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Influence of the Calmodulin Antagonist EBB on Cyclin B1 and Cdc2-p34 in Human Drug-resistant Breast Cancer MCF-7/ADR Cells

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OBJECTIVE To investigate the influence of O-(4-ethoxyl-butyl)-berbamine (EBB) on the expression of cyclin B1 and cdc2-p34 in the human drug-resistant breast cancer MCF-7/ADR cell line.
METHODS The MTT assay was used to assess the cytotoxicity of EBB. Different levels of EBB were added to different cell lines at series of time points solely or combined with doxorubicin (DOX) to detect the effect on the expression of cyclinB1 and cdc2-p34 by Western blots. cdc2-p34 tyrosine phosphorylation was detected by immunoprecipitation. In addition, apoptosis and cytoplastic Ca^{2+} concentrations were systematically examined by laser scanning confocal microscopy (LSCM).
RESULTS EBB showed little inhibitory activity on human umbilical vein endothelial cells (ECV304), whereas EBB inhibited cell growth (IC_{50} range, 4.55~15.74 μmol/L) in a variety of sensitive and drug-resistance cell lines. EBB also down-regulated the expression of cyclin B1 and cdc2-p34 in a concentration and time dependent manner, which was an important reason for the G_2/M phase arrest. EBB was shown to induce apoptosis of MCF-7/ADR cells while increasing the level of cytoplastic Ca^{2+}.
CONCLUSION The low cytotoxicity of EBB suggests it may be useful as a rational reversal agent. The effect of EBB on cell cycle arrest and related proteins, apoptosis, and cytoplastic Ca^{2+} concentration may be involved in reversing multidrug resistance.

KEY WORDS: EBB, cell cycle, cyclinB1, cdc2-p34, apoptosis, Ca^{2+}.

Introduction

EBB, a derivative of bisbenzylisoqunoline alkaloid, was shown to be a potential calmodulin antagonist[1]. We have reported that EBB was able to suppress tumor invasion by down-regulating the expression of matrix metalloproteinase MMP-2 and MMP-9[2]. $IC_{5\text{-}10}$ EBB reversed drug resistance to DOX, and EBB showed a potential application when combined with DOX, especially for the inhibition of the cell cycle in MCF-7/ADR cells[3]. To study the mechanism of EBB on the cell cycle, we chose two proteins which play important roles during cell division—the mitotic kinase cylin B1 and cdc2-p34. These two kinases were thought to be involved in the regulation of G_2/M arrest, and are very important for preventing mitotic entry when DNA is damaged. Ca^{2+} signalling is an essential component of mitogen responses. We also presume there is a close relationship between cytoplastic Ca^{2+} and EBB. So the purpose of this study was: *i*) to evaluate the cytotoxicity of EBB in normal ECV304 cells and in a panel of human tumor-sensitive and MDR cell lines; *ii*) to analyze cell apoptosis and the expression of cyclin B1 and cdc2-p34 under the treatment of EBB and DOX alone and combined. *iii*) to clarify the influence of

EBB on cytoplastic Ca^{2+} concentration in sensitive and MDR cells.

Materials and Methods

Reagents

EBB, 0-(4-ethoxiyl-butyl)-berbamine, was kindly provided by the Institute of Molecular Biology, Nankai University (Tianjin, China). DOX was purchased from Pharmacia & Upjohn Spa (Milan, Italy). MTT, PI and Fluo-3/AM were purchased from Sigma (St. Louis, MO, USA).

