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· 基础研究 ·

Effect and Mechanism of Isotetrandrine to Enhance Doxorubicin Sensitivity in Multidrug Resistance Tumor Cells

WANG Tian-xiao, LEI Kai-jian

Institute of Pharmacy, Henan University, Kaifeng 475004, *China*

异汉防己碱增强多药耐药肿瘤细胞对阿霉素的敏感性及其机制

王天晓,雷凯健

摘　要:目的　以 K562/DOX 和 MCF-7/DOX 细胞为对象,探讨异汉防己碱对化疗药物阿霉素(DOX)的增敏作用及其作用机制。**方法**　采用 MTT 法检测异汉防己碱的内在细胞毒性及其对阿霉素的增敏作用,并以 RF 值评价其增敏效果。应用流式细胞术(FCM)检测细胞膜上 P-gp 的表达以及细胞内 DOX 和罗丹明 123(Rh123)的蓄积量。**结果**　异汉防己碱在 10 μg/ml 的无毒剂量可明显增强 DOX 的细胞毒性。K562/DOX 和 MCF-7/DOX 细胞膜上 P-gp 均呈强阳性表达,但异汉防己碱对该 P-gp 表达水平无明显影响。异汉防己碱可使 K562/DOX 和 MCF-7/DOX 细胞内 DOX 和 Rh123 的荧光密度(FI)均明显增加,由此证明异汉防己碱可有效抑制 P-gp 的功能。**结论**　异汉防己碱可通过抑制 P-gp 的功能而增强阿霉素的敏感性,从而有效逆转肿瘤细胞的多药耐药性(MDR),它可能成为有效多药耐药逆转剂的候选药物。

关键词:异汉防己碱;阿霉素;肿瘤细胞;多药耐药性

Abstract: Objective　To explore the effect and mechanism of Isotetrandrine to enhance doxorubicin (DOX) sensitivity of K562/DOX and MCF-7/DOX cells. **Methods**　The activity of Isotetrandrine to enhance doxorubicin cytotoxicity was tested using MTT [3-(4, 5-dimethylthiazol)-2,5-diphenyltetrazolium bromide] assay and evaluated by the reversal fold (RF) values. The level of P-glycoprotein (P-gp) expression and intracellular accumulation of doxorubicin and rhodamine123 (Rh123) were assessed by flow cytometry (FCM). **Results**　The doxorubicin-induced cytotoxicity was significantly potentiated by isotetrandrine with the concentration of 10 μg/ml. P-gp was expressed in both K562/DOX cells and MCF-7/DOX cells, but the level of P-gp expression was not distinct difference at the absence or presence of isotetrandrine. The intracellular accumulation of DOX and Rh123 was increased in the presence of isotetrandrine, which indicated that the function of P-gp was effectively inhibited. **Conclusion**　Isotetrandrine exhibited potent effect in the reversal of tumor multidrug resistance (MDR) by inhibiting the function of P-gp *in vitro*, suggesting that it may become a candidate of effective MDR reversing agents in cancer chemotherapy.

Key words: Isotetrandrine; Doxorubicin; Tumor cells; Multidrug resistance introduction

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0　Introduction

Cancer multidrug resistance (MDR) is one of the major obstacles for the success of chemotherapy. It is related to a 170 kDa plasma membrane protein, P-glycoprotein (P-gp)[1]. P-gp functions as an ATP-dependent drugs transporter which unilaterally transports the intracellular drugs out of the cells, thereby reducing drug cytotoxicity. So chemotherapy drugs in conjunction with a P-gp inhibitor at the time of tumor treatment will be the effective way to overcome MDR. Accordingly, a variety of drugs have been reported as agents for overcoming MDR. However, side effects of many drugs make them incapable effectively applied in the clinic. Therefore, development of safe and effective MDR reversing agents is eagerly required. Isotetrandrine, a bisbenzylisoquinoline alkaloid ex-

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作者单位:475004 河南开封,河南大学药物研究所

作者简介:王天晓(1975-),女,博士,副教授,主要从事生物制药及肿瘤 MDR 的研究

tracted from Traditional Chinese drug Mahonia, is diastereoisomer of tetrandrine(TET)[2]. Previous reports have shown that TET could exhibit reversal effect on tumor MDR [3-5]. In this study, we were to investigate the reversal effect of Isotetrandrine on MDR in K562/DOX and MCF-7/DOX cells.

