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Reversal effects of droloxifene on multidrug resistance in adriamycin-resistant K562 cell line

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KEY WORDS K562 cells; droloxifene; multiple drug resistance

ABSTRACT

AIM: To study the reversal effects of droloxifene (DRO) on multidrug resistance (MDR) in K562 cell line resistant to adriamycin (ADR). **METHODS**: K562 cell line resistant to ADR (K562/A02) and K562 cell line sensitive to ADR (K562) were treated with DRO. Using MTT assay, chemosensitivity to ADR in DRO-treated K562 cell lines was studied. Before and after the treatment with DRO 10 μmol/L, MDR1 and *GSTπ* gene expression were assayed by reverse transcription-polymerase chain reaction and immunocytochemistry assay. Flow cytometry was used to determine intracellular ADR concentration. **RESULTS**: DRO significantly reversed MDR in K562/A02 ($P < 0.01$). After treatment of DRO 20, 10, and 5 μmol/L, the chemosensitivity to ADR was increased to 14, 13, and 4 folds, respectively. The reversal activity of DRO was similar to that of verapamil (VRP). After treated with DRO 10 μmol/L, both MDR1 and *GSTπ* mRNA expression began to decline on the 2nd day, and significantly decreased on the 5th day ($P < 0.01$). The changes in P-gp and *GSTπ* protein expression were similar to that of their mRNA expression. Two hours after treatment of DRO 20, 10, and 5 μmol/L, intracellular ADR concentration in K562/A02 was increased to 2.9, 2.3, and 1.5 folds, respectively. However, DRO did not markedly increase ADR accumulation in K562. **CONCLUSION**: DRO had strong reversal effect on MDR in K562/A02, which was comparable to that of VRP, but the reversal effect was via different pathways.

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INTRODUCTION

Multidrug resistance (MDR) is a major reason for drug resistance in malignant tumor. Therefore, people have focused their attention on the study of the reversal agents of MDR recently. Although hundreds of compounds were found *in vitro* to be able to modulate MDR phenotype, their clinical application was limited due to high toxicity *in vivo*. Droloxifene (DRO), a derivative of tamoxifen (TAM) that is a classical reversal agent, has low toxicity. There is little liver lesion and estrogen-like activity, which is common in other antiestrogen compounds[1]. DRO might therefore be suitable for clinical use. DRO was firstly synthesized by Pharmaceutical College of Fudan University (China). The present work was aimed to study the reversal effects of DRO on MDR in K562/A02 and compare the effect with verapamil (VRP), a classical reversal agent.

MATERIALS AND METHODS

Drugs and reagents DRO was synthesized by Prof XIA Peng (Department of Organic Chemistry Synthesis, Pharmaceutical College of Fudan University, Shanghai). The product is pure with mp 166 - 167 ℃. VRP was purchased from Sigma Chemical Co.

Cell lines and cell culture K562 and K562/A02 cell lines maintained in RPMI-1640 were obtained from Shanghai Cancer Institute and incubated at 37 ℃ in 5 % CO_2-95 % air with a high humidity, and subcultured every 2 d. Medium for K562/A02 was further supplemented with ADR (Shanghai Hualian Pharmaceutical Co Ltd) 2 μmol/L. Prior to use in experiments, K562/A02 cells were cultured in drug-free medium for two weeks.

MTT assay of cytotoxic activity MTT assay

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was performed as follows[2]. Cell lines were seeded into 96-well plates at 1×10^4 viable cells per well. Blank well and control well were set up. DRO, VRP, and ADR were added alone or with combination in a final volume of 200 μL per well. After 48 h incubation, the medium was removed. Medium containing MTT 0.5 g/L [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] was added to each well in a volume of 200 μL and incubated for 4 h. Afterwards, the medium was removed and 180 μL of Me_2SO (Sigma) was added and incubated for 30 min at room temperature. A 96-well microtiter plate reader (Dynatech, Chantilly VA) was used to determine absorbance values at 570 nm. Mean value of each concentration ($n = 3$ wells) was obtained. Absorbance of untreated controls was taken as 100 % survival and the percentage of inhibition was calculated as follows: Cell survival rate (%) = (T-B)/(U-B) × 100 %, where T (treated) is absorbance of drug treated cell, U (untreated) is the absorbance of untreated cells, and B (blank) is the absorbance when neither the drug nor MTT was added. IC_{50} values were determined graphically from relative survival curves. Reversal fold = IC_{50} (ADR)/IC_{50} (ADR + reversal agents).