Cell lines and culture

MCF-7 was kindly provided by Professor Liu, Cancer Hospital, Peking University, Beijing, China, and KB_{v200} cells were purchased from the Institute of Materia Medica, Chinese Academy of Medical Science, Beijing, China[4]. Human umbilical vein endothelial ECV304 cells and wild type tumor cells PG, BE-1, HL60, SKOV3, M21, COC1, KB and MCF-7/WT were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (Gibco, Grand Island, NY). The multidrug resistant MCF-7/ADR cell line was maintained in a complete RPMI 1640 medium, containing 2 μmol/L DOX, while KB_{v200} and COC1/DDP cells were cultured in 14 μmol/L STI571, 200 nmol/L vincristine and 1.7 μmol/L cisplatin respectively. All cell lines were cultured at 37℃ in a humidified incubator with 5% CO_2 in air. Before experiments, all drug-resistant cells were cultured in drug-free medium for one week.

Chemo-sensitivity assays

The sensitivity of cells to drugs was determined by the tetrazolium dye assay (MTT) as described previously[5]. The cells were trypsinized, counted and seeded into 96-well flat-bottomed plates (Costar, Charlotte, NC) at $1\sim2\times10^4$/well in 180 μl of complete medium. After 12 h of incubation, EBB was added to the medium in a 20 μl aliquots (PBS) to produce a final series of EBB concentrations. Control cells received 20 μl complete medium. Wells for the calculation of back-ground contained only complete medium. Triplicate wells were utilized and incubated for an additional 72 h. MTT (20 μl, 5 mg/ml, in PBS) was added to each well. After another 4 h of incubation, the MTT containing medium was removed and each well was washed gently with PBS. Proliferative cell absorbance was monitored at 546 nm by a spectrophotometer (Model A-5082, SLT Lab Instruments. Grodig, Austria). Concentrations of EBB producing 50% inhibition of cell growth (IC_{50}) were calculated according to a previously described method[6].

TUNEL analysis

Cells were seeded on coverslips in 6-well plates. After 12 h, EBB (6,10 μmol/L) or DOX (2 μmol/L) were added and incubated for another 72 h. Then the cells were washed 3 times with PBS. TUNEL analysis was performed to detect the apoptotic cells according to the manufacturer's instructions of an In Situ Cell Death Detection Kit, AP (Roche Diagnostics GmbH, Germany). The cells were viewed and photographed by fluorescence microscopy.

Immunoblots

Cell pellets were lysed with RIPA-lysis buffer containing 50 μmol/L Tris-HCl (pH 7.4), 150 μmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl-fluoride (PMSF), 5 mmol/L dithiothreithol (DTT), 1 μg/ml aprotinin and 1 μg/ml leupeptin. The suspension was sonicated at 4℃ and centrifuged at 12,000 rpm, 4 ℃ for 10 min. After removal of the supernatants, the protein concentration in each sample was determined using a BCATM protein assay kit (Pierce, Rock ford, USA) using bovine serum albumin (BSA) standards. Aliquots containing 100 μg of protein were separated by 12% SDS-PAGE polyacrylamide gel electrophoresis. For immunoblot analysis, electrophoretically separated protein extrats were electroblotted onto nitrocellulose membranes with a BioRad 200/2.0 semi-dry blotter. The blotted membranes were blocked for 2 h at 25℃ with 3~5% nonfat dry milk in TBS buffer. Blots were then incubated for 2 h in TBST buffer containing affinity purifed mouse anti-cyclin B1 monoclonal IgG, mouse anti-cdc2-p34 monoclonal IgG, or goat anti-β-actin polyclonal IgG antibody (dilution 1:200) (Santa Cruz, CA, USA) at room temperature. Blots were developed using horseradish peroxidase (HRP)-conjugated anti-mouse or anti-goat IgG in TBST buffer at room temperature for 2 h. The signal was detected with the DAB detection system (Bio Basic Inc., Toronto, Canada).

Immunoprecipitation

An assay was carried out to examine the phosphorylation status of Tyr on cdc2-p34. Protein extracts were incubated with anti-cdc2-p34 antibody overnight at 4℃. Protein A-agarose (Roche Diagnoistics GmbH, Mamnheim, Germany) was added (dilution 1:10) and blocked at 4℃ for another 24 h, followed by centrifugation at 7,500 rpm, 4℃ for 5 min. Samples were resuspended and boiled. SDS-polyacrylamide gel and immunoblot analysis were performed as described above with the mouse anti-p-Tyr antibody (Santa Cruz, CA, USA).

