. The cells were exposed to 10 μM doxorubicin in the presence or absence of various concentrations of CJX2 for 1 h. The doxorubicin-associated MFI was measured as described in Materials and Methods. Each bar represents the mean ± SD from four experiments. Significant differences from the control were determined by using analysis of variance (ANOVA) followed by q test ($^*P<0.05$); Significant differences from the verapamil were determined by using analysis of variance (ANOVA) followed by q test ($\Delta P<0.05$).

exposure to 2.5 μM CJX2 and subsequent removal from the incubation medium.

The inhibitory effect of CJX2 on P-gp function indicated that this compound might be able to reverse P-gp-mediated MDR in tumor treatment. Therefore, the ability of CJX2 to reverse P-gp-mediated resistance to doxorubicin, a widely applied topoisomerase II (TOPOII) inhibitor, was further evaluated with K562/DOX cells. TOPOII is an essential nuclear enzyme involved in DNA replication and gene transcription by regulating the topological status of DNA. TOPOII acts by passing a double-stranded DNA helix through a transient double-strand break site and then religating the strand break. There are two TOPOII isoforms, α and β, encoded by the distinct genes that mediate TOPOII activity [Drake et al., 1989; Chung et al., 1989]. It has recently been shown that TOPOIIβ is transiently distributed to the cytoplasma during the mitotic stage, while TOPOIIα is associated tightly with chromosomes constantly throughout the cell cycle [Heck et al., 1988; Nakano et al., 1996]. TOPOII is also a crucial intracellular target of several clinical important anticancer agents. In fact, the anthracycline doxorubicin can inhibit the rejoining action of the enzyme by covalent binging to it, thereby stabilizing the DNA-enzyme complex, resulting in DNA double-strand breaks and apoptosis. However, the sensitivity of these two isoforms of TOPOII against anthracyclines is not fully understood. Although there are other mechanisms of resistance to anthracyclines [Nielsen et al., 1996], P-gp is still a major obstacle to successful treatment of cancer. The present results showed that the decreased IC_{50} and increased RF values were found to be concentration-related, and no cytotoxicity was observed after K562/DOX cells and K562 cells were exposed to 50 μM CJX2 for 48 h, suggesting that CJX2 could reverse P-gp-mediated MDR in a concentration-dependent manner.

Previous studies have demonstrated that P-gp can protect leukemia cells against doxorubicin-induced caspase-dependent cell death [Johnstone et al., 1999]. Doxorubicin can activate undefined intrinsic apoptotic stimuli (i.e., cellular stress), and then excite acid sphingomyelinase (aSMase) [Weisburg et al., 1996], thereby generating ceramide, which putative downstream targets to induce apoptosis include the mitochondria, resulting in release of cytochrome C and apoptosis-inducing factor (AIF), and the stress-activated protein kinase/c-JUN N-terminal kinase (SAPK/JNK) signaling pathway that has been reported to be one of a number of parallel death-signalling pathways to the central caspase pathway [Xia et al., 1995]. P-gp, as an antiapoptotic molecule, can mediate movement of sphingomyelin (SM) out of the inner leaflet of the plasma membrane and decrease ceramide production by reducing the availability of SM to be acted on by either acid sphingomyelinase (aSMase) or neutral sphingomyelinase (nSMase) [Bezombes et al., 1998]. In addition, expression of P-gp correlates with an increased in pHi (intracellular pH) and this has been proposed to result in a state of caspase-inactivity and decreased free drug concentration due to altered transmembrane partitioning or intracellular sequestration [Gottlieb et al., 1996; Belhoussine et al., 1999]. Moreover, the drug-induced cell cycle perturbations such as decrease in the G1 phase fraction and increase in the S phase and/or the G2/M phase fraction have been reported to correlate with a response to chemotherapy and depend on the cell line studied, the drug itself, the exposure time, and the dosage [Briffod et al., 1992; Sorenson and Eastman, 1988]. G2/M is a crucial period for further cellular proliferation. Irreversible drug-induced G2/M arrest is associated with DNA double-strand breaks and extensive chromosome damage [Sorenson and Eastman, 1988]. In contrast, a lack of cell cycle perturbations was found to indicate chemoresistance**.** In the present report, after K562/DOX cells were exposed to 10 μM doxorubicin in the presence of CJX2 for 24 h, there was a pronounced increase in both the apoptosis rate and the fraction of cells in the G2/M phase. In contrast, a remarkable reduction in the fraction of cells in the G1 phase was found. Our results also showed that the intracellular MFI associated with doxorubicin, a substrate of P-gp, was elevated after incubation with various concentrations of CJX2, which can explain the enhanced effects of CJX2 on doxorubicin cytotoxicity in

K562/DOX cells. Taken together, these data suggest that, as a result of inhibition of P-gp function, CJX2 could greatly potentiate doxorubicin cytotoxicity and elevate doxorubicin-induced apoptosis and G2/M block in K562/ DOX cells. The effects of CJX2 on the caspases activities and the intracellular doxorubicin distribution as well as on the P-gp function in vivo will be tested in our laboratory.

In conclusion, the present results showed that CJX2 exhibited potent effects in vitro in the inhibition of P-gp function and the reversal of P-gp-mediated MDR. It may become a candidate for an effective MDR reversing agent in cancer chemotherapy.

REFERENCES

Belhoussine R, Morjani H, Sharonov S, Ploton D, Manfait M. 1999. Characterization of intracellular pH gradients in human multidrug-resistant tumor cells by means of scanning microspectrofluorometry and dual-emission-ratio probes. Int J Cancer 81: 81–89.

