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Reversal of tumor multidrug resistance by 2-phenyl-3-(3′,5′-dimorpholinomethyl-4′-hydroxy)-benzoyl-indole (HWL-12)

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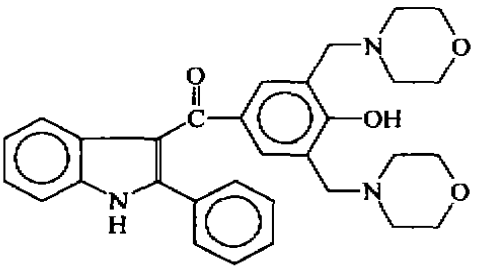
KEY WORDS multiple drug resistance; indoles; HWL-12; doxorubicin; Fura-2; calcium; cultured tumor cells; verapamil; egtazic acid

AIM: To explore the reversal of multidrug resistance (MDR) by indole derivative HWL-12. **METHODS**: Cytotoxicity was determined by tetrazolium (MTT) assay. The function of P-gp was examined by Fura 2-AM assay. Cellular accumulation of doxorubicin (Dox) was measured by fluorescence spectrophotometry. **RESULTS**: HWL-12 10 μmol · L^{-1} markedly increased Fura-2 accumulation and was 17.2-fold reversal of MDR in MCF-7/ADR cells. The cellular Dox accumulation in MDR cells was increased in the presence of HWL-12 on the MCF-7/ADR cells. No effect was observed for Dox accumulation in the presence of high Ca^{2+} (addition of $CaCl_2$) or low Ca^{2+} (addition of egtazic acid). **CONCLUSION**: HWL-12 has a potent MDR reversal action which was associated with the increase of cellular Dox accumulation in MDR cells and not related with calcium ion concentration.

Multidrug resistance (MDR) is characterized by a decreased sensitivity of tumor cells not only to the drug employed for chemotherapy but also to a broad spectrum of anticancer drugs with neither obvious structural homology nor common targets. The resistance is usually associated with the overexpression of a 170-kDa plasma membrane integral protein known as permeability glycoprotein (P-gp) encoded by *mdr*-1 gene. P-gp is an ATPase-dependent active outward transporter of the anticancer drugs and so intracellular drug accumulation is diminished in MDR cells[1].

Verapamil (Ver), a potent MDR chemosensitizer (CS) *in vitro* is usually used as a positive-control CS for research[2]. But few CS could be used in clinical treatment. So it is important to look for new CS in cancer chemotherapy.

2-Phenyl-3-(3′,5′-dimorpholinomethyl-4′-hydroxy)-benzoyl-indole (HWL-12) is a kind of aminomethyl-phenol indole compounds which was synthesized by Prof HUANG Wen-Long and exhibited anti-arrhythmic activity[3]. HWL-12 owns the common physical-chemical properties including lipid solubility, 2 planar aromatic rings, and tertiary nitrogen atom[4]. So the reversal of MDR by HWL-12 was studied.



HWL-12

MATERIALS AND METHODS

Materials The MDR cell lines MCF-7/ADR and the parental sensitive cell line MCF-7 were generously provided by Prof LIU Xu-Yi (the cells from National Cancer Institute, USA). Fura 2-AM and Fura-2 were purchased from Sigma Chemical Co. DMEM was purchased from Gibco BRL. Dox was purchased from Huaming Pharmaceutical Co, Shantou. Ver was generously provided by Ebewe Pharmaceutical Factory (Australia). HWL-12 was synthesized by Prof HUANG Wen-Long (China Pharmaceutical University).

Cell culture The MDR cell line MCF-7/ADR and the parental cell line MCF-7 (human breast carcinoma cell lines)[5] were grown as adherent monolayers on the flasks in DMEM with 10 % fetal bovine serum, benzylpenicillin (50 kU · L^{-1}), and streptomycin (50 mg · L^{-1}) at 37 ℃ in a humidified atmosphere of 5 % CO_2 + 95 % air.