Confocal microscopic analysis

The fluorescent intensity increased with Fluo-3 binding to intracellular free Ca^{2+}. Different fluorescent intensity represents the corresponding alteration of Ca^{2+} in both MDR and parental cells. The samples were prepared as follows: *i*) cells were seeded onto the glass bottom of 0.17 mm tissue culture dishes (Mat Tek, Ashland, MA) with 2 ml complete medium for 12 h. *ii*) EBB (3 and

6 μmol/L) was added and the cells cultured for 24 h. *iii*) cells were washed by D-Hank's buffer (Ca^{2+}-free), and incubated in the buffer with 10 μmol/L fluo-3 acetoxymethyl ester (Fluo-3/AM) at 37℃ for 40 min. *iv*) incubation was stopped with ice-cold D-Hank's buffer. *iv*) cells were visualized (excitation, 480 nm; emission, 560 nm) by laser scanning confocal microscopy (Leica, TCS SP2, Germany). The intensity of fluorescence was quantified by TCS-SP2 software.

Statistical analysis

Levels of statistical significance were evaluated by performing the *t*-test using Prism software with data from at least three independent experiments (GraphPad Prism 4.0).

Results

Cytotoxicity of EBB in vitro

As shown in Table1, EBB caused inhibition of cell growth (IC_{50} range, 4.55~15.74 μmol/L) in a variety of human solid tumor cell lines, including 2 lung cancer cell lines PG and BE-1; 3 ovarian cancer cell lines SKOV3, COC1, COC1/DDP; a melanoma M21 cell line, 2 epidermoid cancer cell lines KB and KBV200; 2 breast cancer cell lines MCF-7 and MCF-7/ADR, and 1 myeloid HL60 leukemias, whereas it had little inhibitory activity on human umbilical vein endothelial ECV304 cells.

Effects of EBB on cyclinB1 and cdc2-p34 expression and cdc2-p34 Tyr phosphorylation

Western blots were used to explain the reasons for EBB-induced G_2/M arrest. The expression of cyclinB1 and cdc2-p34 were both remarkably down regulated by EBB combined with DOX in MCF-7/ADR cells after 24 h of incubation (Fig.1.a). It was apparent that EBB has a greater effect on these two key proteins compared to DOX alone in MCF-7/ADR cells. It was also shown that the inhibitory effects were dependent on the concentration and duration of EBB treatment (Fig.1.b,c). Furthermore, we found that the cdc2-p34 Tyr phosphorylation remained unchanged in MCF-7/ADR cells treated with EBB when compared to untreated ones (Fig.1.d). Similar results were optained 5 times.

Table 1. Cyctotoxicity of EBB on cell lines in vitro[a]

Cell lines	IC_{50} (μmol/L)[b]
Wild type tumor cells	
PG (lung carcinoma)	7.66 ± 2.31
BE-1 (lung carcinoma)	7.36 ± 1.26
HL60 (myeloid leukemia)	4.55 ± 1.74
$SKOV_3$ (ovarian carcinoma)	9.83 ± 1.60
M21 (melanoma)	8.26 ± 0.18
COC1 (ovarian carcinoma)	6.58 ± 2.95
KB (epidermoid carcinoma)	13.86 ± 7.11
MCF-7 (breast carcinoma)	15.74 ± 2.21
Drug resistant tumor cells	
COC1/DDP (ovarian carcinoma)	4.72 ± 0.81
KB_{v200} (epidermoid carcinoma)	9.03 ± 0.71
MCF-7/ADR (breast carcinoma)	14.67 ± 3.42

a: Determined by MTT assays.
b: Each number represents the mean±SD values of triplicate determinations in at least three separate experiments.

