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Function and mechanism of pyronaridine: a new inhibitor of P-glycoprotein-mediated multidrug resistance[1]

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KEY WORDS pyronaridine; multiple drug resistance; cultured tumor cells; glycoproteins

ABSTRACT

AIM: To study the effect and mechanism of pyronaridine (PND) on the reversal of multidrug resistance (MDR) in K562/A02 and MCF-7/ADR cell lines with *mdr*1[+]. **METHODS**: MTT assay was used to determine the cells growth inhibition after incubation for 72 h in the presence of doxorubicin (DOX) with or without PND. Intracellular accumulation of DOX was analyzed by spectrofluorometry. P-glycoprotein (P-gp) activity was investigated by measuring the extrusion of the cationic dye rhodamine 123 (Rh123). The apoptosis of cells and *mdr*1 gene expression were detected using flow cytometry and RT-PCR, respectively. **RESULTS**: PND slightly inhibited the growth of MDR human leukemia, breast cancer cells, and their parental cell lines. The IC_{50} of PND were 5.10 - 18.66 μmol/L depending on the kinds of cell lines. PND at low-toxic concentrations enhanced anti-proliferative effect of DOX on MDR cells and the apoptosis induced by DOX in a concentration-dependent manner. Intracellular accumulation of DOX and Rh123 in MDR cell lines increased after combination with PND. PND did not down-regulate *mdr*1 gene expression in MDR cell lines K562/A02 and MCF-7/ADR. **CONCLUSION**: As the third-generation P-gp inhibitor, PND significantly reversed MDR in MDR cell lines K562/A02 and MCF-7/ADR by inhibiting P-gp function.

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INTRODUCTION

The emergence of multidrug resistance (MDR) in the patients with tumors usually brings the failure of chemotherapy, thus the identification of reversal agents of MDR at non-toxic concentrations or non-MDR drugs should open new perspectives in cancer treatment. In the last 20 years, many studies have clearly showed that various agents were capable of reversing MDR. First-generation MDR inhibitors such as verapamil (VER), quinidine, and cyclosporine required high concentrations to reverse MDR and were associated with unacceptable toxicities[1,2]. Second-generation MDR inhibitors include PSC833, GF120918, VX-710, and so on. However, limitations to the use of these modulators are due to multiple and redundant cellular mechanisms of resistance, alterations in pharmacokinetics (PK) of cytotoxic agents, and clinical toxicities[3]. Recent investigations suggested that third-generation MDR inhibitors such as OC144-093 possessed more advantages such as lack of nonspecific cytotoxicity, P-gp specificity, relatively long duration of action with reversibility, good oral bioavailability, and lack of PK interaction with antitumor drugs[4]. Here, we reported a new inhibitor of P-gp with third-generation characteristics.

MATERIALS AND METHODS

Chemicals Pyronaridine, 2-methoxy-7-chloro-10-[3,5-bis(pyrolidinyl-1-methyl)-4-hydroxyphenyl-aminol] benzo[b]-1,5-naphthyridine was confered by Prof LIU De-Quan (Institute of Parasitic Disease, Chinese Academy of Preventive Medicine, Shanghai, China). Doxorubicin (DOX) was obtained from Wanle Pharmaceutical Co Ltd, Shenzhen. RNA isolated kit (TRIzol), DNA polymerase, rhodamine123 (Rh123), and 3-(4, 5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co, USA. CycleTEST™ plus DNA reagent was bought

from Becton Dickinson, USA. Fetal calf serum and RPMI-1640 were purchased from Gibco, USA. PND, DOX, and MTT were all dissolved in saline.

Cell lines and *in vitro* culture The human leukemic and breast cancer cell lines K562, MCF-7, ane their MDR sublines K562/A02[5], MCF-7/ADR were regularly cultured in our laboratory. All cells were incubated in RPMI-1640 medium at 37 ℃ in a humid atmosphere containing 5 % CO_2/95 % air.

Growth inhibition assay *in vitro*[6] The suspension with sensitive and MDR cells 1×10^8/L were inoculated into 96-well microtiter plates (Costar, Charlott, NC, USA). Twenty-four hours later, normal saline containing either test compound or solvent at the desired concentration was added to each well. After 72 h, 20 μL of MTT solution (5 g/L) was added to each culture well, and then density was read using a spectrophotometer Titertek Multiscan (SLT Labinstruments, Australia) at 570 nm.