Fura 2-AM fluorescence measurements Fura 2-AM was the substrate of the MDR transporter (P-gp)[6]. Fura 2-AM, which can be hydrolyzed to Fura-2 and AM in the cells, was transported out of the cells from the lipid phase of the plasma membrane, so the intracellular accumulation of Fura-2 was reduced in MDR cells. The intracellular accumulation of Fura-2

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was 4.1-fold in the sensitive cells MCF-7 as compared with that in the MDR cells MCF-7/ADR[17]. But the extrusion of Fura 2-AM could be blocked in the MDR cells by reversers, so the cellular accumulation of Fura-2 was increased in MDR cells by the addition of reversers. The more the amount of intracellular Fura-2 in MDR cells in the presence of CS was, the higher the activity of reversing MDR was. The comparison of the amount of intracellular Fura-2 in the absence or presence of CS reflected the reversal activity of MDR. Such the measurement of the accumulation of Fura-2 can be used as a method for studying P-gp function and screening MDR CS[17].

MTT cytotoxicity assay The cells were collected and resuspended at 4×10^5 cells·L^{-1}. 0.13 mL aliquots were seeded in 96-well multiplates. The CS and Dox were added after 24-h incubation. After 72 h, the cell growth inhibition was evaluated by the MTT method on triplicate assays[18]. IC_{50} values were calculated from cytotoxicity curves. The reversal fold was the ratio of the IC_{50} in the absence and presence of CS. The degree of resistance was calculated by dividing the IC_{50} for MCF-7 ADR cells by that for MCF-7 cells.

Cellular Dox accumulation MCF-7 cells and MCF-7 ADR cells were seeded in 1 mL of medium at a density of 4×10^5 cells·L^{-1}, respectively. Dox 10 μmol·L^{-1} was added in the absence or presence of CS 10 μmol·L^{-1}. 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·L^{-1} in 60 % ethanol. Following centrifugation, the supernatant was removed and assayed spectrofluorometrically at λ_{ex} 470 nm and λ_{em} 590 nm[19]. The CS did not affect the absorbance or emission spectra of Dox. To probe the relation of Dox accumulation with extracellular calcium, the Dox accumulation was measured in the presence of high Ca^{2+} (addition of $CaCl_2$) or low Ca^{2+} (addition of egtazic acid). The accumulation fold of Dox was calculated by dividing the value in the presence of HWL-12 by that without HWL-12

Statistical analysis Data were analyzed by t-test.

RESULTS

Cellular accumulation of Fura-2 The Fura-2 accumulation in MCF-7 cells was 4.13 times as much as that in MCF-7/ADR cells. The cellular accumulation of Fura-2 was markedly increased in MCF-7/ADR cells, but not in MCF-7 cells in the presence of HWL-12. The cellular accumulation of Fura-2 in MCF-7/ADR was more in the presence of HWL-12 than Ver at equimolar concentration (10 μmol·L^{-1}) (Tab 1).

Tab 1. The effects of HWL-12 and Ver on Fura-2 accumulation in MCF-7, MCF-7/ADR, respectively. $n = 3$ indenpendent experiments. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* Ver.

Drug	(μmol·L^{-1})	Fura-2 accumulation (pmol/10^6 cells) MCF-7 Cells	MCF-7/ADR Cells
Control	0	921 ± 7	223 ± 10
Verapamil	10	911 ± 25^a	661 ± 36^c
HWL-12	10	938 ± 26^a	815 ± 22^{cf}
	5	929 ± 32^a	678 ± 9^c
	2.5	927 ± 43^a	575 ± 16^c

Cytotoxicity The resistance fold of MCF-7/ADR to Dox was 66.6 times as much as that for MCF-7 cells. HWL-12 and Ver were not cytotoxic at 10 μmol·L^{-1} in MCF-7 cells and MCF-7/ADR cells. HWL-12 and Ver markedly increased the sensitivity of MCF-7/ADR to Dox, but not for MCF-7 cells. At equimolar concentration (10 μmol·L^{-1}), HWL-12 and Ver were 17.2- and 8.2-fold reversal of MDR, respectively (Tab 2).

