Reversal of P-Glycoprotein Mediated Multidrug Resistance in K562 Cell Line by a Novel Synthetic Calmodulin Inhibitor, E6

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The overexpression of P-glycoprotein (P-gp) is associated with multidrug resistance (MDR) of tumor cells to a number of chemotherapeutic drugs. P-gp inhibitors have been shown to effectively reverse P-gp-mediated MDR in both in vitro and in vivo. Our previous studies demonstrated that E6, a novel synthetic calmodulin inhibitor, exhibited potent inhibitory effect on P-gp in rat brain microvessel endothelial cells (RBMECs). In the present study, the effect of E6 on MDR in a K562 MDR cell line (K562/DOX) highly expressing P-gp was studied and compared with that of a conventional P-gp inhibitor, verapamil (VER). E6 at concentrations of 1, 3, 10, 30 μM reduced the IC50 value of doxorubicin in K562/DOX cells from 79.19 μM to 35.18, 21.86, 6.31 and 1.97 μM, respectively. However, the IC50 value of doxorubicin in K562 sensitive subline was not significantly changed by E6. Using a DNA content analysis and an annexin V binding assay, the effects of E6 on doxorubicin-induced apoptosis were also examined. The results indicated that E6 effectively reversed the resistance to doxorubicin-induced apoptosis in K562/DOX cells. In addition, co-treatment of E6 and doxorubicin resulted in a remarkably G2/M blocking effect in K562/DOX cells. Furthermore, the treatment of K562/DOX cells with 10 μM E6 led to increased intracellular accumulation and decreased efflux of doxorubicin. Overall, the pharmacological effects of E6 on P-gp-mediated MDR is much stronger than that of positive control drug VER. These results suggested that E6 is a novel and potent MDR reversal agent and may be a potential adjunctive agent for tumor chemotherapy.

Key words E6; doxorubicin; P-glycoprotein; multidrug resistance

Multidrug resistance (MDR) has been thought to be one of the major obstacles for successful chemotherapy in patients with cancers. The MDR has been associated with overexpression of P-glycoprotein (P-gp) in cancer cells.1) P-gp is a 170- to 180-kDa plasma membrane glycoprotein. P-gp belongs to the superfamily of ATP-binding cassette (ABC) transporters and actively effluxes a wide range of structurally diverse amphipathic anticancer agents. The efflux function of P-gp can decrease drug concentrations in tumor cells and result in chemotherapeutic failure. P-gp mediated MDR has also been associated with inhibition of multiple forms of caspase-dependent tumor cell apoptosis.2—4)

It has been demonstrated that some P-gp inhibitors could effectively reverse P-gp mediated MDR in in vitro experiments. Among the P-gp inhibitors identified, verapamil (VER) was the first which has potent effect on reversal of MDR through inhibition of P-gp.5) Subsequently, a number of other structurally unrelated compounds such as cyclosporin, phenothiazines, antimalarials and antibiotics have also been demonstrated to be effective P-gp inhibitors of P-gp.6,7) However many of these compounds are fail to be introduced to clinical trials due to the unacceptable toxicities for anticancer treatment or nonspecific and weak inhibitory effect on P-gp. Therefore, the development of newer P-gp inhibitors with higher selectivity and stronger potency remains a major goal for this field of research.

Calmodulin is a calcium-binding protein that may play a role in the signaling of insulin- and contraction-stimulated glucose transport.8) Calmodulin inhibitors may therefore inhibit both contraction/hypoxia- and insulin-stimulated glucose transport. Several calmodulin inhibitors have been found to reverse MDR by inhibiting the P-gp mediated drug efflux.9—17) E6, a derivative of berberine which belongs to bi-bezylisoquinolines, was synthesized by China Pharmaceutical University and exhibited potent calmodulin antagonistic activity (Fig. 1).18) Our previous study showed that E6 exhibited strong inhibitory effect on the activity of P-gp in rat brain microvessel endothelial cells (RBMECs).19) However, the possible reversal effect of E6 on MDR cancer cells has not been investigated. In the present study, we studied the effect of E6 on MDR of K562/DOX cell line that has overexpression of P-gp.20) In addition, these effects were compared with those in K562 parent cell line. Furthermore, the effect of E6 on the doxorubicin-induced apoptosis were studied and compared in both K562/DOX and K562 parent cells.