1 Materials and methods

1.1 Drugs and reagents

Isotetrandrine was extracted and prepared by our project group; Doxorubicin(DOX) was purchased from Jintairong Medicine Co. Ltd., Guangdong, China; 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide(MTT) and rhodamine 123 (Rh123) were purchased from Sigma Co., USA; Anti-human-P-gp mouse mAb and FITC-marked goat-anti-mouse IgG were purchased from Maixin Biotechnology Co. Ltd.,Fuzhou, China; Fetal calf serum and RPMI-1640 medium were purchased from Gibco, USA.

1.2 Cell lines and cell culture

Human leukemia (K562) cells and human breast cancer (MCF-7) cells and their DOX-resistant (K562/DOX and MCF-7/DOX) cells were purchased from Institute of haematology, Chinese Academy of Medical Sciences. They were cultured in RPMI-1640 medium with 10% fetal calf serum at 37℃ in a humidified 5% CO_2 atmosphere. K562/DOX and MCF-7/DOX cells were maintained in culture medium with 1.0 μg/ml DOX and incubated in DOX-free medium for 2 weeks before the planned experiment.

1.3 Drugs cytotoxicity assay

The intrinsic cytotoxicity of Isotetrandrine and the ability of it to potentiate DOX cytotoxicity in all kinds of cells were determined with MTT assay [6-8]. Cells were seeded into 96-well plates at 2×10^4/well. Various concentrations of DOX and isotetrandrine were subsequently added and incubated for 48 h. Then 5 μg/ml MTT was added and incubated for 4 h later, the culture fluid was eliminated and DMSO was added. After formazan in the cells was dissolved by DMSO, the absorbance at 570 nm was detected and inhibitor rates of cells were obtained. By regression analysis, IC_{50} which was the concentration of the drug causing 50% inhibition of cells growth was obtained. The reversal fold (RF), the ratio of IC_{50} of cytotoxic drug (DOX) alone and that of cytotoxic drug (DOX) in the presence of modulator (isotetrandrine), embodied the effect of Isotetrandrine to enhance doxorubicin cytotoxicity and reversal potency of Isotetrandrine.

1.4 Detection of expression level of P-gp

Anti-human-P-gp mouse mAb was added in all kinds of cells in logarithmic phase at the absence or presence of Isotetrandrine and incubated for 30 min at 4℃, then added FITC-marked goat-anti-mouse IgG and incubated for 30 min at 4℃ again, the expression of P-gp was determined by FCM.

1.5 Accumulation and efflux of Rh123 assay

Rh123 is the favorable and specific substrate of P-gp, so the accumulation and efflux of Rh123 assay is classical method of determining P-gp function. At the accumulation assay, cells (1×10^6/ml) in logarithmic phase were incubated with medium containing 2 μg/ml Rh123 in the presence or absence of 10 μg/ml Isotetrandrine at 37℃ for 45 min. After two washes with ice-cold PBS, the intracellular Rh123-associated fluorecence intensity (FI) was measured with FCM. At the efflux assay, cells (1×10^6/ml) in logarithmic phase were first incubated with medium containing 6 μg/ml Rh123 at 37℃ for 45 min, washed three times with serum-free RPMI-1640 medium, and then incubated in the presence or absence of 10 μg/ml Isotetrandrine at 37℃ for 45 min. After two washes with ice-cold PBS, the intracellular Rh123-associated FI was measured with FCM.

1.6 Detection of intracellular DOX concentration

The K562/DOX and MCF-7/DOX cells (1×10^6/ml) in logarithmic phase were exposed to 10 μg/ml DOX in the presence or absence of 10 μg/ml Isotetrandrine for 90 min at 37℃. After two washes with ice-cold PBS, intracellular DOX-associated FI was measured with FCM.

