R-dl-Verapamil downmodulates multidrug resistance of KBv200 cells to vincristine and doxorubicin

FANG Gang[1], YANG Yu-Long, LI Jie-Shou, ZHANG Zu-Xuan
(*Nanjing University School of Medicine, Nanjing 210093, China*)

KEY WORDS verapamil; vincristine; doxorubicin; multiple drug resistance; KB cells; toxicity tests

ABSTRACT

AIM: To study the attenuation of multidrug resistance (MDR) by *R*-*dl*-verapamil (*R*-Ver) and the acute animal toxicity of *R*-Ver, and to compare these results of *R*-Ver with the results of *dl*-verapamil (Ver). **METHODS**: Cytotoxicity was determined by tetrazolium (MTT) assay. Cellular accumulation of doxorubicin (Dox) was measured by fluorescence spectrophotometry. Acute animal toxicity was tested by ip drug administration in BALB/c mice. **RESULTS**: *R*-Ver attenuated MDR of KBv200 cells to vincristine (VCR) and Dox. This attenuation ability was dose-related, and was also dependent on drug exposure time. *R*-Ver 1.25 $\mu mol \cdot L^{-1}$ increased the sensitivity of KBv200 cells to VCR ($P < 0.01$) with a 24-h period of drug exposure. *R*-Ver downmodulated MDR and increased cellular Dox accumulation of KBv200 cells as effectively as Ver, but possessed lower acute toxicity in BALB/c mice. While LD_{50} of Ver was 60 (49 - 73) $mg \cdot kg^{-1}$, LD_{50} of *R*-Ver was 166 (137 - 202) $mg \cdot kg^{-1}$. **CONCLUSION**: *R*-Ver downmodulated the MDR to VCR and Dox at 1.25 $\mu mol \cdot L^{-1}$, and this effect on VCR can be realized with drug exposure duration of 24 h.

INTRODUCTION

Chemotherapy of cancers is often hindered by multidrug resistance (MDR) of tumor cells. A 170-kDa transmembrane glycoprotein (P170) enhances the efflux of drugs out of cells and reduces the sensitivity of tumor cells to a number of anticancer drugs, including vincristine (VCR) and doxorubicin (Dox), which was described by the term of MDR. The effects of P170 on drug efflux can be downmodulated by calcium channel-blocking agents. However, the cardiovascular effects of some calcium channel-blocking agents prevent them from reaching and maintaining *in vivo* the concentrations which are required to attenuate MDR *in vitro*.

dl-Verapamil (Ver) is a racemic mixture of equal amounts of two enantiomers. *R*-*dl*-Verapamil (*R*-Ver) (ie, *D*-isomer of Ver) has one-tenth of cardiovascular activity of the racemic mixture[1], but retains a MDR-modulating activity. However, some previous reports showed contradictory results about effectiveness of *R*-Ver and Ver on MDR attenuation[2,3]. In the present study, we observed the attenuating effect of *R*-Ver on MDR, and acute toxicity of *R*-Ver, which was prepared from a Chinese product of Ver.

MATERIALS AND METHODS

Materials The MDR cell line KBv200 and the parental sensitive cell line KB were generously provided by Institute of Materia Medica, Chinese Academy of Medical Sciences. Ver was a gift from Lianyungang Pharmaceutical Factory. *R*-Ver was prepared from Ver with a chemical dissolution method[4]. VCR and Dox were products of Chinese Pharmaceutical Factories.

Cell culture KB cells were cultured as adherent monolayers on the flasks in RPMI-1640 medium with 10 % fetal bovine serum at 37 ℃ in a humidified atmosphere of 5 % CO_2, KBv200 cells were grown well in RPMI-1640 medium containing fetal bovine serum (10 %) and VCR (200 $nmol \cdot L^{-1}$)[5]. They

---

[1] Correspondence to Assistant Researcher FANG Gang.
Phn 86-25-482-3773. Fax 86-25-330-2728.
E-mail yingfeng@nju.edu.cn
Received 1998-08-13 Accepted 1998-12-13

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Jul; 20 (7)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

were cultured in the absence of drug for 3 d before experiments.