Flow cytometry (FCM) analysis of apoptosis[7] K562/A02 and MCF-7/ADR cells were incubated for 72 h in a CO_2 incubator with DOX in the absence or presence of PND (1.1, 2.2, and 3.3 μmol/L). For the stained cells to be analyzed by FCM, 10 mg/L of PI was directly added to the culture medium for 30 min, then the percentages of intact, apoptotic, and dead cells were measured with an Epics Elite FCM (Coulter, USA).

Rhodamine123 efflux assay[8] K562, K562/A02 and MCF-7, MCF-7/ADR cells 5×10^8/L were stained with Rh123 10 μmol/L and PND 2.2 μmol/L for 30 min at 37 ℃ in RPMI-1640 medium. The cells were then rinsed twice with ice-cold phosphate buffer saline (PBS) and resuspended in the same solution to efflux the dye at 37 ℃. At the end, the samples were observed under the fluoro-microscope.

Drug-accumulation measurement *in vitro*[9] K562/A02, MCF-7/ADR cells, and their parental cells 5×10^8/L were incubated with DOX 2 μmol/L in the absence or presence of PND 2.20 μmol/L. At different intervals, cells were harvested. After washed twice in ice-cold PBS and resuspended in the same solution and frozen at −20 ℃ for 12 h, the cells were lysed using F550 ultrasonic breaker (Fisher Scientific, USA). The fluorescence of DOX in the lysates was determined with spectrofluorometer (UV-3000, Japan, excitation wavelength of 475 nm and emission wavelength of 590 nm). The protein content of each sample was correspondingly measured by Lorry's assay, and then DOX concentration was calculated in unit protein.

RT-PCR analysis of *mdr*1 gene expression Total cellular RNA was isolated from K562/A02 and MCF-7/ADR cells and their parental cells using TRIzol reagent. RT-PCR analysis for *mdr*1 and β-actin expression was performed as described[10] with the following modification. cDNA prepared from 20 μg of total cellular RNA was divided into two parts, each for PCR amplification with either β-actin or *mdr*1 specific primers. Subsequently, PCR products were simultaneously analyzed by electrophoresis on 2.0 % agarose gel stained with ethidium bromide.

Statistical analysis All the data were statistically analyzed by t-test. $P < 0.05$ were considered significant.

RESULTS

Nonspecific toxicity of PND From the comparison of drug resistance index (RI) between PND and DOX (Fig 1), it showed that PND was effective to both parental and MDR cells. While the RI were 74 ± 9 and 42 ± 3 for DOX in MDR cells K562/A02 and MCF-7/ADR, respectively, but for PND the index was near to 1. The IC_{50} (concentration of drug inhibiting cell growth by 50 % of untreated cells) values were from (5.1 ± 0.9) to (11.0 ± 2.2) μmol/L depending on different cell lines including both parental and MDR cells.

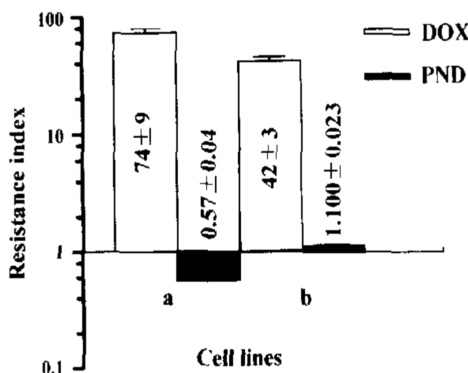

Fig 1. Resistance index of drug in two kinds of MDR cell lines. Comparison of RI between PND and DOX on (a) K562/A02, (b) MCF-7/ADR. RI was calculated as the ratio of the IC_{50} values in resistant sublines and parental cell lines. $n = 3$. $\bar{x} \pm s$.

Reversal of MDR *in vitro* by PND The

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cytotoxicity of PND alone on cells was tested to determine the range of concentration to use in reversal experiments. The IC_{50} values for PND were 5.1 ± 0.9, 11 ± 2.2 μmol/L in K562/A02, MCF-7/ADR cells and 9.1 ± 1.7, 9.9 ± 1.8 μmol/L in their parental cells, respectively. When used in combination with DOX, PND at a series of low-cytotoxic concentrations potentiated the antiproliferative effect of DOX on K562/A02 and MCF-7/ADR cells (Tab 1). Conversely, on sensitive cells K562 and MCF-7 this potentiating effect was only tested at the high concentration. In particular, the reversal activity of PND 4.4 μmol/L was more potent than that of VER 10 μmol/L.

