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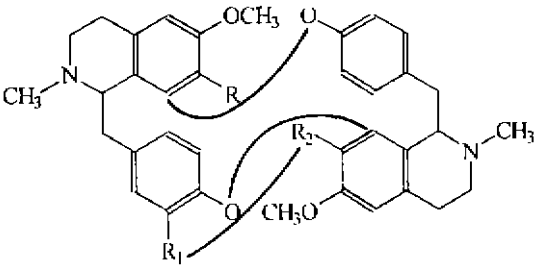
Modulation of multidrug resistance by three bisbenzyl-isoquinolines in comparison with verapamil[1]

TIAN Hui, PAN Qi-Chao[2]
(*Cancer Institute, Sun Yat-sen University of Medical Sciences, Guangzhou 510060, China*)

KEY WORDS cycleanine; insularine; insulanoline; isoquinolines; doxorubicin; vincristine; cultured tumor cells; multiple drug resistance

AIM: To compare cycleanine (Cyc), insularine (Insr), insulanoline (Insn) and verapamil (Ver) in modulation of multidrug resistance (MDR) *in vitro*. **METHODS**: The cytotoxic effect was determined by 3-[4, 5-dimethylthiazol-2-yl], 5-diphenyl tetarzolium bromide (MTT) assay. The intracellular doxorubincin (Dox) accumulation was assayed by spectrofluorometer. **RESULTS**: Cyc, Insr, Insn, and Ver showed significant activities in modulating Dox and vincristine resistances in acquired resistant MCF-7/Adr and KB_{v200} cell lines in a dose-dependent manner. Cyc, Insr, Insn, and Ver increased intracellular Dox accumulation in MCF-7/Adr cells. Cyc and Insr had greater activities than Ver in modulating MDR, while Insn had similar activity to that of Ver. **CONCLUSION**: MDR was modulated by Cyc, Insr, and Insn, due to the increase of intracellular Dox accumulation.

The recognition of multidrug resistance (MDR) as a major reason for failure of chemotherapy has stimulated efforts to identify modulator and improve existing chemotherapy. Recognition of the role of P-glycoprotein (P-gp) as a drug efflux pump has stimulated efforts to antagonize the function of this membrane glycoprotein[1,2]. Many compounds enhanced drug accumulation in MDR cells *in vitro*[3]. It is well known that verapamil (Ver), a calcium channel blocker, possesses significant effect on modulation of MDR[4]. However, its potent cardiovascular effect limited the clinical use of Ver to modulate the MDR. It is therefore necessary to search for novel effective modulator with lower toxicity. We have screened 11 principles in different structure with weaker calcium channel blocking effect than Ver from Chinese herbal medicine on their effect of modulation of MDR. We found that tetrandrine and dauricine, 2 bisbenzylisoquinoline (BBI) alkaloids, showed greater activity in reversing MDR than Ver, a standard MDR reversing agent[5]. There are some reports about the modulation of MDR by the other BBI alkaloids. Cepharanthine was found to overcome MDR by binding to P-gp as the structure similar to photoaffinity substrates for P-gp[6,7]. Thaliblastine, a dimeric aporphine benzylisoquinoline alkaloid, showed activity of overcoming MDR in Dox-resistant murine leukemic P388/R-84 cells, enhancing Dox retention and partially blocking the photolabeling of P-gp with [^{3}H]azidopine[8]. Therefore we studied the effect of cycleanine (Cyc), insularine (Insr), and insulanoline (Insn), another 3 BBI alkaloids with minor difference in structure all derived from *Cyclea racemosa* Oliv[9], on the modulation of MDR in comparison with Ver.



Cycleanine: $R = OCH_3$, $R_1 = H$, $R_2 = CH_3O$
Insularine: $R = OCH_3$, $R_1 = CH_2$, $R_2 = O$
Insulanoline: $R = OH$, $R_1 = CH_2$, $R_2 = O$

Bisbenzylisoquinoline compounds

MATERIALS AND METHODS

Drugs and reagents Cyc, Insr, and Insn were kindly supplied by Prof LAI Sheng, School of Pharmacy, West China University of Medical Sciences[6]. Ver was the gift from Ebewe Pharmaceutical Ltd, Austria. Vincristine (VCR) was made by Guangzhou 10th Pharmaceutical Factory. 3-[4, 5-dimethyl-

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[2] Correspondence to Prof PAN Qi-Chao.
Phn: 86-20-8777-8223, ext 2496. Fax: 86-20-8775-4506.
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thiazol-2-yl], 5-diphenyl tetarzolium bromide (MTT) was made by Jassen Chemica.