1.7 Data analysis

All data were presented as $\bar{x} \pm s$ and analyzed using SPSS statistic software.

2 Results

2.1 Resistance of K562/DOX and MCF-7/DOX

cells to DOX

By MTT assay and regression analysis, IC_{50} of DOX to inhibit the reproduction of MCF-7 cells was 0.22 μg/ml and IC_{50} of DOX to MCF-7/DOX cells was 45.25 μg/ml, thereby the resistant fold was 205.68; IC_{50} of DOX to inhibit the reproduction of K562 cells was 1.48 μg/ml and IC_{50} of DOX to K562/DOX cells was 75.25 μg/ml, thereby the resistant fold was 50.84. Their resistant folds were over 20, so the K562/DOX and MCF-7/DOX cells had phenotype of MDR.

2.2 Intrinsic cytotoxicity of Isotetrandrine

As shown in Tab1, inhibitory rate of Isotetrandrine to cells growth took on dependence of concentrations. Along with the rise of Isotetrandrine concentration, inhibitory rates of cells were gradually increased. When concentration of Isotetrandrine was 20 μg/ml, Inhibitory rate of cells growth were about 10%. So the concentrations under 20 μg/ml were its noncytotoxic dose. We chose 10 μg/ml dose of Isotetrandrine to study the effect of it enhancing DOX sensitivity.

Tab 1 Inhibitory rate of Isotetrandrine on au kinds of cells($\bar{x} \pm s$)

表 1 异汉防己碱对各种细胞的抑制率($\bar{x} \pm s$)

Isotetrandrine concentration	Inhibitory rate of cells (%)			
	K562 cells	K562/DOX cells	MCF-7 cells	MCF-7/DOX cells
10 μg/ml	9.79±0.05	5.82±0.03	8.38±0.07	6.45±0.03
20 μg/ml	12.00±0.16	11.59±0.08	10.08±0.06	9.98±0.05
50 μg/ml	47.21±0.12	38.29±0.06	28.85±0.12	17.75±0.06
100 μg/ml	57.07±0.11	51.39±0.08	45.78±0.11	24.45±0.08

2.3 Effect of Isotetrandrine on DOX cytotoxicity

The activity of Isotetrandrine to enhance doxorubicin cytotoxicity in K562/DOX and MCF-7/DOX cells was shown in Tab 2. Isotetrandrine exhibited a distinct reversal effect at 10 μg/ml concentration. No such activity was found in K562 and MCF-7 cells.

2.4 Effect of Isotetrandrine on expression level of P-gp

In Isotetrandrine-untreated group, positive expression rates of P-gp in K562/DOX and MCF-7/DOX cells were (75.28±4.36)% and (73.25±3.15)% respectively. In Isotetrandrine-treated group, positive expression rates of P-gp in K562/DOX and MCF-7/DOX cells were (72.48±3.45)% and (71.55±3.37)% respectively, no distinctive difference with Isotetrandrine-untreated group ($P>0.05$). The results indicated that Isotetrandrine hardly affected expression level of P-gp.

Tab 2 Effect of isotetrandrine on DOX cytotoxicity in two kinds of cells($\bar{x} \pm s$)

表 2 异汉防己碱对阿霉素细胞毒性的影响($\bar{x} \pm s$)

Cells	IC_{50} (μg/ml)		Reversal Fold (RF)
	DOX	DOX+Isotetrandrine	
K562 cells	1.63±0.05	1.60±0.07	
K562/DOX cells	79.50±0.11	18.12±0.37	4.38
MCF-7 cells	0.22±0.08	0.20±0.03	
MCF-7/DOX cells	51.08±0.17	13.12±0.48	3.89

2.5 Effect of Isotetrandrine on P-gp function

No matter at Rh123 accumulation experiment or at Rh123 efflux experiment, intracellular Rh123 concentration (Rh123-associated FI) of DOX-resistant cells in Isotetrandrine-treated group was significantly higher than that in Isotetrandrine-untreated group($P<0.05$), although it did not reach the level of Rh123 concentration in DOX-sensitive cells, suggesting that Isotetrandrine could promote Rh123 accumulation and inhibit Rh123 efflux and therefore inhibiting P-gp function of "drug efflux bump", as shown in Tab 3.