Apoptosis induced by combination DOX with PND Results showed that the apoptotic percentage induced by DOX in MDR cell lines K562/A02 and MCF-7/ADR, compared with their parental cells K562 and MCF-7, was highly enhanced in the presence of PND. After the cells were treated with DOX 18.4 μmol/L combined with PND (1.1, 2.2, and 3.3 μmol/L) for 72 h, the apoptotic percentage was 12, 19, and 46 % in K562/A02 cells and was 9.4, 11, and 18 % in MCF-7/ADR cells, respectively. The apoptotic percentage in the group treated with DOX alone was only 2.6 % and 3.9 %, respectively.

Cellular DOX accumulation As shown in Fig 2, uptake of DOX was markedly diminished in MDR cells, compared to that of sensitive cells. The results suggested that PND was able to increase the concentration of DOX in both K562/A02 and MCF-7/ADR cells. Specifically, in K562/A02 cells, DOX accumulation almost achieved to the level of sensitive cells K562.

Modulation of rhodamine123 efflux The efflux of Rh123 in K562/K562/A02 and MCF-7/MCF-7/ADR cells was detected. After the cells were exposed to Rh123 10 μmol/L, K562/A02 and MCF-7/

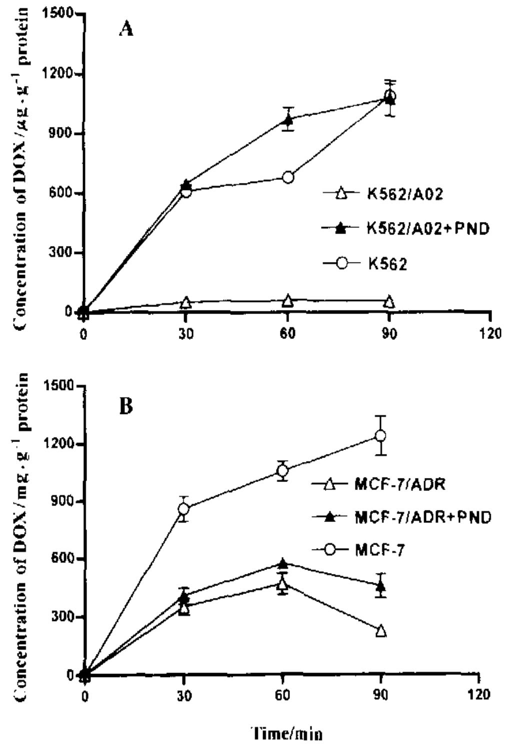

Fig 2. **(A): Effect of PND on the uptake of DOX in K562 and K562/A02 cells. (B): Effect of PND on the uptake of DOX in MCF-7 and MCF-7/ADR cells. DOX uptake by sensitive (white circle) and MDR cells in the absence (white triangle) or in the presence (black triangle) of PND 2.2 μmol/L in different intervals. $n=3$. $\bar{x} \pm s$.**

Tab 1. Specific reversal effect of PND on resistant cells, and the data in the table was IC_{50} values of DOX with or without PND or VER. In parenthesis is the enhanced index (EI), calculated as the ratio of IC_{50} in DOX alone to IC_{50} of combination with PND or VER. $n=3$ independent experiments. $x \pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ vs control. $^eP<0.05$, $^fP<0.01$ vs VER.

Cell line	Control	VER/μmol·L⁻¹ 10	PND/μmol·L⁻¹ 4.4	3.3	2.2	1.1
K562	3.1 ± 0.3	1.82 ± 0.08^b (1.52 ± 0.06)	1.86 ± 0.09^b (1.47 ± 0.07)	2.56 ± 0.04^b (1.16 ± 0.022)	2.4 ± 0.4^a (1.16 ± 0.20)	2.69 ± 0.11^a (1.02 ± 0.04)
K562/A02	231 ± 60	5.1 ± 2.0^c (49 ± 19)	1.3 ± 2.5^{ce} (237 ± 98)	3.2 ± 1.3^c (80 ± 31)	15 ± 6^c (18 ± 8)	89 ± 7^b (2.60 ± 0.22)
MCF-7	1.6 ± 1.0	1.23 ± 0.03^a (1.31 ± 0.03)	0.9 ± 0.5^b (2.2 ± 1.1)	1.21 ± 0.07^a (1.38 ± 0.08)	1.4 ± 0.3^a (1.2 ± 0.3)	1.5 ± 2.6^a (1.12 ± 0.21)
MCF-7/ADR	77.3 ± 2.5	8.9 ± 1.8^c (8.8 ± 1.8)	2.61 ± 0.15^{cf} (29.7 ± 1.7)	23 ± 13^c (4.3 ± 2.4)	35 ± 21^b (2.7 ± 1.4)	28 ± 6^c (2.8 ± 0.6)

