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CJY, an isoflavone, reverses P-glycoprotein-mediated multidrug-resistance in doxorubicin-resistant human myelogenous leukaemia (K562/DOX) cells

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Abstract

In an effort to develop safe and effective multidrug-resistance (MDR) reversing agents, the effect of CJY, an isoflavone, on the P-glycoprotein (P-gp) function and P-gp-mediated MDR was evaluated in doxorubicin-resistant human myelogenous leukaemia (K562/DOX) cells. The results showed that CJY caused a marked increase in accumulation and a notable decrease in efflux of rhodamine 123 (Rh123). The inhibitory effect of the agent on P-gp function persisted for at least 120 min after removal of 2.5 μM CJY from the incubation medium. The doxorubicin-induced cytotoxicity, apoptosis and cell cycle perturbations were significantly potentiated by CJY. The intracellular accumulation of doxorubicin was also enhanced. The compound exhibited potent effects in-vitro on the reversal of P-gp-mediated MDR, suggesting that it could become a candidate as an effective MDR reversing agent in cancer chemotherapy.

Introduction

Resistance to chemotherapy by some human tumours is due, in part, to an over-expression of membrane-associated transport proteins. The best characterized of these is the 170 kDa P-glycoprotein (P-gp) (Gottesman & 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. Cells that over-express P-gp exhibit a multidrug resistance (MDR) phenotype.

A number of substances, such as verapamil and ciclosporin, have been shown to modulate or inhibit the transport function of P-gp, thus enhancing or restoring chemosensitivity of MDR cells to cytotoxic agents (Twentyman 1988). However, the clinical use of these first-generation chemosensitizers has been hampered by the toxic side effects. Accordingly, development of safe and effective MDR reversing agents without other pharmacological activity is eagerly required. It was reported that flavonoid could increase accumulation of rhodamine 123 (Rh123), a P-glycoprotein substrate, in LLC-GA5-COL150 cells and reverse P-gp-mediated MDR in doxorubicin-resistant human myelogenous leukaemia (K562/DOX) cells (Ferte 1999; Patanasethanont 2007). We have investigated the effects of CJY, an isoflavone (see Figure 1), on the P-gp function and P-gp-mediated MDR in human myelogenous leukaemia (K562) cells.

Materials and Methods

Materials

K562 cells and K562/DOX cells were purchased from Shanghai Institutes for Biological Science, Chinese Academy of Sciences. CJY was obtained from Professor Wei-Sheng Tian (Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences). Doxorubicin was obtained from Wanle Pharmaceutical Co Ltd, Shenzhen, China. Rhodamine 123 (Rh123), 3-(4,5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), RNase and verapamil were purchased from Sigma Co., USA. Fetal calf serum and RPMI-1640

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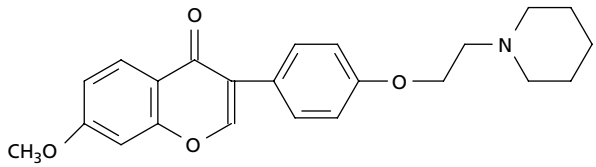

Structure of CJY

Figure 1 The structure of CJY.

medium were purchased from Gibco, USA. All other chemicals used in the experiments were commercial products of reagent grade.

Cell culture

Human myelogenous leukaemia 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 two weeks before the experiments.

Intracellular Rh123 and doxorubicin accumulation

The K562 cells and K562/DOX cells at a density of 5×10^5 mL^{-1} in exponential growth were used for the test. Cells were incubated in the presence or absence of various concentrations of CJY with medium containing 5 μM Rh123 or 10 μM doxorubicin at 37°C for 60 min. Verapamil was used as a positive control for an MDR-inhibitory agent. The intracellular mean fluorescence intensity (MFI) associated with Rh123 or doxorubicin was measured with a FACScan flow cytometer (Becton Dickinson). Excitation was performed by an argon ion laser operating at 488 nm, the emitted fluorescence was collected through a 530 nm and 615 nm pass filter for the Rh123 and the doxorubicin assay, respectively. 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 CJY or verapamil at 37°C for 10, 25, 45, 60 and 90 min, respectively. The rate constant (K) was obtained from fitting the data to $F_t=F_{ss}(1-e^{-kt})$, where F_t is the Rh123-associated MFI at time t, F_{ss} is the MFI at time 90 min. 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 CJY or verapamil at 37°C for 5, 10, 25, 30, 60 and 90 min, respectively. The rate constant (K) was obtained from fitting data to $F_t=F_0e^{-kt}$, where F_t 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 CJY or verapamil at 37°C for 90 min. The cells were then washed three times with rhodamine-free and drug-free medium for 10, 30, 60, 90 and 120 min. The ability of the cells to accumulate Rh123 was assessed by determining cellular Rh123-associated MFI at time points mentioned above, respectively.

