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Reduction of doxorubicin resistance by tetrandrine and dauricine in harringtonine-resistant human leukemia (HL60) cells[1]

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KEY WORDS tetrandrine; dauricine; doxorubicin; harringtonines; drug resistance; cultured tumor cells; leukemia; drug synergism

AIM: To study whether tetrandrine (Tet) and dauricine (Dau) can reduce doxorubicin (Dox) resistance in the harringtonine (Har)-resistant human leukemia cells. **METHODS**: The drug cytotoxities were determined by counting cell numbers and colony formation. Cell cycle phases were assayed by flow cytometry, Dox contents were quantified by Dox fluorescence. **RESULTS**: The non-cytotoxic concentrations of Tet and Dau potentiated the growth-inhibitory actions of Dox in the Har-resistant HL60 cells. The colony formation effiencies were reduced from 60 % by Dox to 0.2 % by Tet + Dox and 9.2 % by Dau + Dox. Retardation of the G_2M phase cells was increased. But Tet and Dau did not potentiate Dox cytotoxities in the sensitive HL60 cells. Dox accumulation in the Har-resistant HL60 cells treated by Tet was increased. **CONCLUSION**: Dox resistance in the Har-resistant HL60 cells treated by Tet or Dau was reduced, due to the increase of Dox accumulation in the cells.

One of the mechanisms of multidrug resistance in tumor cells is overexpression of cell membrane glycoproteins, termed P-glycoprotein (PGP). PGP pumps antitumor drugs out of tumor cells, causing drug resistance[1,2]. Calcium antagonists and some calmodulin inhibitors such as verapamil, nifedepine, trifluorapine have effect on reversion of drug resistance, binding directly to PGP[3,4], but side effect of them is intolerable in clinical use. So searching for other potentiators to overcome drug resistance may be another avenue. Tetrandrine (Tet) effectively circumvented the resistance of Chinese hamster ovary cells to doxorubicin (Dox)[5]. Dauricine (Dau) is a bisbenzylisoquinoline alkaloid from *Stephaia tetrandra*[6]. In this paper we studied whether Tet and Dau could reduce Dox resistance in the harringtonine (Har)-resistant human leukemia 60 (HL60) cells.

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MATERIALS AND METHODS

Cells and drugs The Har-resistant HL60 cells and sensitive HL60 cells were routinely cultured in RPMI-1640 medium (Gibco), supplemented with 10 % - 12 % bovine serum at 37 ℃ in 95 % air + 5 % CO_2. The resources of Tet and Dox were described[5]. Dau, extracted by Prof PENG Si-Xun in China Pharmaceutical University, was dissolved in HCl 1 mol·L^{-1} and adjusted pH to 6.7 with RPMI-1640 medium.

Drug inhibition of cell growth About 5×10^7 cells·L^{-1} at exponential growth stage were seeded into 24-well plate, and treated by the drugs in triplicate for 72 h. The cell viability was detected by trypan blue exclusion and the cell number was counted with Coulter Counter (England).

Colony formation assay About 400 cells/well were seeded in 24-well plate in 0.25 mL of the mixture consisting of RPMI-1640 medium, the drug solution diluted with RPMI-1640 medium and 3 % agarose at the volume of 8:1:1. The final concentration of bovine serum was 25 %. The cells were cultured in a 5 % CO_2 incubator at 37 ℃ with 100 % humidity for 4 - 6 d. The colony numbers were counted under an inverse microscope.

Drug accumulation The accumulation of Dox in the HL60 cells was quantified by Dox fluorescence[5].

Flow cytometry 1×10^9 cells·L^{-1} at exponential growth stage were treated by the drugs for 24 h. The cells were collected and fixed with 3 mL of 70 % ethanol (-20 ℃). Before measurement, the ethanol solution was discarded by centrifugation and the cells were rinsed twice with PBS(NaCl 137, KCl 2.7, Na_2HPO_4 8.1, KH_2PO_4 1.3 mmol·L^{-1}). Having been digested with RNase A 10 mg·L^{-1} at 37 ℃ for 30 min, the cells were stained with propidium iodide (PI, Sigma) 50 mg·L^{-1} at 4 ℃ for at least 1 h. The fluorescences in the cells were measured with FACS 420 (Becton-Dickinson). The data were calculated by the com-

puter with DNA cell-cycle analysis software-Ver C (Becton-Dickinson).

RESULTS

Inhibition of cell growth The Har-resistant HL60 cells had cross-resistance to Dox. The viability of the sensitive HL60 cells treated with Dox 20 $\mu g \cdot L^{-1}$ was about 35 %, and that of the Har-resistant HL60 cells was about 29 % in Dox 4 $mg \cdot L^{-1}$. Having been treated with Dox 4, 2, 1 and 0.5 mg $\cdot L^{-1}$, the viability rates of the Har-resistant HL60 cells were 24 %, 56 %, 82 % and 87 %, and, in adding Tet 0.25 $mg \cdot L^{-1}$, reduced to 10 %, 10 %, 9 % and 19 %, respectively (Fig 1).