Drug sensitivity assay Drug sensitivity was determined by a tetrazolium-based chemosensitivity assay as described previously[6]. Briefly, cells were plated out at a density of 3×10^3 per well in 96-well flat bottomed plates and allowed to attach and grow for 2 d. Drugs were added at the required concentration. After a 3-d incubation in continuous drug exposure, MTT (50 μL, 5 $g \cdot L^{-1}$) was added to each well. Plates were kept in the dark at 37 ℃ for 4 h, medium and MTT were removed, and MTT-formazan crystals were dissolved in Me_2SO (150 μL/well). Glycine buffer (25 μL/well pH 10.5) was added and the absorbance was measured at 570 nm in a multi-well plate reader (Model DG3022A). Cytotoxicity curves were made with percentage of cell growth obtained at 6 or 7 drug concentrtions. Percentage of cell growth = (absorbance value at tested well/absorbance value at control well) × 100 %. IC_{50} value was the drug concentration required to kill 50 % of cells. The potentiation fold was the ratio of the IC_{50} in the absence and presence of *R*-Ver or Ver. To evaluate cell growth and growth inhibition after drug exposure for a variety of time, cells were exposed to drug for 6, 10, 24, 48, and 72 h, respectively, and then fed with fresh medium and incubated until the end of 72 h. The adding of MTT and measuring of absorbance were done as mentioned above. Three wells were used for each drug concentration.

Cellular Dox accumulation KB cells and KBv200 cells were each seeded in 5 mL of medium at a density of 1.3×10^9 cells $\cdot L^{-1}$. Dox 10 $\mu mol \cdot L^{-1}$ was added in the absence or presence of 5 $\mu mol \cdot L^{-1}$ of *R*-Ver or Ver. Cells were incubated at 37 ℃ for 3 h. After centrifugation the cellular suspension was washed 3 times with cold PBS. The cells were resuspended in HCl 0.3 $mol \cdot L^{-1}$ in 60 % ethanol. Following centrifugation, the supernatant was removed and assayed spectrofluorometrically at λ_{ex} 470 nm and λ_{em} 585 nm[7,8]. Ver did not affect the absorbance or emission spectra of Dox. The accumulation fold of Dox was calculated by dividing the value in the presence of Ver or *R*-Ver by that in control wells.

Mouse toxicity test A dose-ranging experiment was carried out in BALB/c mice (♂ & ♀, weighing 18 – 22 g) for *R*-Ver and Ver. The mice were supplied by Experimental Animal Centre of Nanjing Military Unit, PLA (Grade Ⅱ, Certificate № 95047). Drugs were administered ip and mice were observed for 7 d. The doses ranged from 102 to 250 $mg \cdot kg^{-1}$ for *R*-Ver and from 38.4 to 93.8 $mg \cdot kg^{-1}$ for Ver. Ratio of dose interval was 0.8. LD_{50} values were derived from Bliss' weighted probit analysis.

Statistical analysis Results were expressed in terms of inhibitory rate (%) of cell proliferation: inhibitory rate (%) = (1 – percentage of cell growth) × 100 %. Controls were provided by cells plus culture medium. Comparisons of the results were made with *t*-test.

RESULTS

Cytotoxicity of *R*-Ver and Ver alone
Percentage of cell growth after exposure to *R*-Ver or Ver alone was more than 90 %, compared with 100 % of the control. *R*-Ver or Ver at 5 and 10 $\mu mol \cdot L^{-1}$ had no significant inhibitory effect on the growth of both KB and KBv200 cells. There was also no significant difference between the values of *R*-Ver and of Ver ($P > 0.05$) (Tab 1).

Tab 1. Percentage of cell growth after exposure to *R*-Ver or Ver for 3 d. $n = 3$ experiments. $\bar{x} \pm s$. [a]$P > 0.05$ *vs* control.

Drug/$\mu mol \cdot L^{-1}$		KB	KBv200
Control	0	100 ± 0	100 ± 0
R-Ver	5	100 ± 4[a]	97 ± 6[a]
	10	96 ± 6[a]	98 ± 5[a]
Ver	5	98 ± 6[a]	97 ± 5[a]
	10	95 ± 7[a]	95 ± 6[a]

Attenuation of MDR by *R*-Ver and Ver
The resistance fold of KBv200 cells to VCR was 174.7 times as much as that for KB cells, and to Dox was 9.1 times as much as that for KB cells. *R*-Ver and Ver increased the sensitivity of KBv200 cells to VCR and Dox, but not for KB cells. At equimolar concentration, *R*-Ver had the same attenuating effect as Ver (Tab 2, 3).

Dox accumulation After incubation with Dox

Tab 2. Potentiation by *R*-Ver and Ver of VCR cytotoxicity to KB cells and KBv200 cells as determined by MTT assay. $n = 3$ experiments. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control. $^dP > 0.05$ *vs* Ver.

Drug/μmol·L⁻¹		IC₅₀ of VCR/nmol·L⁻¹		Potentiation fold	
		KB	KBv200	KB	KBv200
Control	0	6.2±0.6	1 083±16		
R-Ver	1.25	6.5±0.5[a]	127±11[c]	0.95	8.5
	2.5	5.9±0.4[a]	76±8[c]	1.05	14.3
	5	6.7±0.8[a]	42±6[c,d]	0.93	25.8
Ver	5	6.4±0.6[a]	41±6[c]	0.97	26.4

Tab 3. Potentiation by *R*-Ver and Ver of Dox cytotoxicity to KB cells and KBv200 cells as determined by MTT assay. $n = 3$ experiments. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control. $^dP > 0.05$ *vs* Ver.