Effects of EBB on apoptosis

TUNEL staining revealed different levels of DNA breakage in MCF-7/ADR and MCF-7 cells after 72 h of exposure to EBB and DOX respectively. The results indicated that in sensitive cells apoptosis is more easily induced compared to multidrug-resistant cells by DOX treatment (Fig.2. d, e). Apparent DNA breakage was observed with EBB 10 μmol/L treatment for 72 h, indicating that EBB induced cell apoptosis in a concentration-dependent manner.

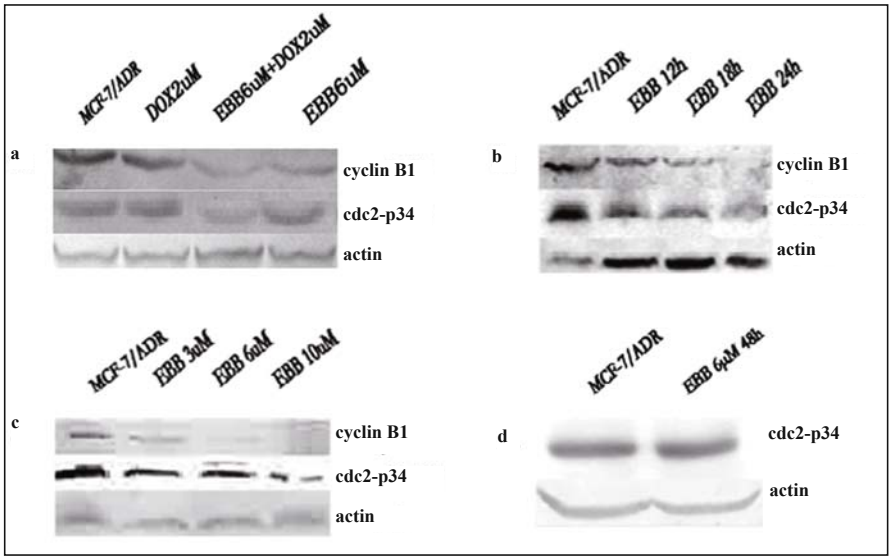

Fig.1. Effect of EBB on the expression of cyclinB1 and cdc2-p34 (a~c), and the activity of Tyr phosphorylation of cdc2-p34 (d). (a) MCF-7/ADR treated with EBB and DOX alone and combined; (b) MCF-7/ADR cells exposed to EBB 6 μmol/L for a series of time points; (c) MCF-7/ADR cells treated for 24 h with increasing concentrations of EBB; (d) the level of cdc2-p34 Tyr phosphorylation in the presence of EBB.

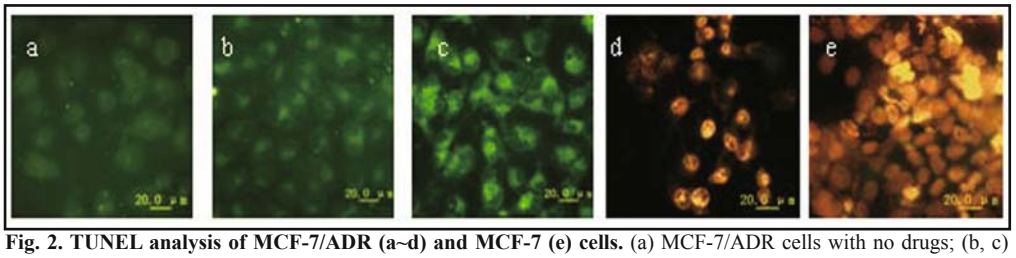

Fig. 2. TUNEL analysis of MCF-7/ADR (a~d) and MCF-7 (e) cells. (a) MCF-7/ADR cells with no drugs; (b, c) MCF-7/ADR cells with EBB 6 μmol/L (b), 10 μmol/L (c) for 72h; (d, e) MCF-7/ADR cells (d) and MCF-7 cells (e) were exposed to DOX for 72 h. Notice that the fluorescein tag of the In Situ Cell Death Detection Kit is green, and the yellow color (d, e) came from the overlap of red (DOX) and green. The bright green staining indicated DNA breaks in apoptotic cells.