Dox accumulation After incubation with Dox 10 μmol·L^{-1}, cellular Dox accumulation in MCF-7/ADR cells was 4.9 times as much as that in MCF-7 cells. HWL-12 10 μmol·L^{-1} and Ver 10 μmol·L^{-1}

Tab 2. Potentiation by HWL-12 and Ver of Dox cytotoxicity to MCF-7/ADR cells and MCF-7 cells as determined by MTT assay. The cellular growth inhibition rate by the reverser itself was less than 10 %. 95 % confidence limits in parentheses. $n = 3$ indenpendent experiments. $^aP > 0.05$, $^cP < 0.01$ *vs* control, $^fP < 0.01$ *vs* Ver.

Drug	(μmol·L^{-1})	IC_{50} of Dox (nmol·L^{-1}) MCF-7	MCF-7/ADR	Reversal fold MCF-7	MCF-7/ADR
Control	0	119 (73 – 166)	7 950 (5 375 – 10 525)		
Verapamil	10	103 (93 – 113)^a	974 (729 – 1219)^c	1.2	8.2
HWL-12	10	91 (50 – 132)^a	460 (433 – 487)^{cf}	1.3	17.2
	5	119 (107 – 131)^a	696 (647 – 745)^c	1.0	11.4
	2.5	119 (70 – 168)^a	1 060 (1 017 – 1 103)^c	1.0	7.5

increased cellular Dox accumulation by 3.2- and 2.9-fold in MCF-7/ADR cells, but not in MCF-7 cells. Cellular Dox accumulation was not altered by high or low extracellular Ca^{2+} (Tab 3).

Tab 3. Effect of HWL-12, Ver, $CaCl_2$, or egtazic acid on Dox accumulation. $n = 3$ **independent experiments,** $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* **control.** $^fP < 0.05$ *vs* **Ver.**

Drug ($\mu mol \cdot L^{-1}$)		Dox accumulation (nmol/10^6 cells)		Accumulation fold of Dox	
		MCF-7	MCF-7/ADR	MCF-7	MCF-7/ADR
Control	0	2.16 ± 0.06	0.43 ± 0.03	1.0	1.0
Ver	10	2.17 ± 0.09^a	1.26 ± 0.04^c	1.0	2.9
HWL-12	10	2.17 ± 0.05^a	1.38 ± 0.15^{cf}	1.0	3.2
Egtazic acid	2000	2.16 ± 0.11^a	0.45 ± 0.06^a	1.0	1.1
$CaCl_2$	2000	2.13 ± 0.06^a	0.44 ± 0.05^a	1.0	1.0

DISCUSSION

We established a new method for screening the CS of MDR with Fura-2/AM[7]. Actually, Fura-2/AM, a kind of fluorescent indicators for measuring intracellular calcium ion, is a hydrophobic compound and can rapidly diffuse through the cell membrane into the cells and is rapidly hydrolyzed into free acids (Fura-2) and acetoxymethyl ester (AM) in cytoplasm by non-specific esterases. Fura 2-AM was the substrate of the MDR transporter (P-gp), but Fura-2 was not the substrate of P-gp[9,10]. And the extrusion of Fura 2-AM was blocked in the MDR cells by MDR CS. Our experimental results showed that HWL-12 increased MDR cellular Fura-2 accumulation. This suggested that HWL-12 blocked the Fura 2-AM extrusion in MDR cells. The Fura-2 accumulation in MDR cells was more in the presence of HWL-12 than Ver, suggesting that HWL-12 was more potent in reversing MDR than Ver. HWL-12 10 $\mu mol \cdot L^{-1}$ caused a 17.2-fold reversal of MDR and was more effective than Ver which was only 8.2-fold reversal of MDR at equimolar concentration for cytotoxicity assay in MCF-7/ADR. In our opinion, HWL-12 was a potent MDR reversal agent and more potent than Ver in reversing MDR activity.