Cytotoxicity assay

The ability of CJY to potentiate doxorubicin cytotoxicity was evaluated in K562/DOX cells and K562 cells by the conversion of MTT to a purple formazan precipitate as described by Hansen et al (1989). Cells were seeded into 96-well plates at 5×10^4/well. Various concentrations of doxorubicin and CJY were subsequently added and incubated for 48 h. IC50 values for doxorubicin (concentration resulting in 50% inhibition of cell growth) were calculated from plotted results using untreated cells as 100%. The resistance factor (RF) values, as potency of reversal, were obtained from fitting the data to RF=IC50 of cytotoxic drug alone/IC50 of cytotoxic drug in the presence of modulator.

Intrinsic cytotoxicity assay

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

Flow cytometric apoptosis assay and cell cycle analysis

The K562/DOX cells at a density of 5×10^5 mL^{-1} in exponential growth were exposed to 10 μM doxorubicin in the presence or absence of various concentrations of CJY at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. The cells were then collected, fixed with 70% ethanol (–20°C) and stored at –20°C for 24 h. The internucleosomally fragmented DNA was removed from apoptotic cells by incubation in 0.2 M citrate-phosphate buffer (pH 7.8) containing 0.2 mg mL^{-1} RNase for 30 min. Propidium iodide 100 μg mL^{-1} was then added. The remaining DNA content was measured using FACScan flow cytometry (Becton Dickinson) (Gong et al 1994). Data acquisition and analysis were controlled by Cell Quest software.

Data analysis

All data were presented as mean ± s.d. and analysed using analysis of variance followed by q test.

Results

Effect on the uptake and intracellular accumulation of Rh123

K562/DOX cells and the parental K562 cells were used to study the effects of CJY on P-gp function by determining intracellular Rh123-associated MFI. As shown in Figure 2, in

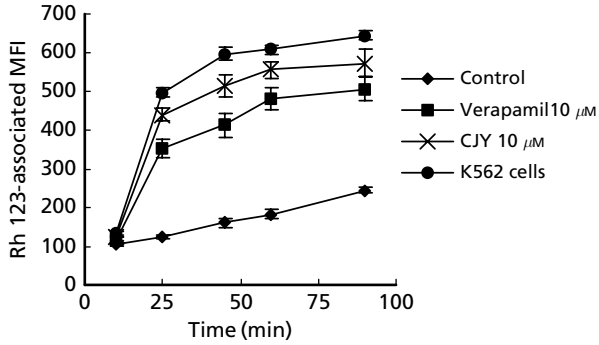

Figure 2 Effect of CJY on the uptake of Rh123 in K562 cells and K562/DOX cells. K562 cells and K562/DOX cells were incubated with medium containing 5 μM Rh123 in the presence or absence of 10 μM CJY at 37°C for 10, 25, 45, 60 and 90 min, respectively. The Rh123-associated MFI was measured as described in "Materials and Methods". Each point represents the mean ± s.d. from four experiments.

the uptake study, after K562 cells were incubated with Rh123, the MFI increased in a time-dependent manner over 90 min. After the K562/DOX cells were incubated in the presence of CJY, it accumulated Rh123 in a manner similar to that of untreated K562 cells. In contrast, untreated K562/DOX cells accumulated the substrate at lower degree over this same time period. The rate constant (K) for the uptake in CJY-treated K562/DOX cells was 0.067 ± 0.0031, higher than in control cells (0.032 ± 0.0012) and in verapamil-treated K562/DOX cells (0.048 ± 0.0034). CJY enhanced Rh123 accumulation in a concentration-dependent manner (Table 1), no such increase in MFI was observed in CJY-treated K562 cells.

