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Circumvention of tumor multidrug resistance by a new annonaceous acetogenin: atemoyacin-B[1]

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KEY WORDS annonaceous acetogenin; bullatacin; atemoyacin-B; multiple drug resistance; Fura-2; doxorubicin; vincristine; apoptosis; cultured tumor cells; phytogenic antineoplastic agents

ABSTRACT

AIM: To explore the effect of atemoyacin-B (Ate) on overcoming multidrug resistance (MDR). **METHODS**: Bullatacin (Bul) was used as a positive control. Cytotoxic effects of Bul and Ate were studied with cell culture of human MDR breast adenocarcinoma cells, MCF-7/Dox and human KB_{V200} cells, and their parental sensitive cell lines MCF-7 and KB. Cytotoxicity was determined by tetrazolium (MTT) assay. The function of P-glycoprotein (P-gp) was examined by Fura 2-AM assay. Cellular accumulation of doxorubicin (Dox) was determined by fluorescence spectrophotometry. Apoptosis was measured by flow cytometry. **RESULTS**: IC_{50} of Ate for MCF-7/Dox, MCF-7, KB_{V200}, and KB cells were 122, 120, 1.34, and 1.27 nmol·L^{-1}, respectively. IC_{50} of Bul for MCF-7/Dox, MCF-7, KB_{V200}, and KB cells were 0.60, 0.59, 0.04, and 0.04 nmol·L^{-1}, respectively. The cytotoxicities of Bul and Ate to MDR cells were similar to those to parental sensitive cells. Bul and Ate markedly increased cellular Fura-2 and Dox accumulation in MCF-7/Dox cells, but not in MCF-7 cells. The rates of apoptosis in MDR cells were similar to those in sensitive cells induced by Ate. **CONCLUSION**: There was no cross-resistance of P-gp positive MCF-7/Dox and KB_{V200} cell lines to Bul and Ate as compared with their sensitive P-gp negative MCF-7 and KB cell lines. The mechanism of the circumvention of MDR was associated with the decrease of P-gp function and the increase of cellular drug accumulation in MDR cells.

INTRODUCTION

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].

Chemotherapy is successful for the treatment of several neoplasias, yet many patients continue to perish due to intrinsic or acquired chemoresistance of tumor cells. MDR is a main obstacle of tumor chemotherapy. The circumvention of MDR has 2 pathways. A promising approach is to utilize nontoxic and potent agents with the reversal of MDR, the combination of anticancer drug with the modulator increases the anticancer effect. Several compounds such as verapamil, bepridil, trifluoperazine, tetrandine[2], ciclosporin, S9788[3], and other hydrophobic compounds[4] reversed MDR *in vitro*, but could not be used in clinic because of side effects. The other way is to look for new anticancer drugs with no resistance to MDR cells.

Bullatacin (Bul) is an annonaceous acetogenin, which had potent cytotoxicities to various tumor cell lines and anticancer activity *in vivo* and being used as a

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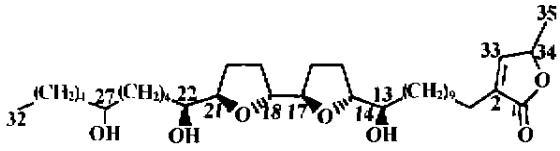
positive control[5]. Atemoyacin-B (Ate) is a novel bis-tetrahydrofuranyl annonaceous acetogenin from atemoya plant[6].



Atemoyacin-B (M_r = 594)

The inhibition of NADH dehydrogenase (Complex I) in the oxidative phosphorylation pathway in mitochondria and ATP depletion was the mode of action of annonaceous acetogenins[7]. P-gp was an energy-dependent agent exflux pump. So we infer that ATP depletion could decrease or loss the activity of P-gp. The MDR cellular accumulation of acetogenin and other anticancer drugs was increased. MDR cells could be sensitive to annonaceous acetogenins. This study was to explore the effect of atemoyacin-B (Ate) on tumor MDR.

MATERIALS AND METHODS

Materials The MDR cell lines MCF-7/Dox and the parental sensitive cell line MCF-7 were generously provided by Prof LIU Xu-Yi (the cells from National Cancer Institute, USA). KB_{V200} cells and parental sensitive KB cells were obtained from Chinese Academy of Medical Sciences, Beijing. 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. Bul and Ate were generously provided by Prof CHEN Wen-Shen, South China Institute of Botany, Chinese Academy of Sciences.

Cell culture The MDR cell line MCF-7/Dox and the parental cell line MCF-7 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. KB_{V200} cells and KB cells were cultured with RPMI-1640 culture medium. Both MCF-7/Dox cells and KB_{V200} cells were overexpression of P-gp which was main cause for inducing MDR[8].