MATERIALS AND METHODS

Chemicals E6 was kindly provided by Dr. Wen-Long Hwang (China Pharmaceutical University). 3-(4,5-Dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT), doxorubicin and VER were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). FITC-annexin V and propidium iodide

Fig. 1. Chemical Structure of E6

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(PI) were obtained from Becton Dickinson (Franklin Lakes, NJ, U.S.A.). All other drugs and reagents were of analytical grade and commercially available.

**Cell Lines** The drug-sensitive human leukemia cell line, K562, and its drug-resistant variant, K562/DOX, were obtained from Institute of Hematology of Chinese Academy of Medical Sciences (Tianjin, China). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO2 in air. The K562/DOX cells were grown in the presence of 1 μM doxorubicin, which was withdrawn for two generations before the experiments.

**Cytotoxicity Assay** Cytotoxicity was assessed by the MTT assay. Briefly, cells were seeded into 96-well culture plates at a density of 5×10^4 cells/ml. Doxorubicin were added alone or with E6 or VER in a final volume of 200 μl per well. After the drugs treatment for 44 h, the medium was replaced with an equal volume of fresh medium containing 0.5 mg/ml MTT and incubated for 4 h at 37 °C. After that, the medium was removed and 100 μl DMSO were added and incubated for 30 min at room temperature. The cytotoxic effects of drugs were determined according to the OD values using a microplate reader (Victor^2 1420, Perkin Elmer Life Sciences, Turku, Finland) at absorption wavelength of 570 nm. The concentrations required to inhibit growth by 50% (IC50) values were calculated from the cytotoxicity curves using a Bliss's software. The fold-reversal of MDR was calculated by dividing the IC50 values in the absence of the P-gp inhibitors by those in the presence of the P-gp inhibitors.

**Flow Cytometric Apoptosis Assay and Cell Cycle Analysis** The DNA content analysis and annexin-V binding assay were used to determine the reversal effect of E6 on the resistance to apoptosis in K562/DOX cells.

Apoptotic cells show diminished DNA content below the G2/M population of normal diploid cells. In order to count the cells in sub-diploid and analyze the cell cycle, flow cytometry was used by the method of cell staining with PI. Briefly, after treatment with doxorubicin alone or combined with E6, the cells were centrifuged at 1000 rpm for 5 min, washed twice with Hank’s solution; then chilled with cool 70% ethanol and the samples were kept at 4 °C for 1 h. Thereafter, internucleosomally fragmented DNA was removed from apoptotic cells by incubation in 0.2 M citrate-phosphate buffer (pH 7.8) containing 0.2 mg/ml RNase for 30 min at 37 °C. Finally, samples were stained with 25 μg/ml PI at room temperature in the dark. The fluorescence of the remaining DNA content was analyzed in the FL2H, FL2A channels using a flow cytometry (Becton Dickinson, U.S.A.). Data were analyzed with CellQuest software (Becton Dickinson). Apoptosis rate was determined by evaluating the percentage of events accumulated in the pre-G1/G0 position. Cell cycle analysis was done by Modfit LT version 3.0 software (Becton Dickinson).

The second apoptotic method depends on annexin-V binding assay. Briefly, cells were collected by centrifugation, washed twice in PBS, and resuspended in binding buffer at a density of 10^6 cells/ml, then stained with FITC-annexin V and PI for 15 min at room temperature in the dark. The samples were then analyzed by a flow cytometry using CellQuest software (Becton Dickinson). The proportion of apoptotic cells was estimated by percentage of cells that stained positive for annexin V while remaining impermeable to PI (annexin V+ and PI−); necrosis was determined as positive stain with both annexin V and PI (annexin V+ and PI+); and viability was defined as annexin V− and PI−.