Cell lines and culture MCF-7 human breast cancer cell line and its MDR subclone MCF-7/Adr, selected by stepwise exposure of parental cells to increasing concentration of doxorubicin (Dox), were maintained in DMEM supplemented with 10 % fetal bovine serum in a humidified atmosphere of 5 % CO_2 + 95 % air. MCF-7/Adr cell line was approximately 100-fold more resistant to Dox than the parental cell line and was known to overexpress P-gp and maintained a stable MDR phenotype while grown in drug-free medium for a period of at least 3 months[10]. Both cell lines from NCI, USA, were the gifts from Prof LIU Xu-Yi, Beijing Cancer Institute.

KB_{v200} cell line was selected by stepwise exposure of parental sensitive KB cell to increasing concentrations of (VCR) and ethylmethane sulfonate mutagenesis. KB_{v200} cell line was approximately 100-fold more resistant to VCR than the parental KB cell line and was known to possess an increased *mdr*-1 mRNA expression and a decreased intracellular VCR accumulation[11]. Both cell lines were kindly supplied by Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing.

Cytotoxicity *in vitro*: MTT assay Cells were seeded in 96-well plates at the density of 10×10^3 MCF-7/Adr cells per well, 5×10^3 MCF-7 cells/well, 7×10^3 KB_{v200} and KB cells/well, and incubated in a humidified atmosphere of 5 % CO_2 + 95 % air for overnight, then drugs were added. For the control the mediam was added instead of the drug solution. After a 72-h incubation, MTT 20 μL (5 $g \cdot L^{-1}$ in PBS) was added for an additional 4-h incubation. IC_{50} was the concentration of drug that reduced the absorbance to 50 % of controls. Resistance modulating factor (RMF) = IC_{50} drug/IC_{50} drug + modulator.

Intracellular accumulation of Dox Drug accumulation studies were performed as previously described[5]. The presence of the 3 BBI alkaloids were shown not to affect the absorbance or emission spectra of Dox.

RESULTS

Modulation of Dox resistance *in vitro* in MCF-7/Adr cells Cyc, Insr, and Insn increased the cytotoxicity of Dox to P-gp positive MCF-7/Adr cell in a dose-dependent manner, but not to its parental MCF-7 cells, which do not overexpress P-gp. RMF of Cyc and Insr 5 $\mu mol \cdot L^{-1}$ (noncytotoxic dose) was 21.2 and 11.8 respectively, while that of Insn and Ver 5 $\mu mol \cdot L^{-1}$ was 5.3 and 6.5, respectively. Thus, Cyc and Insr were 3.3-fold and 1.8-fold more potent than Ver, while the potency of Insn was similar to that from Ver (Tab 1).

About Cyc 1.3 $\mu mol \cdot L^{-1}$, Insr 3.7 $\mu mol \cdot L^{-1}$, Insn 6.3 $\mu mol \cdot L^{-1}$, and Ver 9.1 $\mu mol \cdot L^{-1}$ were necessary to cause 10-fold sensitization of cytotoxicity (Fig 1).

Modulation of VCR resistance *in vitro* in KB_{v200} cells Cyc, Insr, and Insn increased the cytotoxicity of VCR to P-gp positive KB_{v200} cell in a dose-dependent manner, but not to its parental KB cells, which do not overexpress P-gp (Tab 2, Fig 2). As shown in the curve of dose-effect relationship (Fig 3), about Cyc 4.5 $\mu mol \cdot L^{-1}$, Insr 5.5 $\mu mol \cdot L^{-1}$ were necessary to cause a complete (100-fold) reversal of VCR resistance while dose of Insn and Ver needed were more than 10 $\mu mol \cdot L^{-1}$ (Tab 2).

Intracellular accumulation of Dox MCF-7/Adr cells accumulated 6.6-fold less Dox than sensitive MCF-7 cells. The addition of 10 $\mu mol \cdot L^{-1}$ of Cyc, Insr, Insn, and Ver caused 4.6-fold, 4.7-fold, 4.4-fold, and 4.2-fold ($P < 0.01$, compared with control

Tab 1. Effect of Cyc, Insr, Insn, and Ver on modulation of Dox resistance in MCF-7/Adr cells *in vitro*. **Resistance modulating factor = IC_{50} drug/IC_{50} drug + modulator; The modulator itself was at relative cytotoxic concentration (The inhibitory rate was more than 10 %). 95 % confidence limits in parentheses.** **$n = 3$ experiments (each 8 wells). [a]$P > 0.05$, [b]$P < 0.05$, [c]$P < 0.01$ *vs* control.**