Semiquantitative RT-PCR analysis

DRO treatment Cells were treated with DRO 10 μmol/L. Cells were passaged as usual. After 2 and 5 d, DRO-containing medium was removed and cells were harvested.

Reverse transcription reaction Total RNA was extracted from treated cell lines or control samples using TRIzol system (Gibco/BRL), according to the manufacturer's instructions. The samples were stored at −70 ℃. The reverse transcription reaction was performed using Promega products. The cDNA was stored at −20 ℃ until required for analysis.

PCR Two sets of primer were used in all reactions to result in amplification of an endogenous control (383 bp of β-actin, primer sequences: 5′ GAA ATC GTG CGT GAC ATT AAG GAG AAG CT 3′, 5′ TCA GGA GGA GCA ATG ATC TTG A 3′) and a specific target gene of interest (157 bp of MDR1, primer sequences: 5′ GTT GCC ATT GAC TGA AAG AAC 3′, 5′ ACA GGA GAT AGG CTG GTT TGA 3′, a gift of Jian LIN, MD; Cancer Center/Institute of Cancer Research, Columbia University, USA; 270 bp of *GSTπ*, primer sequences: 5′ ATG CTG CTG GCA GAT CAG 3′, 5′ GTA GAT GAG GGA GAT GTA TTT GCA 3′[3]). PCR amplification was performed on 1 μL of RT product (25 μmol/L) incubated with 0.5 U of *Taq* polymerase (Promega) in a 25 μL reaction mixture containing 0.5 μL of deoxynucleoside triphosphate 10 mmol/L, 1.5 μL of $MgCl_2$ 25 mmoL, 2.5 μL of 10 × *Taq* polymease buffer from Promega, internal standard gene upstream and downstream primers 10 pmol/L to minimize tube-to-tube variations in amplification efficiency. PCR was performed in Gene Amp PCR Systems 9700 (PE, USA). For MDR1 and *GSTπ* genes, after an initial denaturation at 94 ℃ for 1.5 min, 30 cycles of PCR amplification were performed. Each cycle consisting of denaturing at 94 ℃ for 1.5 min, annealing at 54 ℃ for 1 min, and extending at 72 ℃ for 3 min, followed by final step at 72 ℃ for 10 min. The amplified fragments were visualized by 2 % agarose gel electrophoresis and ethidium bromide (Sigma) staining 0.3 mg/L. Each band was analyzed on image analysis system IS1000 (Alpha Innotech). Specific gene expression level was determined semiquantitatively by calculating the ratio of density metric value from specific gene expression in relation to the internal standard (specific gene expression/β-actin expression).

Immunocytochemistry staining Treatment with DRO 10 μmol/L was performed as above (see RT-PCR). A smear was made from each cell sample. Cells were fixed in acetone for 10 min. Immunocytochemistry was performed with an avidin-biotin complex immuno-peroxidase method as described by Zhou[4] with some modifications. Monocolonal antibody against P-gp was purchased from Boehringer Mannheim Biochemica, and *GSTπ* obtained from Dako Coperation. In each assay, five categories of staining were observed according to Xu and Yang[5]. Immunocytochemistry assays standard was determined as following: grade 0, negative; grade 1-3, feeble positive; grade 4 or 5, weak positive; grade 6 or 7, moderate positive; grade more than 7, strong positive.

ADR accumulation Cell count was modulated to 5×10^8/L. Accumulation of ADR was monitored using a standard procedure by incubating K562/A02 cells for 2 h at 37 ℃ in the presence of ADR (4 μmol/L) alone or in combination with DRO (20, 10, and 5 μmol/L). Cells were then harvested and washed twice by cold PBS (0 ℃). Cells were placed in ice-water to block the reaction until analysis. In 30 min, fluorescence intensity of 1×10^7/L cells was determined by flow cytometry (FACSCalibar, BE Company, USA).