**Doxorubicin Accumulation and Efflux Assay** Doxorubicin accumulation and efflux were analyzed by flow cytometry. 

In doxorubicin accumulation studies, K562 or K562/DOX cells were incubated at 37 °C with 5 μM doxorubicin in the absence or presence of 1, 3, 10 μM E6 or 10 μM VER for 60 min. After incubation, the cells were washed twice with ice-cold PBS, resuspended in 200 μl PBS and then analyzed by flow cytometry.

In doxorubicin efflux studies, cells (1×10^6) were incubated with 10 μM doxorubicin at 37 °C for 30 min (substrate-loading phase) and then washed twice with ice-cold PBS. Thereafter, cells were resuspended in doxorubicin-free RPMI 1640 in the absence or presence of 10 μM E6 or 10 μM VER at 37 °C for 0, 5, 15, 30, 60 min. At the defined intervals, cells were harvested, centrifuged and washed in ice-cold PBS. Cell pellets were then resuspended in 200 μl PBS and used immediately for flow cytometric analysis for intracellular doxorubicin retention.

**Statistical Methods** All results were represented as mean±S.D. Statistical analysis was performed using Student t-test.

**RESULTS**

**Effect of E6 on Doxorubicin Cytotoxicity** As shown in Table 1, K562/DOX cells were resistant to doxorubicin compared with K562 cells. The ability of E6 to reverse the drug resistance of K562/DOX was examined and compared with that of VER. 30 μM of either E6 or VER had no cytotoxic effects on K562/DOX and K562 cells. After K562/DOX cells

<table>
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<tr>
<th>Group</th>
<th>Concentration (μM)</th>
<th>K562</th>
<th>K562/DOX</th>
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<tr>
<td></td>
<td>IC50 (μM)</td>
<td>Fold-reversal</td>
<td>IC50 (μM)</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0.54±0.04</td>
<td>1.00</td>
</tr>
<tr>
<td>VER</td>
<td>30</td>
<td>0.33±0.03</td>
<td>1.02</td>
</tr>
<tr>
<td>E6</td>
<td>1</td>
<td>0.58±0.03</td>
<td>0.93</td>
</tr>
<tr>
<td>3</td>
<td>0.55±0.02</td>
<td>0.98</td>
<td>21.86±1.05**</td>
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<tr>
<td>10</td>
<td>0.56±0.04</td>
<td>0.96</td>
<td>6.31±0.42**</td>
</tr>
<tr>
<td>30</td>
<td>0.51±0.03</td>
<td>1.06</td>
<td>1.97±0.06**</td>
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IC50 values are the mean±S.D. of six independent experiments. **p<0.01 vs. control group.
were treated with 30 μM E6 or VER for 48 h, the percentages of viability were 101.3 ± 3.6 and 99.5 ± 2.4, respectively. As for K562 cells, 102.1 ± 2.4 and 100.4 ± 1.0 of percentages of viability were found after the treatments with 30 μM of E6 and VER for 48 h, respectively. Therefore, 1, 3, 10, 30 μM E6 and 30 μM VER were used in the experiments. E6 at 1, 3, 10, 30 μM and VER at 30 μM significantly reversed the resistance of K562/DOX to doxorubicin, and reduced the IC50 values (Table 1). E6 was about 4-fold potent than VER in reversing the resistance to doxorubicin in K562/DOX cells. In contrast, E6 and VER, at the concentrations tested, had no effect on doxorubicin cytotoxicity in K562 cells (Table 1).