HWL-12, Ver increased the intracellular Dox accumulation up to 3.2- and 2.9-fold in MCF-7/ADR, respectively, but not in sensitive cells MCF-7. The results suggested that reversal mechanism of HWL-12 was related to the increase of cellular anticancer drug Dox accumulation in MDR cells. Cellular accumulation of Dox was not changed at high or low calcium ion concentration on MCF-7/ADR cells and MCF-7cells. And HWL-12 did not change cytosolic free calcium ion concentration (data was not shown). Dox accumulation was not enhanced either by increasing the concentration of cellular calcium with the calcium ionophore ionomycin nor by chelating the cytosolic free Ca^{2+} by the membrane permeable Ca^{2+}-buffering agents BAPTA or MAPTAM[11]. These suggested that calcium was not involved directly in drug transport processes and the level of calcium ion had no influence on drug accumulation.

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2-苯-3-(3′,5′-双吗啉甲基-4′-羟基)-苯甲酰吲哚(HWL-12)逆转肿瘤多药抗药性

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关键词 多种抗药性; 吲哚类; HWL-12; 阿霉素; Fura-2; 钙; 培养的肿瘤细胞; 维拉帕米; 依他酸

目的: 探讨吲哚衍生物 HWL-12 逆转 MDR 的作用及其作用机制. **方法**: 细胞毒的测定用 MTT 法; P-糖蛋白的测定用我们新近建立的 Fura 2-AM 法. 细胞内阿霉素(Dox)的积累测定用荧光分光光度计法. **结果**: HWL-12 能显著地增加 MDR 细胞内 Fura-2 的积累和逆转 MDR 17.2 倍. 在 HWL-12 的作用下, MDR 细胞内阿霉素的积累亦明显增加, 但在介质中高钙或低钙离子浓度对 Dox 积累均无影响. **结论**: HWL-12 具有较强的逆转作用, 它的逆转机制可能与增加 MDR 细胞内阿霉素的积累有关, 而与钙离子浓度无关.

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Effect of catecholamic acid on detoxication and distribution of $NiCl_2$ in mice and rats[1]

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KEY WORDS chelating agents; catecholamic acid; nickel; lethal dose 50; autoradiography; tissue distribution

AIM: To study the effect of catecholamic acid (CBMIDA) on detoxication of $NiCl_2$. **METHODS**: Mice and rats were injected sc or im CBMIDA immediately after ip $NiCl_2$. Each mouse was injected ip CBMIDA after iv $^{63}NiCl_2$ 185 kBq, and radioactivities of various tissues were measured with liquid scintillation counter at 24 h. The localization of ^{63}Ni was shown by the whole-body autoradiography. **RESULTS**: CBMIDA sc 0.5 – 1.5 $g \cdot kg^{-1}$ markedly reduced the mortality from acute poisoning of ip $NiCl_2$ 500 $mg \cdot kg^{-1}$. After ip $NiCl_2$ in mice, the LD_{50} was 82.7 $mg \cdot kg^{-1}$. Mice were injected sc CBMIDA 1.5 or 2.5 $g \cdot kg^{-1}$ after Ni poisoning, the LD_{50} of $NiCl_2$ were raised to 789 or 820 $mg \cdot kg^{-1}$, respectively. The LD_{50} of $NiCl_2$ was 39 $mg \cdot kg^{-1}$ in rat. If CBMIDA was injected im 0.5 $g \cdot kg^{-1}$ after ip $NiCl_2$, the LD_{50} was 332 $mg \cdot kg^{-1}$. CBMIDA 1.5 $g \cdot kg^{-1}$ im after iv $^{63}NiCl_2$, decreased the contents of ^{63}Ni in blood and lung of mice *vs* control mice at 24 h. The contents of ^{63}Ni in brain, heart, spleen, and kidney were similar to those of the control mice. The content of ^{63}Ni in bone was more than the control. The excretions of ^{63}Ni through urine and feces were not increased by CBMIDA at 24 h. The whole-body autoradiograpy showed that the radioactivity was highly localized in the kidney, lung, and Harder's gland. There was a moderate level of ^{63}Ni in the liver, bone, skin, and blood. A pronounced accumulation occurred in the bone. There was a marked reduction of ^{63}Ni in the lung, skin, liver, and blood after ip CBMIDA. **CONCLUSION**: The CBMIDA markedly raised the survival rate of nickel-poisoned mice and rats, and decreased ^{63}Ni levels in lung and blood.

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