Drug/μmol·L⁻¹		IC₅₀ of Dox/nmol·L⁻¹		Potentiation fold	
		KB	KBv200	KB	KBv200
Control	0	6.7±0.7	61±8		
R-Ver	1.25	7.2±0.8[a]	23±3[c]	0.9	2.7
	2.5	6.1±0.8[a]	18±2[c]	1.1	3.4
	5	6.6±0.5[a]	13±2[cd]	1.0	4.7
Ver	5	6.7±0.9[a]	12±2[c]	1.0	5.1

10 μmol·L⁻¹, cellular Dox accumulation in KB cells was 4.2 times as much as that in KBv200 cells. *R*-Ver and Ver were equally effective in increasing Dox accumulation in KBv200 cells, but not in KB cells (Tab 4).

Tab 4. Effect of *R*-Ver and Ver on Dox accumulation. $n = 3$ experiments. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control. $^dP > 0.05$ *vs* Ver.

Drug/μmol·L⁻¹		Dox (nmol/10⁶ cells)		Accumulation fold	
		KB	KBv200	KB	KBv200
Control	0	5.13±0.19	1.22±0.07	1.0	1.0
R-Ver	5	5.15±0.26[a]	2.79±0.12[cd]	1.0	2.3
Ver	5	5.16±0.24[a]	2.83±0.18[c]	1.0	2.3

Effect of drug exposure time Exposed to VCR and *R*-Ver at three concentrations for 6 and 10 h, KBv200 cells changed into round shape and stoped from division. After removal of drug, most cells returned to spindle shape and began to proliferate. Cells were not killed by cooperation of *R*-Ver and VCR for 6 and 10 h. However, *R*-Ver at 1.25 μmol·L⁻¹ for 24 h had an effect on drug sensitivity, resulting in 58 % inhibitory rate, which was different from that of control wells ($P < 0.01$). While most of cells were dead and swollen, some of cells kept alive and possessed the ability to proliferate after exposed to VCR and *R*-Ver at 1.25 μmol·L⁻¹ for 72 h. Cells were totally killed at the wells containing VCR and *R*-Ver at 5 μmol·L⁻¹ for 72 h (Tab 5).

Tab 5. Inhibitory rate (%) of cell proliferation after exposure to *R*-Ver and VCR. $n = 3$ experiments. The final concentration of VCR was 200 nmol·L⁻¹ each well. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control (0 %).

Time of exposure/h	*R*-Ver exposure/μmol·L⁻¹		
	5	2.5	1.25
6	7±6[a]	5±5[a]	1±4[a]
10	16±7[b]	8±5[a]	4±4[a]
24	92±1[c]	81±2[c]	58±4[c]
48	99±0[c]	94±1[c]	69±3[c]
72	100±0[c]	97±1[c]	83±2[c]

Toxicity *R*-Ver had a lower toxicity than Ver ($P < 0.05$). For *R*-Ver and Ver deaths were observed on the first day after administration of drugs. LD_{50} (95 % confidence limits) of *R*-Ver and Ver in Balb/C mice were 166 (137 – 202) mg·kg⁻¹ and 60 (49 – 73) mg·kg⁻¹ respectively.

DISCUSSION

Ver is able to restore the sensitivity of MDR cell lines to Vinca alkaloids and anthracyclines *in vitro*, but its clinical application is limited by cardiovascular side-effects. *R*-Ver is a candidate for the clinical downmodulation of MDR, for it has less effects on the cardiovascular system[1], and can be safely maintained *in vivo* at concentrations of 2 – 3 μmol·L⁻¹[9]. However, one prior report implied that *R*-Ver was less effective in MDR attenuation than Ver[3]. Another paper indicated that they were equally effective[2]. Our experimental results showed that *R*-Ver and Ver were equally potent in attenuating MDR to VCR and Dox. This effect was dose-related. *R*-Ver 1.25 μmol·L⁻¹ increased the sensitivity of KBv200 cells to

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Jul; 20 (7)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

VCR and Dox.