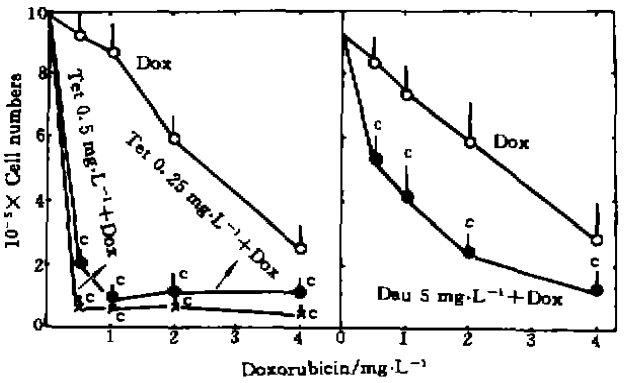

Fig 1. Growth-inhibitory actions of Har-resistant HL60 cells by drugs. $n = 9$, $\bar{x} \pm s$. $^cP < 0.01$ *vs* Dox alone.

Dox showed a stronger growth-inhibitory effect on the cells when the concentration of Tet was increased to 0.5 $mg \cdot L^{-1}$. The viability rate of the cells treated with Dox 0.5 $mg \cdot L^{-1}$ was 87 %, but reduced to 5 % in combination with Tet. These concentrations of Tet had little inhibitory effect on the Har-resistant HL60 cells. Dau enhanced the growth-inhibitory effect of Dox on the Har-resistant HL60 cells. The viability rates of the cells treatment with Dox were 33 %, 59 %, 80 %, and 91 %, but reduced to 14 %, 26 %, 44 %, and 55 %, respectively, in adding Dau 5 $mg \cdot L^{-1}$. The non-inhibitory concentrations of Tet and Dau slightly potentiated the Dox growth-inhibitory actions on the sensitive HL60 cells.

Inhibition of colony formation Tet 0.5 mg $\cdot L^{-1}$ and Dau 5 $mg \cdot L^{-1}$ had little effect on the colony formation of the Har-resistant HL60 cells, but reduced the colony formation efficiencies of Dox from 60 % to 0.2 % and 9.2 % in the groups of Tet + Dox and Dau + Dox, respectively. The potentiating effect of Tet was stronger than that of Dau. The colony formation efficiencies on the sensitive HL60 cells revealed little difference between Dox and Tet + Dox or Dau + Dox (Tab 1).

Tab 1. Inhibition of colony formations by doxorubicin, tetrandrine, and dauricine in harringtonine-resistant and sensitive HL60 cells. $n = 6$, $\bar{x} \pm s$.
$^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.
$^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* Dox.

Cell	Drugs	Colony numbers	Percent (%)
HL60/Har	Control	318 ± 20	100 ± 6
	Dox 1 $mg \cdot L^{-1}$	189 ± 18	60 ± 6^c
	Tet 0.5 $mg \cdot L^{-1}$	305 ± 22	96 ± 7^a
	Dau 5 $mg \cdot L^{-1}$	293 ± 26	93 ± 8^a
	Tet + Dox	1.0 ± 0.8	0.20 ± 0.25^f
	Dau + Dox	30 ± 8	9.2 ± 2.7^f
HL60	Control	316 ± 15	100.0 ± 2.9
	Dox 10 $\mu g \cdot L^{-1}$	128 ± 13	40 ± 3^c
	Tet 0.5 $mg \cdot L^{-1}$	318 ± 22	101 ± 6^a
	Dau 2.5 $mg \cdot L^{-1}$	293 ± 9	93 ± 4^b
	Tet + Dox	131 ± 8	41.5 ± 2.2^d
	Dau + Dox	109 ± 10	34 ± 3^e

Retardation of cell cycle progression In contrast with the control, the cell numbers in the G_2M phase slightly increased by Dox and the cell cycle phases were treated by Tet or Dau had no change. The cells treated by Tet + Dox or Dau + Dox were retardated in the G_2M phase, which was increased from 17 % by Dox to 49% by Tet + Dox or 30 % by Dau + Dox (Fig 2).

Increase of Dox accumulation The Dox accumulation in the Har-resistant HL60 cells treated by Tet was increased about 0.2 μg in contrast with Dox alone. Dau exhibited no effect on the Dox accumulation in the cells (Tab 2).

Tab 2. Effect of tetrandrine and dauricine on doxorubicin accumulation in harringtonine-resistant HL60 cells. $n = 9$, $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* Dox.