ADR cells retained much less Rh123 than sensitive cells. Treating with PND for 30 min did not change the amount of Rh123 retained in sensitive cells K562 and MCF-7, but caused a significant increase in accumulation of rhodamine 123 in K562/A02 and MCF-7/ADR cells (Fig 3).

RT-PCR analysis of *mdr*1 gene The

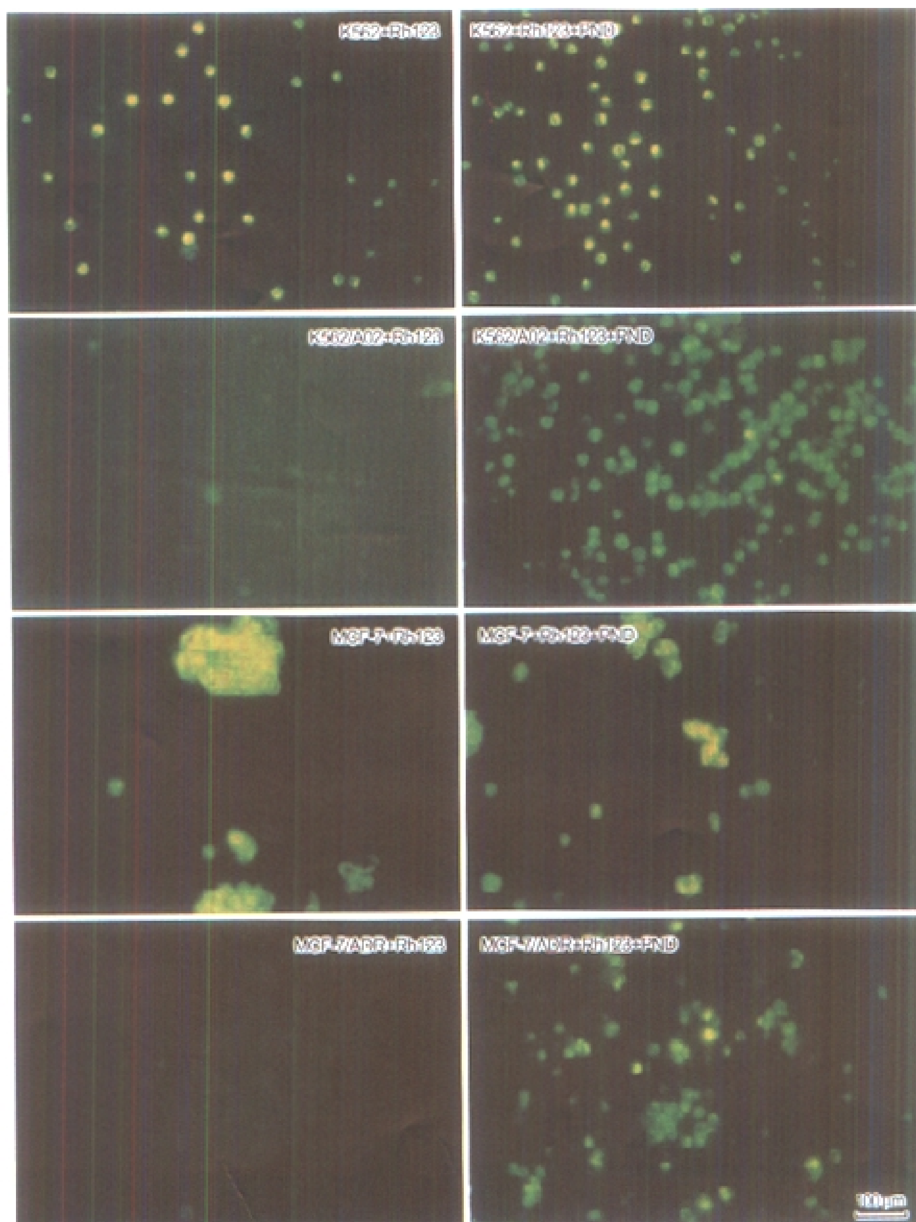

Fig 3. Comparison of efflux of rhodamine123 with or without 2.2 μmol/L PND in K562, MCF-7, and their MDR line K562/A02, MCF-7/ADR cells. × 100.

expression of *mdr*1 gene in K562/A02 and MCF-7/ADR cells was significantly higher than in sensitive cells, and PND did not down-regulate *mdr*1 expression in both K562/A02 and MCF-7/ADR cells (Fig 4). The same effect was observed by measuring P-glycoprotein (P-gp) encoded by *mdr*1 gene (data not shown).