Bezombes C, Maestre N, Laurent G, Levade T, Bettaieb A, Jaffrezou JP. 1998. Restoration of TNF-alpha-induced ceramide generation and apoptosis in resistant human leukemia KG1a cells by the P-glycoprotein blocker PSC833. FASEB J 12:101–109.

Briffod M, Spyratos F, Hacene K, Tubiana-Hulin M, Pallud C, Gilles F, Rouesse J. 1992. Evaluation of breast carcinoma chemosensitivity by flow cytometric DNA analysis and computer assisted image analysis. Cytometry 13:250–258.

Chen G, Jaffrezou JP, Fleming WH, Duran GE, Sikic BI. 1994. Prevalence of multidrug resistance related to activation of the mdr1 gene in human sarcoma mutants derived by single-step doxorubicin selection. Cancer Res 54:4980–4987.

Chung TD, Drake FH, Tan KB, Per SR, Crooke ST, Mirabelli CK. 1989. Characterization and immunological identification of cDNA clones encoding two human DNA topoisomerase II isozymes. Proc Natl Acad Sci USA 86:9431–9435.

Drake FH, Hofmann GA, Bartus HF, Mattern MR, Crooke ST, Mirabelli CK. 1989. Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II. Biochemistry 28: 8154–8160.

Gong J, Traganos F, Darzynkiewicz Z. 1994. A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. Anal Biochem 218:314–319.

Gottesman MM, Pastan I. 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 62:385–427.

Gottlieb RA, Nordberg J, Skowronski E, Babior BM. 1996. Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification. Proc Natl Acad Sci USA 93:654–658.

Green LJ, Marder P, Slapak CA. 2001. Modulation by LY335979 of P-glycoprotein function in multidrug-resistant cell lines and human natural killer cells. Biochem Pharmacol 61:1393–1399.

Heck MM, Hittelman WN, Earnshaw WC. 1988. Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. Proc Natl Acad Sci USA 85:1086–1090.

Horio M, Lovelace E, Pastan I, Gottesman MM. 1991. Agents which reverse multidrug-resistance are inhibitors of [3H]vinblastine transport by isolated vesicles. Biochim Biophys Acta 1061: 106–110.

Ikegawa T, Ushigome F, Koyabu N, Morimoto S, Shoyama Y, Naito M, Tsuruo T, Ohtani H, Sawada Y. 2000. Inhibition of P-glycoprotein by orange juice components, polymethoxyflavones in adriamycin-resistant human myelogenous leukemia (K562/ ADM) cells. Cancer Lett 160:21–28.

Ikegawa T, Ohtani H, Koyabu N, Juichi M, Iwase Y, Ito C, Furukawa H, Naito M, Tsuruo T, Sawada Y. 2002. Inhibition of P-glycoprotein by flavonoid derivatives in adriamycin-resistant human myelogenous leukemia (K562/ADM) cells. Cancer Lett 177:89–93.

Johnstone RW, Cretney E, Smyth MJ. 1999. P-glycoprotein protects leukemia cells against caspase-dependent, but not caspase-independent, cell death. Blood 93:1075–1085.

Katoh M, Nakajima M, Yamazaki H, Yokoi T. 2000. Inhibitory potencies of 1,4-dihydropyridine calcium antagonists to P-glycoprotein-mediated transport: comparison with the effects on CYP3A4. Pharmaceut Res 17:1189–1197.

Morten B, Hansen SE, Nielsen K, Berg. 1998. Re-examination and further development of a precise and rapid dye method for measuring cell growth /cell kill. J Immunol Methods 119:203–210.

Nakano H, Yamazaki T, Miyatake S, Nozaki N, Kikuchi A, Saito T. 1996. Specific interaction of topoisomerase II beta and the CD3 epsilon chain of the T cell receptor complex. J Biol Chem 271: 6483–6489.

Nielsen D, Maare C, Skovsgaard T. 1996. Cellular resistance to anthracyclines. Gen Pharmacol 27:251–255.

Ohbayashi Y, Tsutamoto T, Sakaguchi T, Tanaka T, Kanamori T, Yokohama H, Sichiri G, Hukai D, Okabayashi T, Ozawa T, Ishii C, Tsutsui T, Ohno K, Ohnishi M, Wada A. 2003. Effect of an angiotensin II type 1 receptor blocker, valsartan, on neurohumoral factors in patients with hypertension: comparison with a long-acting calcium channel antagonist, amlodipine. J Cardiovasc Pharmacol 42(Suppl 1):S71–74.

Page RL, Hughes CS, Huyan S, Sagris J, Trogdon M. 2000. Modulation of P-glycoprotein-mediated doxorubicin resistance in canine cell lines. Anticancer Res 20:3533–3538.

Sorenson CM, Eastman A. 1988. Mechanism of cis-diamminedichloroplatinum(II)-induced cytotoxicity: role of G2 arrest and DNA double-strand breaks. Cancer Res 48:4484–4488.

Tatu CA, Paunescu V, Stanescu DI, Schneider F. 1997. P-glycoprotein mediated multidrug resistance assessment by flow-cytometry in malignant hemopathies. Anticancer Res 17:2583–2586.

Twentyman PR. 1988. Modification of cytotoxic drug resistance by non-immuno-suppressive cyclosporins. Br J Cancer 57:254–258.

Weisburg JH, Curcio M, Caron PC, Raghu G, Mechetner EB, Roepe PD, Scheinberg DA. 1996. The multidrug resistance phenotype confers immunological resistance. J Exp Med 183: 2699–2704.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326–1331.