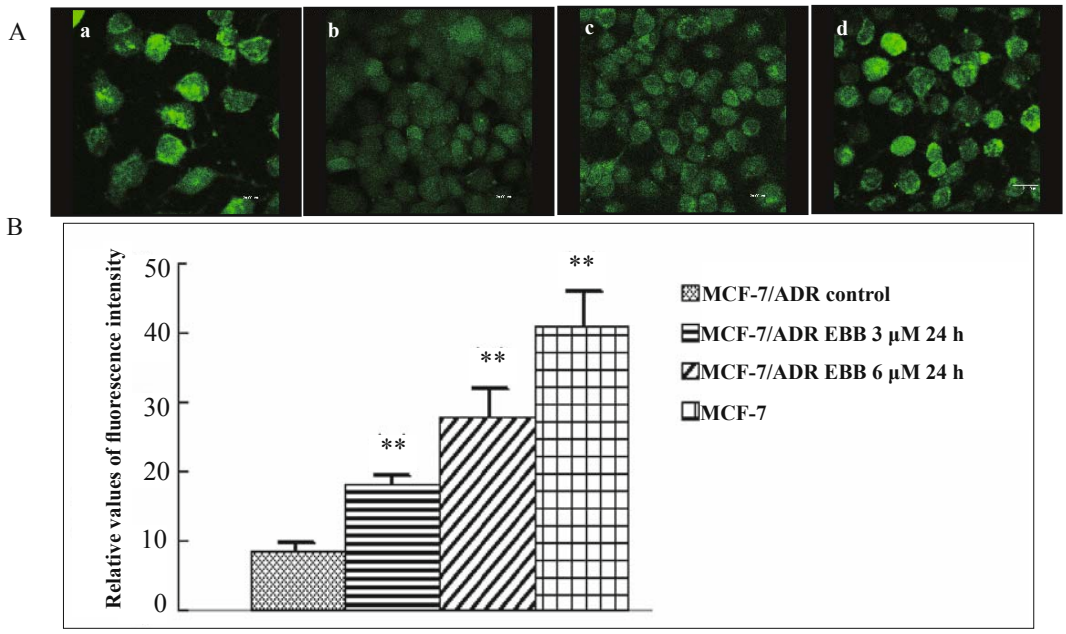

Fig.3. A, Mircrocopic analysis of intracellular Ca^{2+} in MCF-7 (a) and MCF-7/ADR (b, c, d) cells under laser scanning confocal microscopy. MCF-7 cells (a) and MCF-7/ADR cells (b) incubated with drug-free medium; (c, d) MCF-7/ADR cells exposed to EBB 3 μmol/L (c), 6 μmol/L (d) for 24 h. **B, Relative values of fluorescence intensity obtained from TCS-SP2 software analysis.** **, $P < 0.005$, compared with MCF-7.

Effect of EBB on intracellular free Ca^{2+}

Pronounced different concentrations of intracellular Ca^{2+} between MCF-7 and MCF-7/ADR cells are shown in Fig.3.A. Fig.3.B displays the relative values of fluorescence intensity analyzed using TCS-SP2 software. Fluorescent values of intracellular Ca^{2+} in sensitive cells were significantly higher than that in drug-resistant cells ($P < 0.005$) (Fig.3. A. [a, b]), and EBB treatment resulted in an increase of Ca^{2+} concentration in drug-resistant cells in a concentration-dependent manner (Fig.3. A. [b, c, d]). In fact, the increased fluorescence value of MDR cells treated with EBB (6 μM) was almost the same as that of sensitive cells.