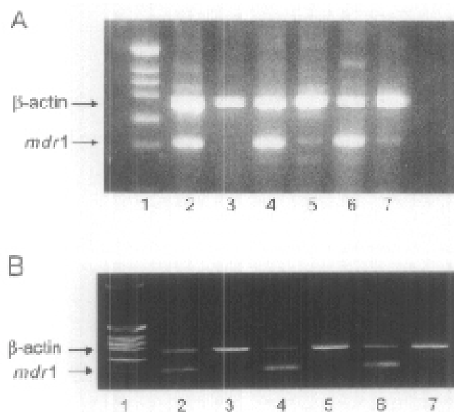

Fig 4. RT-PCR analysis of *mdr*1. The expression of *mdr*1 gene in K562, K562/A02 cells (A) and MCF-7, MCF-7/ADR cells (B) was observed by RT-PCR after exposed to PND (2.2, 3.3 μmol/L) on MDR cells (lane 4, 6) and sensitive cells (lane 5, 7). Lane 1, 2, 3 showed MW marker (pBR322/*Hinf* I), untreated MDR and sensitive cells.

DISCUSSION

Pyronaridine, a synthetic hydroxyanilino-benzonaphthyridine derivative as an antimalarial compound has been used in China for more than 20 years. The drug is characterized by the effectiveness for the multidrug-resistant including chloroquine-resistant infection. The previous study reported that PND caused mild side-effects and was generally well tolerated by patients in clinic[12-14].

At present, we first reported that PND exhibited specific reversal effect on MDR cells K562/A02 and MCF-7/ADR with RI at 74 ± 9 and 42 ± 3 for DOX. The results showed that VER at concentration of 10 μmol/L, the EI was 49 ± 19, whereas at PND 3.3 μmol/L, the EI was 80 ± 31 for K562/A02. At the same concentration of VER, the EI was 8.8 ± 1.8, whereas the EI of PND at 4.4 μmol/L was up to 29.7 ± 1.7 for MCF-7/ADR. Especially, it was worthy to note that PND not only completely or partially reversed P-gp-mediated MDR and showed higher reversal activity than that of the first-generation reversal agent VER at low toxicity concentration, but also was low-cytotoxic by itself at concentration up to 5 μmol/L in MDR cell lines. In addition, the result from flow cytometry analysis demonstrated that PND enhanced the cell apoptosis caused by DOX. Altogether, PND exhibited markedly specific MDR reversal activity.

In order to elucidate the mechanisms of PND reversing MDR, we had used both the DOX accumulation and Rh123 efflux assay as measures of P-gp efflux pump function. Our investigation suggested that in the K562/A02 and MCF-7/ADR P-gp-overexpressing cells, incubation with PND 2.2 μmol/L enhanced the accumulation of DOX which led to cellular concentrations high enough to kill the MDR cells. Furthermore, Rh123, a substrate of P-gp was tested to reflect the activity of P-gp in MDR cancer cells. PND has been found to block the efflux of Rh123 in K562/A02 and MCF-7/ADR cells almost completely, resulting in restoration of sensitivity of MDR cells to DOX. Additionally, RT-PCR showed that *mdr*1 gene expression was not down-regulated by PND. The present study showed that the mechanism of PND reversing tumor MDR was to block P-gp function as an efflux pump, resulting in increase cellular concentration of antitumor drug in MDR cells for killing them, instead of the loss of *mdr*1 expression.