Statistical analysis The levels of statistical

significance were evaluated with data from at least three independent experiments using paired t-test. $P < 0.05$ was set as statistically significant.

RESULTS

Chemosensitivity in K562/A02 and K562 cell lines IC_{50} concentrations of ADR for K562/A02 and K562 cell lines were 8 μmol/L and 0.58 μmol/L, respectively. It means that K562/A02 was 14-fold resistant to the effects of ADR as compared with K562.

Chemosensitivity in DRO-treated cells Cytotoxicity was expressed as the percentage of growth inhibition compared with untreated control cells. MTT assay showed that DRO 20 μmol/L or VRP showed no toxicity to the two cell lines. After treated with DRO 10 μmol/L over 10 d, K562/A02 cell growth rate was still normal compared with control by Trypan blue staining. The results showed that DRO 20, 10, and 5 μmol/L significantly increased the sensitivity of K562/A02 to ADR, and reversal fold was 14, 13, and 4, respectively. Reversal activity of DRO 20, 10, and 5 μmol/L was similar to that of the classical reversal agent VRP. In K562 cells either DRO or VRP did not significantly increase chemosensitivity to ADR (Tab 1, Fig 1).

MDR1 and GSTπ expression in DRO-treated cells RNA from untreated cells served as control. In K562/A02 cells, MDR1 and *GSTπ* gene expression levels were high. In the control K562 cells, MDR1 genes showed negative results, while *GSTπ* gene expressed positively. The effects of DRO on MDR1 and *GSTπ* gene mRNA levels were evaluated by calculating the ratio of the resistant gene expression to β-actin expression by semiquantitative analysis (at least three independent DRO treatment experiments). After treatment with DRO 10 μmol/L, the MDR1 and *GSTπ* expression levels of K562/A02 cells began to decrease on the second day and significantly declined on the fifth day ($P < 0.05$ and $P < 0.01$ respectively) (Tab 2). The change in *GSTπ* mRNA expression in K562 cells is similar to that in K562/A02 cells.

P-gp and GSTπ protein expression K562/A02 cells had high expression levels of P-gp and GSTπ protein. K562 cells only expressed a high level of GSTπ, and P-gp expression was undetectable in K562. After treatment with DRO, P-gp expression levels in K562/A02 cells gradually declined, the lowest level being on the fifth day, and GSTπ expression in K562/A02 and K562 cells also gradually declined. The effects of DRO on MDR-related protein expression were similar to that of DRO on mRNA expression.

ADR accumulation The accumulation of ADR in K562/A02 cells was much less than that in K562 cells. Both cell lines were treated with DRO 20, 10, and 5 μmol/L. A marked increase of intracellular ADR accumulation was found in K562/A02 cells and the increase was 2.9, 2.3, and 1.5 times, respectively. However, DRO did not markedly increase ADR accumulation in K562 cells.

DISCUSSION

TAM, an antiestrogen agent, can directly bind P-gp and inhibit P-gp function[6]. Because high doses of TAM will induce estrogen-like activity and liver lesion, ideal effective concentration *in vivo* is difficult to obtain. DRO is a second generation of antiestrogen agents and has low toxicity[1].

The present study demonstrated that DRO significantly increased the chemosensitivity of K562/A02 cell line to ADR in a concentration-dependent manner. The enhancement of chemosensitivity to ADR by DRO was similar to VRP, a classical reversal agent with a high reversal activity. The results indicated that DRO also had a strong reversal activity.