Apoptosis Induced by Combining Doxorubicin and E6

The appearance of sub-diploid DNA peak is a specific marker of apoptosis; necrosis induced by metabolic poisons or lysis produced by complement did not induce any sub-diploid peak in the DNA fluorescence histogram. Results of DNA content analysis showed that the sub-diploid DNA peak percentage induced by doxorubicin in K562/DOX was greatly enhanced in the presence of E6. After the cells were treated with 3 μM doxorubicin combined with 1, 3 and 10 μM E6 for 24 h, the sub-diploid DNA peak percentages of K562/DOX cells were approximately 2-, 5- and 7-fold higher, respectively, than that treated with doxorubicin alone. The percentage of cells arrested in the G2/M cell cycle phase showed good agreement with the trends of the apoptosis induced by the drugs (Table 2). In sensitive K562 cells, E6 or VER has no significant effects on the apoptosis and G2/M cell cycle phase arrest induced by doxorubicin (data not shown).

Annexin-V is a Ca2+ dependent phospholipid-binding protein with high affinity for phosphatidylserine (PS). When cell death occurs, PS is translocated from the inner side of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell. This occurs in the early phase of apoptotic cell death during which the cell membrane itself remains intact. Therefore, the measurement of annexin-V binding, executed simultaneously with a dye exclusion test (PI staining), provided a useful assay to detect apoptotic cells and to discriminate between apoptosis and necrosis.

According to the annexin-V binding assay, E6 and VER obviously enhanced doxorubicin-induced apoptosis. After treatment of 10 μM VER or 10 μM E6 for 24 h, the apoptotic percentages of K562/DOX induced by 3 μM doxorubicin were increased from 6.97% to 15.15 or 25.77%, respectively (Fig. 2).

E6 or VER has no significant effect on apoptosis induction or alteration of cell cycle in K562/DOX cells in the absence of doxorubicin (Table 3).

| Table 2. Effect of E6 on Sub-diploid DNA Peak Percentage and G2/M Block Induced by 3 μM Doxorubicin in K562/DOX Cells |
|---|---|---|
| Group | Concentration (μM) | Sub-diploid (%) | G2/M phase (%) |
| Control | 0 | 2.4±0.2 | 12.4±2.7 |
| VER | 10 | 11.1±1.6** | 24.2±2.5** |
| E6 | 1 | 6.9±1.0** | 17.4±2.6** |
| | 3 | 13.7±1.6** | 26.3±1.3** |
| | 10 | 18.7±1.5** | 32.0±1.9** |

Values are the mean±S.D. of three independent experiments. **p<0.01 vs. control group.

| Table 3. Effect of 10 μM of E6 and VER on Sub-diploid DNA Peak Percentage, G2/M Block and Apoptosis Rate in K562/DOX Cells |
|---|---|---|---|
| Group | Sub-diploid (%) | G2/M phase (%) | Apoptosis rate (%, annexin V binding assay) |
| Control | 0.20±0.10 | 10.13±1.68 | 0.53±0.15 |
| E6 | 0.19±0.09 | 9.57±2.25 | 0.45±0.18 |
| VER | 0.22±0.15 | 9.80±2.74 | 0.57±0.20 |

Values are the mean±S.D. of three independent experiments.

Fig. 2. Apoptotic Analysis of K562/DOX Cells Treated with 3 μM Doxorubicin for 24 h in the Absence or Presence of P-gp Inhibitors

Values are the mean±S.D. of three independent experiments.
Effect of E6 on Intracellular Accumulation and Efflux of Doxorubicin  The effect of E6 on the intracellular accumulation of doxorubicin was examined using flow cytometry. The accumulation levels of doxorubicin in K562/DOX cells were examined after the cells were incubated with 5 μM doxorubicin at 37 °C for 60 min in the absence or presence of E6 (1—10 μM) or VER (10 μM). Co-administration of E6 or VER markedly enhanced the doxorubicin accumulation in K562/DOX cells, and E6 had stronger effects that of VER at the same concentration (Fig. 3). However, E6 or VER had no such effect in K562 sensitive cells (data not shown).