Tab 3 Effect of Isotetrandrine on intracellular Rh123 concentration (Rh123-associated FI) ($\bar{x} \pm s$)

表 3 异汉防己碱对细胞内 Rh123 浓度的影响($\bar{x} \pm s$)

Groups	Rh123-associated FI	
	Accumulation experiment	Efflux experiment
K562 cells	98.45±1.08	98.15±1.03
K562/DOX cells (ITD-untreated)	28.21±2.49	27.09±2.20
K562/DOX cells(ITD-treated)	81.73±2.17[a]	80.85±2.07[a]
MCF-7 cells	99.41±0.45	98.65±0.72
MCF-7/DOX cells(ITD-untreated)	26.09±0.71	25.42±0.82
MCF-7/DOX cells(ITD-treated)	86.03±0.72[a]	66.58±0.34[a]

a: significantly different from Isotetrandrine-untreated group ($P<0.05$)

2.6 Effect of Isotetrandrine on intracellular accumulation of DOX

At the absence of Isotetrandrine, intracellular DOX-associated FI in K562/DOX and MCF-7/DOX cells was (30.47±0.25) and (33.25±0.12) re-

spectively. At the presence of 10 μg/ml Isotetrandrine, intracellular DOX-associated FI in K562/DOX and MCF-7/DOX cells was rised to (65.48 ± 0.47) and (65.38 ± 0.35) respectively. This also explained why Isotetrandrine enhanced DOX cytotoxicity in DOX-resistant cells.

3 Discussion

MDR is a common problem in cancer chemotherapy. It is a phenomenon that tumor cells resistant to a kind of anticancer drug were also resistant to a variety of in functionally and(or) structurally unrelated anticancer agents. MDR is related to the actions of one or more membrane transport proteins. Among them, P-gp that promote the expulsion of anticancer drugs is classical MDR mechanism. A number of studies have tried to find MDR modulators which increase anticancer drug accumulation in cancer cells [9-11]. But so far, the modulators have not been successfully applied in the clinic because of their toxicity and side effects. So the search for new drugs with low toxicity is in progress to satisfy an urgent need for clinical applications.

In this study, the ability of Isotetrandrine to enhance DOX cytotoxicity in DOX-resistant cells was discussed. The present results showed that Isotetrandrine of 10μg/ml (noncytotoxic dose) concentration could make IC_{50} of DOX to K562/DOX and MCF-7/DOX cells distinctly decreased. The intracellular DOX-associated FI was significantly increased in the presence of Isotetrandrine of 10 μg/ml concentration. The results suggested that Isotetrandrine could enhance DOX cytotoxicity, therefore effectively reversing MDR of K562/DOX and MCF-7/DOX cells.

In the present report, we further investigated the mechanism of Isotetrandrine. By FCM assay, Isotetrandrine hardly affected the expression level of P-gp in K562/DOX and MCF-7/DOX cells. So we further investigated the inhibitory effects of Isotetrandrine on P-gp function. The accumulation and efflux experiment of Rh123 is an important method of determining P-gp function in drug-resistant cell lines expressing P-gp[12], because the efflux of fluorescent dye Rh123 is P-gp-dependent. In this study, after cells were incubated with Rh123, the intracellular Rh123-associated FI in DOX-resistant cells was lower than that in DOX-sensitive cells and intracellular Rh123-associated FI in DOX-resistant cells was distinctly increased by Isotetrandrine. So Isotetrandrine could inhibit P-gp efflux function and therefore reversing MDR of DOX-resistant cells.

In a word, Isotetrandrine possessed potent reversal effect on tumor MDR. It may become a candidate of tumor MDR reversing agents.

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