Tab 1. Reversal effects of DRO and VRP on drug-resistance in K562 and K562/A02 cell lines. IC_{50} of ADR was mesured by MTT assay. $n = 5$ independent experiments. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Dose/μmol·L^{-1}	K562		K562/A02	
	IC_{50}/μmol·L^{-1}	Reversal fold	IC_{50}/μmol·L^{-1}	Reversal fold
Control	0.58 ± 0.04	–	8.00 ± 0.12	–
DRO 20	0.35 ± 0.03	1.7	0.56 ± 0.23^c	14
10	0.45 ± 0.03	1.3	0.62 ± 0.08^c	13
5	0.59 ± 0.06	1.0	2.0 ± 0.6^c	4
VRP 20	0.34 ± 0.07	1.7	0.43 ± 0.06^c	19
10	0.47 ± 0.04	1.2	0.51 ± 0.10^c	16
5	0.54 ± 0.05	1.1	1.4 ± 0.3^c	6

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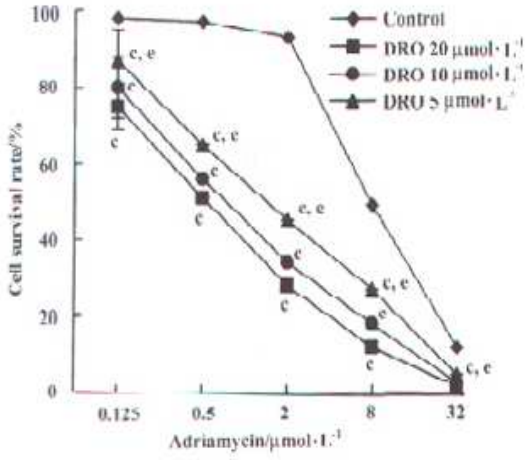

Fig 1. Reversal effects of DRO (20, 10, and 5 μmol/L) on drug resistance in K562/A02 cell line. $n = 5$ repeated times. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^eP < 0.05$ *vs* DRO 20 μmol/L group.

Tab 2. MDR1 and *GST*π gene expression in DRO-pretreated K560/A02 and K562 cells. $n = 3$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* before treatment.

Cell lines	Genes	Before treatment	After treatment (10 μmol·L⁻¹) 2 d	5 d
K562/A02	MDR1	1.75 ± 0.21	1.61 ± 0.15	0.84 ± 0.20^b
	*GST*π	3.4 ± 0.8	2.8 ± 0.8	0.70 ± 0.12^c
K562	*GST*π	2.8 ± 0.6	2.8 ± 0.9	0.66 ± 0.11^c

Fig 2. RT-PCR analysis of MDR1 and *GST*π expression in DRO-pretreated K562/A02, amplifying β-actin (as internal control) with MDR1 and *GST*π, respectively. Lane M: DNA marker. Cell line was treated with DRO before (MDR1: lane 1, 3; *GST*π: lane 5, 7; as control) and after 2 d (MDR1: lane 2; *GST*π: lane 6) and 5 d (MDR1: lane 4; *GST*π: lane 8).

Semiquantitative RT-PCR analysis is a quite sensitive and specific method[7,8]. In order to correct for tube-to-tube variations, we chose to use internal standards in this study as many other studies. The reference mRNA and target mRNA are usually processed together throughout the experiments, ie, from RNA extraction until PCR amplification. This tends to minimize difference of RNA yields between samples. It has been considered that over expression of MDR1/P-gp is a major mechanism of MDR in tumor cells[9]. A few studies have reported that some reversal agents can inhibit MDR1/P-gp expression. Stein *et al*[10] have found that cytokines such as interleukin 2 (IL-2), interferon γ (IFNγ) and tumor necrosis factor α (TNFα) were capable of decreasing MDR1 mRNA expression in colon carcinoma cell line HCT15 and HCT16. Liu *et al*[11] treated MCF7/ADR cells with Chinese herb Fructus Psoraleae for 48 h, and found that P-gp expression became negative. The present study demonstrated that DRO significantly inhibited MDR1/P gp expression on the fifth day. MDR mRNA expression level was low and P-gp expression almost ceased.