The efflux of doxorubicin from K562/DOX and K562 cells was detected by flow cytometry. After incubation with 10 μM doxorubicin at 37 °C for 30 min, the cells were resuspended in doxorubicin-free medium in the absence or presence of 10 μM E6 or VER at 37 °C for the indicated times. The levels of doxorubicin in K562/DOX cells were lower than that in K562 cells at 15, 30, 60 min. E6 as well as VER obviously inhibited the efflux of doxorubicin from K562/DOX cells, but not from K562 cells. At 10 μM, E6 was more effective than VER (Fig. 4). The results from both accumulation and efflux experiments indicated that E6 could effectively increased the intracellular concentration of doxorubicin in K562/DOX cells.

DISCUSSION

Previous studies have demonstrated that structurally unrelated calmodulin inhibitors, such as berbamine, phenothiazine, trifluorouridine, trifluoperazine and nifedipine, exhibited various extent of inhibitory effects on P-gp-mediated drug efflux and reversed MDR in various cancer cells. 9—17,26) Recently, we demonstrated that E6, a strong calmodulin inhibitor derived from berbamine, 17,18) exhibited potent inhibitory effect on activity of P-gp in primary cultured RBMECs. 19) In the present study, it was further demonstrated that E6 strongly reversed MDR in a K562 subline, K562/DOX. In addition, we provided direct evidence that E6 enhances the doxorubicin-induced apoptosis by markedly decreasing the intracellular doxorubicin concentration in K562/DOX cells.

While understanding the structural and functional relationships of P-gp and its substrates and inhibitors remains a major challenge for pharmacological and pharmaceutical research, many newer MDR reverters have been developed and their in vitro effects have been verified. 27) However, few of these MDR reverters achieve clinical success. Actually, there are currently no reversal agents clinically available. A major reason for this lack of clinical success is owing to the deleterious effect of the reversal agents on normal tissues expressing P-gp and their intrinsic toxicities in vivo. 27,29) This has been the major problem encountering for the second and third generations of MDR reversal agents. Therefore, development of potent yet selective P-gp inhibitors remains to be a major task for this field of research. 28—30)

In the present study, E6 was verified to have greater potency than that of positive-control VER in reversing the MDR in a drug resistance cancer cell line, K562/DOX. According to our preliminary data, E6 also has feasible in vivo features which allow it to be safely administered in high doses without apparent toxicities. These may favor E6 to be further tested as a reversal agent in clinical trials. However, considering E6 exhibits potent calmodulin inhibitory activity, the possible side effects caused by calmodulin inhibition must be taken into account in the further MDR reversal studies of E6.

A number of mechanisms have been implicated in the development of MDR by cancer cells, including over-expression of P-gp, 31) multidrug resistance related protein (MRP), 32) lung resistance protein (LPR), 33) and breast cancer resistance protein (BCRP). 34) Recently, it has been reported that a post-transcriptional upregulation of P-gp is responsible for the MDR in K562/DOX, and MRP and LRP may play only minor role. 35) Therefore, reversal of the over-expressed P-gp function in K562/DOX is the most likely explanation for the observed E6 effect in the K562/DOX cell line. This is further evidenced by our previous results in RBMEC, in which the cellular transport of a typical P-gp substrate, rhodamine 123, was markedly inhibited by E6, indicating a P-gp inhibitory effect of E6. 19) However, we acknowledge that the present study can not provide any firm evidence into the extract molecular mechanisms involved in the MDR reversal effect of E6. Considering that multiple mechanisms are involved in the MDR of cancer chemotherapy, comparing the MDR reversal effects of E6 in different cancer cell lines overexpressing different levels of MDR transporters may deserve further investigation.

In conclusion, the present study provides evidence that E6...
effectively reversed MDR in a K562/DOX cell line. The doxorubicin-induced apoptosis was enhanced and the intracellular concentrations of doxorubicin were greatly increased by the treatment of the cells with E6. The strong MDR reversal effects of E6 as well as its favorable in vivo features make it a possible choice for further mechanistic and pharmacokinetic experiments.

REFERENCES