Fura 2-AM fluorescence measurements This was a new method to examine the function of P-gp because Fura 2-AM was the substrate of the MDR transporter (P-gp). 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. But the extrusion of Fura 2-AM could be blocked in the MDR cells when the function of P-gp was decreased or lost and the cellular accumulation of Fura-2 was increased in MDR cells. Such measurement of the accumulation of Fura-2 can be used as a method for studying P-gp function and screening the modulators of MDR induced by P-gp[9].

MTT cytotoxicity assay The cells were collected and resuspended at 4×10^8 cells·L^{-1}. The aliquots (0.19 mL) were seeded in 96-well *multiplates*. *The acetogenin was added after 24-h* incubation. After 72 h, the cell growth inhibition was evaluated by the MTT method on triplicate assays[10]. IC_{50} (95 % condidence limits) were calculated from cytotoxicity curves with Bliss-Finney's weighted probit analysis. The degree of resistance was calculated by dividing the IC_{50} for MCF-7/Dox cells by that for MCF-7 cells.

Cellular Dox accumulation MCF-7 cells and MCF-7/Dox cells were each collected in 1 mL of medium at a density of 4×10^{10} cells·L^{-1}, respectively. Dox 10 μmol·L^{-1} was added in the absence or presence of Ate 168 nmol·L^{-1} or Bul 160 nmol·L^{-1}. The cells were incubated at 37 ℃ for 3 h, and the cellular suspension was centrifuged and 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 assayed spectrofluorometrically at λ_{ex} 470 nm and λ_{em} 590 nm[11]. The annonaceous acetogenins 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 Ate or Bul by that without Ate or Bul. The cellular Dox accumulation reflexed the accumulation of annonaceous acetogenin in cells.

Cellular apoptosis The agent was added in growth adherent monolayers cells and incubated for 24 h. And then the cells were collected and washed 3 times with PBS. The cellular apoptosis was

determined by flow cytometry[12]. MCF-7/Dox cells were resistant to apoptosis induced by Dox[11].

RESULTS

Cytotoxicity The resistant extents of MCF-7/Dox cells to Dox and KB_{V200} cells to vincristine were 66.6-fold and 57.0-fold compared to their parental sensitive cell lines MCF-7 and KB cells, respectively. Bul and Ate exhibited potent cytotoxicities to MCF-7/Dox, MCF-7, KB_{V200}, and KB cells. The IC_{50} of Bul and Ate for MDR cells were similar to those for their parental sensitive cells ($P > 0.05$). This suggested that the sensitivities of MCF-7/Dox and KB_{V200} cells to Bul or Ate were similar to those of their parental cells MCF-7 and KB cells. (Tab 1).

Tab 1. Cytotoxic effects of bullatacin and atemoyacin-B to MCF-7/Dox, MCF-7, KB_{V200}, and KB cells.
$n = 3$ independent experiments.
[a]$P > 0.05$ *vs* parental sensitive cell group.

	IC_{50} (95 % confidence limits)/nmol·L^{-1}	
	Bullatacin	Atemoyacin-B
MCF-7	0.59 (0.38 – 0.81)	120 (112 – 128)
MCF-7/Dox	0.60 (0.33 – 0.87)[a]	122 (104 – 139)[a]
KB	0.04 (0.03 – 0.05)	1.27 (1.18 – 1.37)
KB_{V200}	0.04 (0.03 – 0.05)[a]	1.34 (1.18 – 1.45)[a]

Cellular accumulation of Fura-2 The Fura-2 accumulation in MCF-7 cells was 4.13-fold compared with that in MCF-7/Dox cells. The cellular accumulation of Fura-2 was markedly increased in MCF-7/Dox cells, but not in MCF-7 cells in the presence of Bul or Ate (Tab 2).

Cellular Dox accumulation After 3-h incubation of cells with Dox 10 μmol·L^{-1}, cellular Dox accumulation in MCF-7 cells was 4.9-fold compared to that in MCF-7/Dox cells. Bul 160 nmol·L^{-1} and Ate 168 nmol·L^{-1} increased cellular Dox accumulation by 3.2- and 2.9-fold in MCF-7/Dox cells, but not in MCF-7 cells. (Tab 2)

Apoptosis induced by acetogenin The apoptosis induced by Dox 10 μmol·L^{-1} was 74.6 % and 14.3 % in the MCF-7 and MCF-7/Dox cells, respectively. These results suggested that MCF-7/Dox cells were resistant to apoptosis induced by Dox.

Tab 2. Effects of Bul and Ate on the accumulation of Fura-2 and Dox in MCF-7 and MCF-7/Dox cells.
$n = 3$ independent experiments. $\bar{x} \pm s$.
[a]$P > 0.05$, [b]$P < 0.05$, [c]$P < 0.01$ *vs* control.