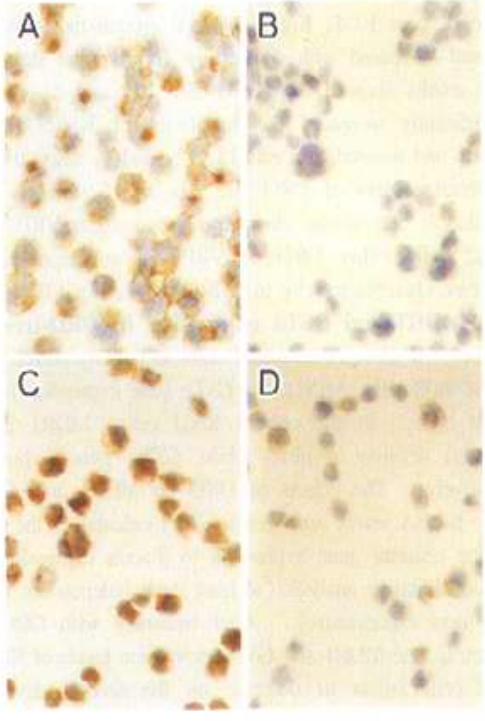

Fig 3. P-gp and GSTπ protein expression in DRO-pretreated K562/A02 cell line, detected with monoclonal antibody. DRO before (A: P-gp control; C: GSTπ control) and after 5 d (B, P-gp; D: GSTπ) treatment. × 400.

We also observed the expression of MDR related genes *GST*π. We found that DRO markedly decreased the level of *GST*π mRNA and protein expression, which was similar to MDR1/P-gp. The results suggest that DRO possibly act as a reversal agent of *GST*π. P-gp belongs to ATP-dependent transporter. It acts as a pump or hydrophobic vacuum cleaner, effectively increasing drug efflux and

decreasing drug influx. Many studies demonstrated that antiestrogen compounds directly bound P-gp, and blocked the function of pump-efflux. We studied the effect of DRO on intracellular ADR accumulation in K562/A02 by flow cytometry assays. ADR can emit fluorescence and the fluorescence intensity represents its accumulation[12]. After 2 h DRO treatment, the intracellular fluorescence intensity of ADR markedly enhanced, suggesting that DRO directly inhibit P-gp pump-efflux function as other antiestrogen compounds do.

In brief, our results indicated that DRO could markedly enhance the chemosensitivity to ADR in K562/A02 cells, down-regulate MDR1/P-gp and *GST*π expression levels, and markedly increase intracellular ADR accumulation. At present, although a variety of agents inhibit the function of P-gp *in vitro*, their clinical use is also limited due to the toxicity associated with the doses required to reverse MDR. Since DRO can be safely administered in a high dose, it may be a good candidate as a MDR reversal agent in clinics.

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屈洛昔芬对 K562 耐阿霉素细胞株耐药性的逆转作用

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关键词 K562 细胞; 屈洛昔芬; 多种抗药性

目的: 研究屈洛昔芬(DRO)对耐阿霉素(ADR)K562 细胞株(K562/A02)多药耐药性(MDR)的逆转作用及逆转机制. **方法**: 用 DRO 分别处理 K562/A02 和 K562 敏感株. MTT 法观察 DRO 影响 K562/A02 对 ADR 化学敏感性的变化. DRO 10 μmol/L 处理 K562/A02 前后, 通过 RT-PCR 和免疫细胞化学染色, 分析 MDR1、*GST*π 基因表达的变化, 采用流式细胞技术测定细胞内 ADR 浓度的变化. **结果**: DRO 显著逆转 K562/A02 的 MDR, 在 20、10 和 5 μmol/L 浓度时, 对 ADR 的化学敏感性分别增加到 14、13 和 4 倍, 逆转活性与维拉帕米相当. MDR1 和 *GST*π 的 mRNA 和蛋白表达在 DRO 10 μmol/L 处理后第 2 天开始下降, 第 5 天明显降低. 用 20、10 和 5 μmol/L 浓度的 DRO 处理两株细胞, K562/A02 细胞内 ADR 积累分别增加到 2.9、2.3 和 1.5 倍. 但 DRO 不能明显增加 K562 细胞内的 ADR 的浓度. **结论**: DRO 对 K562/A02 的 MDR 有较强的逆转活性, 逆转强度与维拉帕米相当, 其逆转机制有多种不同的途径.

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