Modulator/ $\mu mol \cdot L^{-1}$	1		2.5		5		10	
	IC_{50} of Dox $\mu mol \cdot L^{-1}$	RMF	IC_{50} of Dox $\mu mol \cdot L^{-1}$	RMF	IC_{50} of Dox $\mu mol \cdot L^{-1}$	RMF	IC_{50} of Dox $\mu mol \cdot L^{-1}$	RMF
Contol	11.02 (6.04 – 20.08)							
Cyc	3.28 (1.86 – 5.79)[b]	3.4	0.62 (0.37 – 2.68)[c]	17.7	0.52 (0.30 – 0.90)[c]	21.2	0.40 (0.26 – 0.62)[c]	27.5
Insr	3.57 (1.78 – 7.14)[b]	3.1	1.41 (0.80 – 2.49)[c]	7.8	0.94 (0.57 – 1.54)[c]	11.8	0.38 (0.26 – 0.56)[c]	28.9
Insn	4.62 (2.35 – 9.04)[a]	2.4	2.49 (1.52 – 4.07)[c]	4.4	2.06 (1.14 – 3.72)[c]	5.3	0.65 (0.41 – 1.04)[c]	16.9
Ver	4.54 (2.29 – 9.01)[a]	2.4	3.40 (1.99 – 5.81)[b]	3.2	1.70 (0.97 – 2.97)[c]	6.5	1.02 (0.58 – 1.79)[c]	10.8

Tab 2. Effects of Cyc, Insr, Insn, and Ver on modulation of VCR resistance in KB_{v200} cells *in vitro*. * Resistance modulating factor = IC_{50} drug/IC_{50} drug + modulator; The modulator itself was at relative cytotoxic concentration (The inhibitory rate was more than 10 %). 95 % confidence limits in parentheses. $n = 3$ experiments (each 8 wells). $^aP > 0.05$, $^cP < 0.01$ *vs* control.

Modulator/ $\mu mol \cdot L^{-1}$	1		2.5		5		10	
	IC_{50} of VCR $nmol \cdot L^{-1}$	*RMF	IC_{50} of VCR $nmol \cdot L^{-1}$	RMF	IC_{50} of VCR $nmol \cdot L^{-1}$	RMF	IC_{50} of VCR $nmol \cdot L^{-1}$	RMF
Contol	2 938 (1 671 – 5 164)							
Cyc	279 (157 – 495)^c	10.5	101 (61 – 168)^c	29.1	20 (11 – 36)^c	146.9	13 (7 – 24)^c	226.0
Insr	284 (160 – 501)^c	10.3	92 (52 – 163)^c	31.9	20 (11 – 36)^c	146.9	18 (10 – 32)^c	163.2
Insn	947 (524 – 1 709)^a	3.1	336 (188 – 601)^c	8.7	198 (108 – 363)^c	14.8	31 (18 – 55)^c	94.8
Ver	1 204 (667 – 2 177)^a	2.4	422 (237 – 751)^c	12.3	71 (39 – 130^c	41.3	37 (17 – 79)^c	79.4

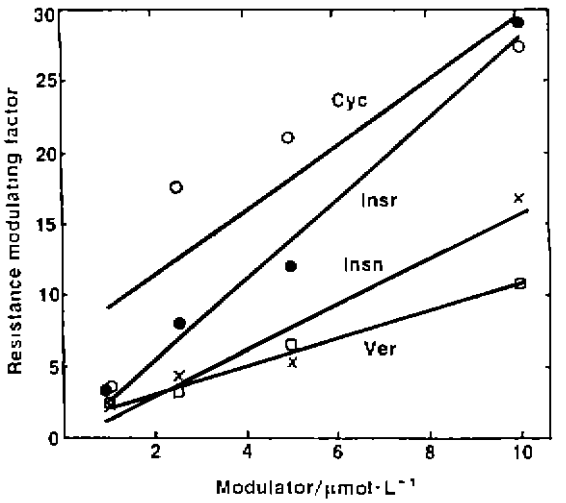

Fig 1. Potency of modulating activities for Cyc, Insr, Insn, and Ver in MCF-7/Adr cells.