A generally accepted mechanism of MDR agents is that reversal agents block hydrophobic binding of drugs to P-gp *via* competing against antitumor drugs for binding-site on P-gp[15]. Therefore, they should be more hydrophobic than the drugs. As a measure of hydrophobicity, the partition coefficient, log P is used. Comparing the log P between MDR reversal agents and antitumor drugs (Tab 2), higher log P values were found among MDR reversal agents. It was observed that their log P values were more than 4 in MDR reversal agents, while the highest log P in antitumor drugs was less than 3.3. The log P of PND was 5.46, while DOX and Rh123 were −1.34 and 1.20, respectively. As a result of higher hydrophobic, PND replaced DOX and Rh123 from binding site to P-gp competitively to block P-gp function as a drug efflux pump, so that intracellular concentration of DOX and Rh123 was increased, and restored sensitivity in MDR cells to the drugs. Taken together, we hypothesized that log P value should be generally more than 4 for MDR reversal agents except other structural requirements.

More recently, it was reported that altering the plasma pharmacokinetics (PK) of coadministratered anti-

Tab 2. Comparison of log P between MDR reversal agents and antitumor drugs, calculated from Chemdraw Pro Version 4.5 (Combridge Soft Corporation, USA) and reference 11.

MDR-reversal agent	log P	MDR-drug	log P	Non-MDR-drug	log P
The first generation		Daunorubicin	-0.64	Nitrogen mustard	-0.27
Verapamil	4.79	Doxorubicin	-1.34	Cyclophamide	-0.46
Tamoxifen	4.03	Rhodamine123	1.20	Melphalan	-0.52
Trifluoperazine	4.36	Mitoxantrone	-1.16	Thiotepa	0.53
Cyclosporin	6.00	Colchicine	0.05	Dacarbazine	-0.62
The third generation		Actinomycin D	3.21	BCNU	1.11
VX-710	4.18	Vinblastine	0.26	CCNU	1.96
CGP41215	4.38	Vincristine	2.57	Me-CCNu	2.28
LY335979	4.75	Vindesine	0.67	Mitomycin	-2.62
GF-120918	5.03	Camptothecin	0.68	Bleomycin	-2.38
XR9501	5.13	Topotecan	0.88	6-Mercaptopurine	0.52
XR9576	5.16	Irinotecan	2.64	5-Fu	-1.31
PND	5.36	Etoposid	1.12	Gemcitabicine	-0.46
S9788	5.92	Teniposid	2.75	Methotrexate	0.94
OC144-093	6.16	Paclitaxel	3.06	Ara-c	-2.04

tumor agents, increasing the accumulation of drugs by decreasing clearance of chemotherapy drugs were the blocks for using the first and second-generation P-gp inhibitors in clinic[16]. PK interaction study showed that PND did not produce a significant PK interaction with DOX *in vivo*. Accordingly, PND belongs to a third-generation inhibitor of P-gp.

In conclusion, PND, as a third-generation reversal agent, had potent reversal effect and low toxicity *in vitro* and *in vivo*, and lacked PK interaction with DOX. Although the mechanism of PND reversing MDR was not completely clear, the present study revealed that due to the high hydrophobicity, PND competitively bound to P-gp to replace the binding of drug coadministered, therefore, the intracellular drug accumulation and cytotoxic effect were restored. Our results indicated that as third generation P-gp inhibitor, PND should be used in clinic to overcome tumor MDR.

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咯萘啶逆转肿瘤多药耐药及其作用机制[1]

R96　A

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关键词　咯萘啶；多种抗药性；培养的肿瘤细胞；糖蛋白类

目的：利用 *mdr*1$^+$的人白血病和乳腺癌多药耐药(MDR)细胞系 K562/A02 和 MCF-7/ADR 研究咯萘啶(pyronaridine，PND)对 MDR 的逆转作用及其机制．**方法**：采用 MTT 法、荧光分光光度法、荧光显微镜法、流式细胞仪法和 RT-PCR 法分别测定 PND 单独或与阿霉素(DOX)合用，对肿瘤细胞的生长抑制、诱导凋亡、细胞内药物浓度、*mdr*1 基因表达的影响．**结果**：PND 对敏感及耐药细胞均具有生长抑制作用，半数抑制剂量(IC_{50})根据不同细胞在 5.10 – 18.66 μmol/L 之间；低毒剂量 PND 显著增强 DOX 对耐药细胞的细胞毒和诱导凋亡作用，且增加 DOX 在耐药细胞内的蓄积及减少罗丹明(Rh123)的外排．RT-PCR 结果显示，PND 对 *mdr*1 基因无下调作用．**结论**：PND 可作为第三代 P-糖蛋白(P-gp)抑制剂，通过下调 P-gp 药物外排泵功能而产生强大的逆转 MDR 效应．

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