Attenuation ability of *R*-Ver was also dependent on the time of drug exposure. Cells were not killed after exposed to VCR and *R*-Ver for 10 h. The inhibitory rate (%) of cell proliferation was increased to 58 % by cooperation of VCR and *R*-Ver (1.25 $\mu mol \cdot L^{-1}$) for 24 h. This phenomenon implied that the attenuating effect of *R*-Ver was realized by increasing intracellular concentration of cytotoxics and maintaining the higher concentration for a certain period of time. The duration of drug exposure in previous studies was always several days, even 7 d[10]. Obviously, it is rather difficult to maintain *R*-Ver in blood plasm at concentration of 2 $\mu mol \cdot L^{-1}$ for a longer duration. Our data indicate that *R*-Ver can be used for the clinical downmodulation of MDR to VCR and Dox, and *R*-Ver has an attenuating effect on MDR with 24 h of drug exposure time, which would facilitate the clinical use of *R*-Ver as a potential chemosensitizer.

REFERENCES

1 Keilhauer G, Emiling F, Raschack M, Gries J, Schlick E. The use of *R*-Ver is superior to racemic Ver in breaking multidrug resistance of malignant cells. Proc Am Assoc Cancer Res 1989; 30: 503-6.

2 Gruber A, Peterson C, Reizenstein P. *D*-Verapamil and *L*-verapamil are equally effective in increasing vincristine accumulation in leukemic cells *in vitro*. Int J Cancer 1988; 41: 224-6.

3 Eliason JF, Ramuz H, Kaufmann F. Human multi-drug-resistant cancer cells exhibit a high degree of selectivity for stereoisomers of verapamil and quinidine. Int J Cancer 1990; 46: 113-7.

4 Yang YL, Fang G, Song BS. Preparation of *R-dl*-verapamil hydrochloride.
Chin J Pharm 1997; 28: 537-8.

5 Zhang XH, Zhang FR, Ji XJ, Li ZY. Vincristine-resistant human KB cell line and mechanism of multidrug resistance. Acta Pharm Sin 1994; 29: 246-51.

6 Plumb JA, Wishart GC, Setanoians A, Morrison JG, Hamilton T, Bicknell SR, *et al*. Identification of a multidrug resistance modulator with clinical potential by analysis of synergistic activity *in vitro*, toxicity *in vivo* and growth delay in a solid human tumour xenograft. Biochem Pharmacol 1994; 47: 257-66.

7 Ford JM, Bruggemann EP, Pastan I, Gottesman MM, Hait WN. Cellular and biochemical characterization of thioxanthenes for reversal of multidrug resistance in human and murine cell lines. Cancer Res 1990; 50: 1748-56.

8 Fu LW, Pan QC, Huang WL, Yang XP. Reversal of tumor multidrug resistance by 2-phenyl-3-(3',5'-dimorpholinomethyl-4'-hydroxy)-benzoyl-indole (HWL-12). Acta Pharmacol Sin 1998; 19: 77-80.

9 Bissett D, Kerr DJ, Cassidy J, Meredith P, Traugott U, Kaye SB. Phase I and Pharmacokinetic study of *D*-verapamil and doxorubicin. Br J Cancer 1991; 64: 1168-71.

10 Damiani D, Michieli M, Michelutti A, Melli C, Cerno M, Baccarani M. *D*-verapamil downmodulates P170-associated resistance to doxorubicin, daunorubicin and idarubicin. Anticancer Drugs 1993; 4: 173-80.

647-650 17 R965.2

R-dl-型维拉帕米调低 KBv200 肿瘤细胞对长春新碱和多柔比星的多药抗药性

方 刚[1], 杨玉龙, 黎介寿, 张祖暄
(南京大学医学院, 南京 210093, 中国)

关键词 维拉帕米; 长春新碱; 多柔比星; 多种抗药性; KB 细胞; 毒性试验

目的: 研究 *R*-型维拉帕米对多药耐药的降低作用及其急性动物毒性, 并与消旋维拉帕米的相应结果作比较. **方法**: 细胞毒性的测定用 MTT 法; 细胞内多柔比星积累的测定用萤光分光光度计法. 急性毒性试验用 BALB/c 小鼠腹腔注射法. **结果**: *R*-型维拉帕米部分调低 KBv200 细胞对长春新碱和多柔比星的耐药性, 其调低效应与作用浓度和作用时间有关. 1.25 $\mu mol \cdot L^{-1}$的 *R*-型维拉帕米与长春新碱对细胞作用 24 h, 能够显著增加 KBv200 细胞对长春新碱的敏感性. 在增敏和增加细胞内多柔比星累积方面, *R*-型维拉帕米与消旋维拉帕米效果一样, 但 *R*-型维拉帕米的急性动物毒性明显低于消旋维拉帕米. **结论**: *R*-型维拉帕米 1.25 $\mu mol \cdot L^{-1}$提高耐药肿瘤细胞对长春新碱和多柔比星的敏感性, 增加对长春新碱敏感性所需的药物作用时间可缩短至 24 h.

(责任编辑 刘俊娥)