Group		Dox *accumulation*, $\mu g/5 \times 10^6$ cells
Dox	10 $mg \cdot L^{-1}$	0.68 ± 0.08
Dox + Tet	0.5 $mg \cdot L^{-1}$	0.89 ± 0.18^c
Dox + Dau	5 $mg \cdot L^{-1}$	0.67 ± 0.13^a

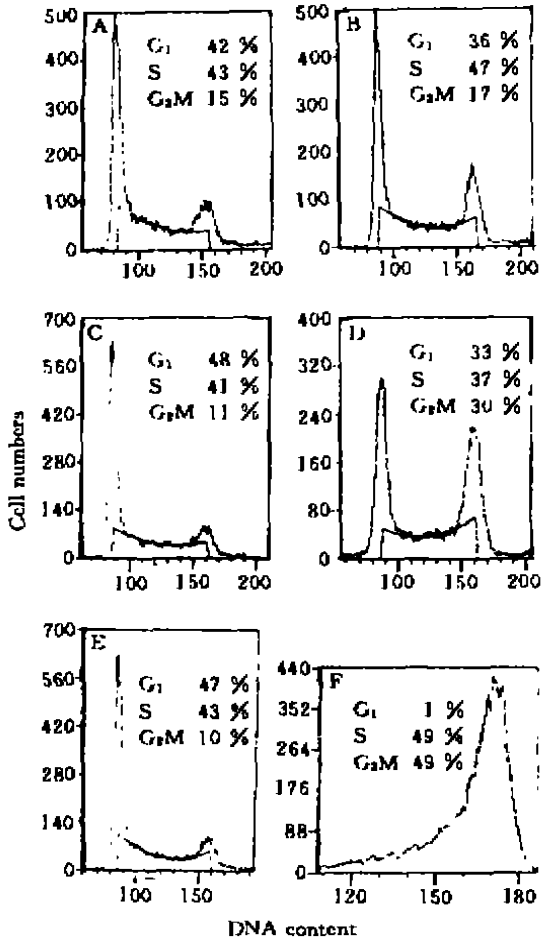

Fig 2. Cell cycle phases in Har-resistant HL60 cells treated with Dox + Tet or Dox + Dau. A) Har-resistant HL60 cell as control, B) Dox 1 mg·L^{-1}, C) Dau 5 mg·L^{-1}, D) Dau 5 mg·L^{-1} + Dox 1 mg·L^{-1}, E) Tet 0.5 mg·L^{-1}, F) Tet 0.5 mg·L^{-1} + Dox 1 mg·L^{-1}.

DISCUSSION

In this report we have confirmed that Tet and Dau could reduce the Dox resistance in the Har-resistant HL60 cell line, and that the mechanism of reversal of the drug resistance by tetrandrine was to increase the Dox accumulation in the resistant cells. However, the molecular mechanisms of reversal of multidrug resistance by tetrandrine remains to be further investigated. Verapamil, a calcium antagonist which effectively overcomes the drug resistance, can directly bind the PGP (170 kDa) in many drug-resistant cell lines[3,4]. We did not detect the over-expression of PGP (170 kDa) in the Har-resistant HL60 cell line. There was no detection of PGP (170 kDa) in the Dox-resistant HL60 cell[7]. Tet overcame the doxorubicin resistance in Chinese hamster resistant cell line[5], and so did in the human leukemia cells. Taken together, Tet may be regarded as an effective drug for overcoming multidrug resistance.

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粉防己碱和蝙蝠葛碱减低抗三尖杉酯碱的人白血病 HL60 细胞对阿霉素的抗性

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关键词 粉防己碱；蝙蝠葛碱；阿霉素；三尖杉酯碱类；抗药性；培养的肿瘤细胞；白血病；药物协同作用

目的：研究粉防己碱(Tet)和蝙蝠葛碱(Dau)能否减低抗三尖杉酯碱(Har)的人早幼粒白血病 HL60 抗性株对阿霉素(Dox)的抗性. **方法**：细胞计数法和克隆形成法测定药物毒性，流式细胞光度术分析细胞周期变化，荧光法测定 Dox 含量. **结果**：无细胞毒性的 Tet 和 Dau 明显地增强 Dox 对 HL60 抗性细胞的生长抑制作用，使克隆形成率从 Dox 单药的 60 % 分别降低到 0.2 %，9.2 %，使阻断在 G_2M 期的抗性细胞增多，但 Tet 和 Dau 不增强 Dox 对敏感的 HL60 细胞的毒性. Tet 使胞内 Dox 积聚增加. **结论**：Tet 和 Dau 减低抗 Har 的 HL60 细胞对 Dox 的抗性，其机制是增加 Dox 在细胞内积聚.