Drug	(nmol·L^{-1})	Accumulation (pmol/10^6 cells)		Accumulation fold	
		MCF-7	MCF-7/Dox	MCF-7	MCF-7/Dox
Fura-2					
Control	0	1022 ± 29	266 ± 39	1.00	1.00
Bul	160	1029 ± 46[a]	834 ± 27[c]	1.00	3.14
Ate	168	1022 ± 51[a]	706 ± 22[c]	1.00	2.61
Doxorubicin					
Control	0	2099 ± 94	408 ± 46	1.00	1.00
Bul	160	2074 ± 48[a]	1671 ± 144[c]	0.99	4.10
Ate	168	2089 ± 68[a]	1339 ± 122[c]	1.00	3.28

But Bul and Ate induced apoptosis of both sensitive cells and MDR cells (Tab 3)

Tab 3. Apoptosis induced by Bul, Ate, and Dox in MCF-7 and MCF-7/Dox cells. $n = 3$ independent experiments. [a]$P > 0.05$, [c]$P < 0.01$ *vs* parental sensitive cell group.

Acetogenin/ nmol·L^{-1}		Apoptosis/ %	
		MCF-7	MCF-7/Dox
Control	0	5.4 ± 2.5	4.9 ± 2.1
Dox	10000	74.6 ± 12.3	14.3 ± 3.2[c]
Bullatacin	160	40.2 ± 5.2	40.2 ± 1.1[a]
Atemoyacin-B	168	20.2 ± 3.6	17.4 ± 2.7[a]

DISCUSSION

The annonaceous acetogenins are a series of apparently polyketide-derived fatty acid derivatives that possess tetrahydrofuran ring and a methylated γ-lactone with various hydroxyl, acetoxyl, and/or ketoxyl groups along the hydrocarbon chain. They exhibit a broad range of potent biological activities including cytotoxicity, antitumor, antimalarial, antimicrobial and pesticidal and so on.

P-gp plays an important role in various tumor resistance to multiple drugs[1]. Actually, P-gp is an energy-dependent efflux pump responsible for reducing intracellular drug accumulation in resistant cells. We infer that the function of P-gp is obstacle or loss if the

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intracellular energy is depleted. The action target of bullatacin is the complex I in mitochondria[7]. The inhibition of complex I blocked up the production of ATP and then the intracellular energy was depleted and the function of P-gp was lost. The results of this research showed that the cytotoxicities of Bul and Ate to MDR cells were similar to those to their parental sensitive cells. Bul was effectively cytotoxic to MCF-7/Dox cells, but more to MCF-7 cells[13]. These results suggested that annonaceous acetogenins were promising in the treatment of tumor with MDR.

The extrusion of Fura 2-AM was reduced or blocked in the decrease or loss of the function of P-gp, and cellular accumulation of Fura-2 was increased in MDR cells. Our experimental results showed that Bul and Ate increased Fura-2 accumulation in MDR cells, but not in MCF-7 cells. This suggested that the annonaceous acetogenins blocked the Fura 2-AM extrusion in MDR cells.

The intracellular accumulation of anticancer agents, such as Dox, dauxorubicin, vincristine was energy-dependent in MDR cells. The accumulation was reduced when the cellular energy was depleted in MDR cells[14]. Annonaceous acetogenins depleted the energy in cells[7]. Our experimental results showed that the accumulation of Dox was increased in the presence of Bul or Ate in MCF-7/Dox cells. These suggested that the annonaceous acetogenins depleted cellular energy and resulted in the decrease or loss of the function of P-gp, and blocked the efflux of agents in MDR cells. So MDR cells were sensitive to annonaceous acetogenins with no cross-resistance.

MDR cells were resistant to apoptosis induced by natural anticancer drugs[12]. The results of this research showed that MCF-7/Dox cells were resistant to apoptosis induced by Dox, but not by Bul and Ate. These results suggested that annonaceous acetogenins were potent to sensitive tumors and promising in the treatment of MDR tumors.

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一种新番荔枝内酯单体 atemoyacin-B 克服肿瘤多药抗药性[1]

R979.1

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关键词 番荔枝内酯；bullatacin；atemoyacin-B；多种抗药性；Fura-2；多柔比星；长春新碱；细胞凋亡；培养的肿瘤细胞；植物性抗肿瘤药

目的：探讨 atemoyacin-B（Ate）克服肿瘤多药抗药性（MDR）作用及其机制．**方法**：Bullatacin（Bul）为阳性对照物．细胞毒测定以 MTT 法；P-gp 功能测定以 Fura 2-AM 法；细胞内药物积累测定以荧光分光光度计法；细胞凋亡测定以流式细胞仪法．**结果**：Ate 对 MCF-7/Dox、MCF-7、KB_{V200} 和 KB 细胞的 IC_{50} 分别为 122、120、1.34、1.27 nmol·L^{-1}．Ate 显著增加 MDR 细胞内 Fura-2 及多柔比星（Dox）的积累，但不增加相应敏感细胞的细胞内 Fura-2 及 Dox 的积累．Ate 也能诱导 MDR 细胞凋亡．**结论**：MDR 细胞对 Ate 同样敏感，不受抗药性影响，其机制与降低 P-gp 功能及增加细胞内药物积累有关．

（责任编辑 杨雪芳）