Discussion

Multidrug resistance (MDR) is a main impediment for successful cancer chemotherapy. EBB was found to reverse multidrug resistance in vitro more effectively than verapamil. In our research, we further demonstrated that EBB caused little inhibition of human umbilical vein endothelial cells (ECV304), whereas it resulted in extensive cytotoxicity in a variety of human tumor cell lines, including drug-resistance cell lines. Though the MDR reversal mechanism of EBB is not clearly understood, we believe it involves complicated regulation not only with regard to the classical P-gp over-expression, but also apoptosis and other possible metabolic alterations.

The combined effect of EBB and DOX on the cell cycle has been reported in our prior work[3]. We found the ratio of cells in the G_2/M transition was obviously enhanced. Cells in the G_0/G_1 transition were reduced at the same time, whereas the S phase cell number had no significant changes in the MCF-7/ADR cells. In the contrast, the effect was not so remarkabe when treated with either EBB or DOX alone. That is to say, EBB combined with DOX led to G_2/M transition arrest, suggesting that EBB and DOX may enhance each other's effect.

To explore further the potential mechanism of this cooperation on cell cycle arrest, cyclinB1 and cdc2-p34 proteins were taken into consideration since the cell cycle check point of the G_2/M transition is under the

direct control of cdc2-p34 and cyclinB1[7]. Treatment of the cells with EBB and DOX individually and simultaneously showed that cyclinB1 and cdc2-p34 were both down-regulated markedly by the combined treatment. However EBB seemed to played a more important role as Dox alone had only a minor effect on the expression of these key proteins in MCF-7/ADR cells. In addition, cyclinB1 and cdc2-p34 were regulated by EBB in a time and concentration dependent manner. The catalytic subunit cdc2-p34 and the regulatory subunit cyclinB1 can form a compound which controls the entry into mitosis. Cdc2-p34 is inactive in a phosphorylated form, and is dephosphorylated to form an active complex with cyclinB1[8-10]. We suggest that EBB can down-regulate the expression of both cyclin B1 and cdc2-p34, but not participate in the dephosphorylation of cdc2-p34 in MCF-7/ADR cells. Such effect of EBB did directly result in G_2/M transition arrest and induce apoptosis especially when it was combined with DOX.

Many reports have suggested that cell cycle arrest is often followed by, or associated with apoptotic death of cancer cells[11], a process important in drug resistance [12]. Apoptosis or cell suicide is a form of cell death that is morphologically and biochemically distinct from necrosis, and is regarded as an efficient way to eliminate cells. Therefore, agents that can induce apoptosis may be useful in management and therapy of cancer[13]. Our data revealed that apoptosis induced by DOX in MDR cells was much lower compared to sensitive cells. EBB showed a good potential to induce cell apoptosis in a concentration-dependent manner.

It has been reported that the cycling of intracellular calcium ions between endoplasmic reticulum and mitochondria likely acts as a switch in the initiation of apoptosis[14]. Studies have revealed that the sensitivity to Ca^{2+} in multidrug-resistant cells is different from drug-sensitive cells[15]. Some soluble resistance-related calcium-binding proteins were shown to be up-regulated in MDR cells[16]. In our study, LSCM was performed to examine the influence of EBB on the intracellular Ca^{2+} concentration in MCF-7/ADR and MCF-7 cells. The results demonstrated that the level of intracellular Ca^{2+} concentration was much higher in sensitive cells than that in MDR cells. Connections between calcium levels and multidrug resistance need to be elucidated clearly as well as the relationship of calcium and cell cycle regulation. Up to now, we have associated the influence of calcium and calmodulin with the classical Ca^{2+}-CAM-CAMK pathway, which is involved in almost all intracellular events including cell proliferation and differentiation[17,18]. Many reports have indicated that the CaM-related signal-transduction pathway plays a significant role in the regulation and control of the cell cycle and apoptosis in tumor cells[19,20]. Therefore cell apoptosis and reversal of multidrug resistance induced by the calmodulin antagonist, EBB, relates to this classical pathway, and is worthy of study[20,21].

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