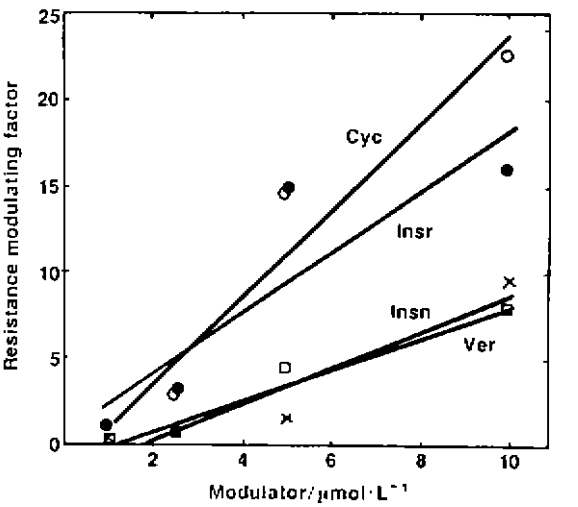

Fig 2. Potency of modulating activities for Cyc, Insr, Insn, and Ver in KB_{v200} cells.

group) increases in Dox accumulation in MCF-7/Adr cells, and slight increase of Dox accumulation in the sensitive MCF-7 cell line ($P > 0.05$, compared with control group) (Tab 3).

DISCUSSION

In this study, we first find that Cyc, Insr, and Insn significantly enhanced the cytotoxic effect of Dox or VCR to P-gp positive MCF- 7/Adr cell and KB_{v200} cell in a dose-dependent manner, but not to their parental sensitive MCF-7 cells and KB cells, which do not overexpress P-gp, and caused the increased intracellular Dox accumulation in MCF- 7/Adr cells, but not in their parental sensitive MCF- 7 cells. The activities of Cyc and Insr on modulation of MDR were

Tab 3. Effect of Cyc, Insr, Insn, and Ver on intracellular Dox accumulation in MCF-7/Adr and MCF-7 cells. *vs* control; †*vs* intracellular Dox accumulation in MCF-7/Adr cells. $n = 3$ experiments (each 8 wells), $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control.

Drug/ $\mu mol \cdot L^{-1}$		Cellular Dox ($nmol/10^6$ cells)		Fold increased*	
		MCF-7/Adr	MCF-7	MCF-7/ Adr	MCF-7
Control		160 ± 23	1 060 ± 145	1.0	1.0 (6.6†)
Cyc	10	745 ± 36^c	1 078 ± 71^a	4.6	1.0
Insr	10	752 ± 23^c	1 210 ± 108^a	4.7	1.1
Insn	10	705 ± 53^c	1 095 ± 95^a	4.4	1.0
Ver	10	670 ± 44^c	1 157 ± 130^a	4.2	1.1

greater than Ver, and that of Insn was similar to that of Ver. These suggest that the 3 BBI alkaloids may at least block the function of drug efflux of P-gp, and result in modulation of MDR.

Between 2 benzylisoquinoline units of these alkaloids, there are two diphenyl ether linkages with head to tail coupling. But in the cases of Insr and Insn, there is another one phenyl-benzyl ether linkage. The number of ether linkage seems to have no relation to activity of these BBI alkaloids on modulation of MDR. Insn differs from Insr and Cyc in nature of oxygenated substituents (OH substitution instead of OCH_3) in position C_7. This minor difference confers water solubility as well as appreciable difference in activity of enhancing Dox or VCR cytotoxicities from Insr and Cyc. Similar structure-activity relationship are also seen between tetrandrine and berbamine, dauricine and daurisoline. The data will be published elsewhere.

As the effect of the BBI alkaloids on Dox accumulation in MCF-7/Adr cells was not paralleled with that on cytotoxicity, the modulating mechanism of MDR by these compounds remains to be investigated further.

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三种双苄基异喹啉生物碱调节多药耐药性的作用及与维拉帕米的比较

田 晖, 潘启超
(中山医科大学肿瘤研究所, 广州 510060, 中国)

关键词 轮环藤碱; 海岛轮环藤碱; 海岛轮环藤酚碱; 异喹啉类; 阿霉素; 长春新碱; 培养的肿瘤细胞; 多种耐药性

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目的: 比较轮环藤碱(Cyc)、海岛轮环藤碱(Insr)和海岛轮环藤酚碱(Insn)与维拉帕米(Ver)体外调节多药耐药性(MDR)的作用. 方法: 细胞毒试验采用 MTT 法, 细胞内阿霉素(Dox)积累采用荧光分光光度法测定. 结果: Cyc, Insr, Insn 和 Ver 在 MDR 细胞系 MCF-7/Adr 和 KB_{v200} 能显著调节 Dox 和长春新碱的耐药性, 且其作用呈剂量依赖性. Cyc, Insr, Insn 和 Ver 均能增加 MCF-7/Adr 细胞内 Dox 的积累. Cyc 和 Insr 调节 MDR 作用明显优于 Ver, 而 Insn 的作用类似于 Ver. 结论: Cyc, Insr 和 Insn 能通过增加 MDR 细胞内 Dox 的积累而调节 MDR.