Inhibition of Rh123 efflux and persistence of modulatory activity

CJY 10 μM could inhibit the efflux of Rh123 from K562/DOX cells (Figure 3). The rate constant (K) for the efflux in CJY-treated K562/DOX cells was 0.0075 ± 0.00064, lower than in control cells (0.0244 ± 0.0017) and in verapamil-treated K562/DOX cells (0.0111 ± 0.0015). The intracellular Rh123-associated MFI at 10, 30, 60, 90 and 120 min in 2.5 μM CJY-treated cells was higher than in control and in 2.5 μM verapamil-treated cells, indicating that the modulatory activity remained even after 2.5 μM CJY was removed from the medium for 120 min.

Effect on doxorubicin cytotoxicity

CJY gave a significant reversal of resistance to doxorubicin in a concentration-dependent manner. The mean IC50 values of doxorubicin for 1, 2.5, 5 and 10 μM CJY-treated K562/DOX cells were 5.5 ± 0.27, 2.8 ± 0.09, 1.28 ± 0.06 and 0.58 ± 0.06 μM, respectively, significantly lower than 19.8 ± 0.25 μM for control cells. The RF of 10 μM CJY was 34.1, comparable with 14.45 for 10 μM verapamil. No such activity was found in K562 cells.

Intrinsic cytotoxicity

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

Effect on doxorubicin-induced apoptosis and cell cycle perturbations

The apoptosis rate in 10 μM doxorubicin-treated cells was approximately 5.6%; the fractions of the cells in the G1 phase and the G2/M phase were approximately 89% and 2.5%, respectively. The exposure of K562/DOX cells to 10 μM doxorubicin in the presence of various concentrations of CJY was associated with an enhancement of internucleosomal DNA fragment and the CJY elevated the doxorubicin-induced apoptosis in a concentration-dependent manner; a reduction of cells in the G1 phase and the accumulation of cells in the G2/M phase were also observed (shown in Figure 4). No apoptosis was found in the cells incubated with 10 μM CJY alone.

Table 1 Effect of CJY on the intracellular accumulation of Rh123 and doxorubicin in K562/DOX cells

Group	Rh123-associated MFI		Doxorubicin-associated MFI
	K562 cells	K562/DOX cells	K562/DOX cells
Control	627 ± 70	249 ± 30	200 ± 20
CJY 1 μM	638 ± 66	327 ± 41*	271 ± 41*
CJY 2.5 μM	634 ± 28	379 ± 34*	294 ± 35*
CJY 5 μM	633 ± 43	428 ± 29*	432 ± 26*
CJY 10 μM	634 ± 45	544 ± 19*†	540 ± 54*†
Verapamil 10 μM	631 ± 85	482 ± 36*	456 ± 36*

Each value represents the mean ± s.d. from four experiments. Significant differences from the control were determined by using analysis of variance followed by q test (*$P < 0.05$); significant differences from the verapamil were determined by using analysis of variance followed by q test (†$P < 0.05$).

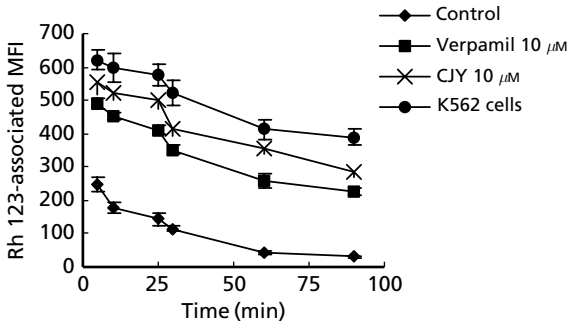

Figure 3 Effect of CJY on the efflux of Rh123 from the K562/DOX cells. Cells were first incubated with medium containing 5 μM Rh123 at 37°C for 90 min, washed three times with Rh123-free medium, and then incubated in the presence or absence of 10 μM CJY and verapamil at 37°C for 5, 10, 25, 30, 60 and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

Effect on intracellular accumulation of doxorubicin

As shown in Table 1, the intracellular doxorubicin associated-MFI was increased in the presence of various concentrations of CJY, which could explain the enhanced effects of CJY on doxorubicin cytotoxicity in K562/DOX cells.

Discussion

Classical first-generation chemosensitizers display side effects at doses required for clinical effectiveness. Thus, the search for chemosensitizers which have lower toxicity and higher selectivity has led much of the research interest to polyphenols with advantages of physiological safety (Kitagawa 2006). In this study, based on flow cytometric technology, we have investigated the effects of CJY, an isoflavone, on the P-gp function and the reversal of P-gp-mediated MDR in K562/DOX cells.

The fluorescent dye Rh123 has been used extensively to determine efflux rate from drug-resistant cell lines expressing P-gp (Green et al 2001). In our study, the changes of the cellular Rh123 in the uptake time-course was the result of passive inward diffusion and outward transport; however, the changes of the cellular Rh123 in the efflux time-course was the result of outward diffusion and transport, which were used to study the P-gp function in continual and dynamic methods. The results showed clearly that the rate constant for the uptake of Rh123 in CJY-treated K562/DOX cells was greater than that in verapamil-treated cells. In contrast, in the Rh123 efflux assay, the rate constant for the efflux of Rh123 in CJY-treated cells was reduced significantly. This indicated that CJY could inhibit P-gp-mediated transport of Rh123 and the inhibitory effect of CJY on P-gp function was more potent than that of verapamil. Previous studies reported modulatory effects of flavonoid derivatives and polymethoxyflavones on P-gp-mediated MDR in K562/DOX cells (Ferte 1999; Ikegawa et al 2000). These results revealed that CJY could

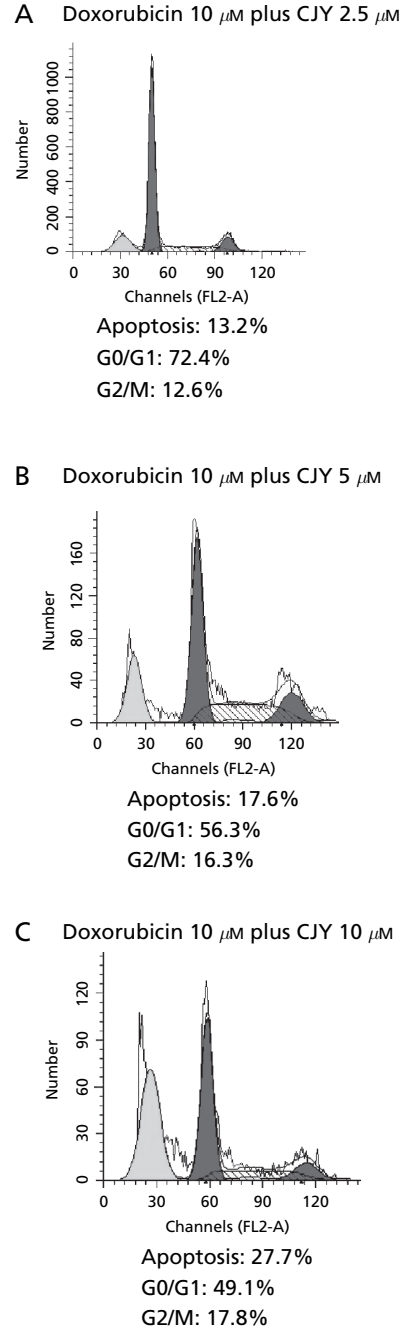

Figure 4 Effect of CJY on doxorubicin-induced apoptosis and cell cycle perturbations in K562/DOX cells.

reverse P-gp-mediated MDR at a dose of 1 μM, and the RF value for 10 μM CJY was approximately two-times higher than that for 10 μM verapamil, suggesting that CJY showed a more potent modulatory effect than the compounds mentioned above.

It was reported that P-gp protected leukaemia cells against doxorubicin-induced caspase-dependent cell death (Johnstone et al 1999). P-gp can mediate movement of sphingomyelin

out of the inner leaflet of the plasma membrane and decrease ceramide production by reducing the availability of sphingomyelin 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 increase in 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 (Sorenson & Eastman 1988; Briffod et al 1992). Irreversible drug-induced G2/M arrest is associated with DNA double-strand breaks and extensive chromosome damage. This study has shown a pronounced increase in the apoptosis rate and the fraction of cells in the G2/M phase, and a remarkable reduction in fraction of cells in the G1 phase after K562/DOX cells were exposed to 10 μM doxorubicin in the presence of CJY. Further more, our results showed that the intracellular MFI associated with doxorubicin was elevated after incubation with various concentrations of CJY, which could explain the enhanced effects of CJY on doxorubicin cytotoxicity. So far, this report is the only data on the pharmacodynamics of CJY. The effects of CJY on the expression of the MDR1 gene and the P-gp function in-vivo requires investigation.

Conclusion

CJY exhibited potent effects in-vitro in the reversal of P-gp-mediated MDR. The compound could become a candidate as an effective MDR reversing agent in cancer chemotherapy.

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