EXPERIMENTAL STUDY

Reversal effect of bufalin on multidrug resistance in K562/VCR vincristine-resistant leukemia cell line

Xiaofeng Zhai, Jianying Lu, Ying Wang, Fanfu Fang, Bai Li, Wei Gu

Xiaofeng Zhai, Fanfu Fang, Bai Li, Wei Gu, Department of Traditional Chinese Medicine, Changhai Hospital Affiliated to the Second Military Medical University, Shanghai 200433, China

Jianying Lu, Ying Wang, Department of Oncology, Anhui Jingan Hospital of Integrative Medicine, Hefei 230000, China

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Correspondence to: Associate Prof. Wei Gu, Department of Traditional Chinese Medicine, Changhai Hospital Affiliated to the Second Military Medical University, Shanghai 200433, China. sam6116@163.com

Telephone: +86-21-31161950

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Abstract

OBJECTIVE: To probe insights into the reversal effect of bufalin on vincristine-acquired multidrug resistance (MDR) in human leukemia cell line K562/VCR.

METHODS: Proliferative inhibition rate and the reversal index (RI) of bufalin were determined by Methyl thiazolyl tetrazolium assay. The uptake of Adriamycin (ADM) in K562/VCR cells, cell cycle and apoptosis rate were determined by flow cytometry (FCM). Cell morphologic changes were observed with Wright-Giemsa staining. The expression of P-glycoprotein (P-gp), multidrug-associated protein-1 (MRP1), Bcl-xL and Bax protein were measured by immunocytochemistry.

RESULTS: The human leukemia multidrug resistant K562/VCR cells showed no cross-resistance to bufalin. The RIs of bufalin at concentrations of 0.0002, 0.001 and 0.005 μmol/L were 4.85, 6.94 and 14.77, respectively. Preincubation of 0.001 μmol/L bufalin for 2 h could increase intracellular ADM fluorescence intensity to 28.07% (P<0.05) and down-regulate MRP1 expression simultaneously, but no remarkable effect was found on P-gp protein. Cell cycle analysis indicated increased apoptosis rate and apparent decreased G2/M phase proportion after treatment with bufalin. When exposed to 0.01 μmol/L bufalin, typical morphological changes of apoptosis could be observed. Down-regulation of Bcl-xL and up-regulation of Bax expression in K562/VCR cells could be detected by immunocytochemistry.

CONCLUSION: Bufalin could partly reverse the MDR of K562/VCR cells, with a possible mechanism of down-regulating MRP1 expression and activating apoptosis pathway by altering Bcl-xL/Bax ratio.

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Key words: Bufalin; Drug resistance, multiple; Apoptosis; Multidrug resistance-associated protein 1; Human leukemia cell line K562/VCR

INTRODUCTION

Chemotherapy is a primary therapy for leukemia, while multidrug resistance (MDR) of leukemia cells to chemotherapeutic drugs could largely compromise the effectiveness of chemotherapy and thus becomes one of the important causes of a therapeutic failure. MDR refers to spontaneous or acquired drug resistance of tumor cells to various anticancer medications of unrelated structures. Clinical application of classic multi-drug resistance modifiers (RM) is somewhat limited due to
serious toxicities developed when the working concentration is reached. RMs among traditional Chinese medications have caught researchers' eyes by their fewer toxic side effects and more working targets. Bufalin is a toxic ligand extracted from Chansu (Venenum Bufo- nis) a medicinal used in Traditional Chinese Medicine (TCM). Research has revealed a multi-target anti-tumor effect of bufalin on tumor cell differentiation and apoptosis induction, as well as related gene expression and signal transduction. Studies also found that bufalin could reverse the drug resistance of leukemia cells to retinoic acid, VP-16 and other agents, but the underlying mechanism is unknown. The purpose of this study is to investigate the mechanism of bufalin possibly underpinning its reversal effect on human leukemia K562/VCR multi-drug resistant cell line and its.

METHODS

Cell culture
Human chronic myeloid leukemia cell line K562 purchased from Shanghai Institute of Cell Biology (Shanghai, China) and human leukemia multidrug resistant cell line K562/VCR introduced from the Institute of Hematology, Chinese Academy of Sciences (Tianjin, China) were cultured in RPMI-1640 complete medium containing 10% fetal calf serum at 37°C, 5% CO2 and saturated humidity.

Drugs and reagents
Ten milligram of bufalin powder (Sigma-Aldrich Co., Santa Clara, CA, USA) was dissolved into 200 μL of anhydrous ethanol, and injectable water was added to prepare a 1000 μmol/L concentration solution. Methyl thiazolyl tetrazolium (MTT) (Amresco), RPMI-1640 medium (Gibco), fetal calf serum (Hangzhou Evergreen Biological Corporation, Hangzhou, China), vincristine (VCR, Zhejiang Haizheng Pharmaceutical Co., Ltd., Zhejiang, China), adriamycin (ADM, Shenzhen Wan Yue Pharmaceutical Co., Ltd., Shenzhen, China), multidrug resistance-associated protein-1 (MRP1), P-glycoprotein (P-gp) single monoclonal antibody (Fuzhou New Biotechnology Development Company, Fuzhou, China) were purchased; Bcl-xL (SI-B6054) and Bax (SI-B8429) monoclonal antibodies were products of Sigma-Aldrich Co., (Santa Clara, CA, USA).

Reversal index calculation using the MTT method
K562 and K562/VCR cell lines were collected in the logarithmic phase and underwent trypan blue staining before adjusting of the cell concentration to 1×10^6/mL. Each well of the 96 well culture plates was seeded with 100 μL cell solution, with a blank cell group and a pure culture medium group as control groups; cells in each well were treated with 10 μL of bufalin (with a gradient of 7 consecutive concentrations, each diluted 10 times than the previous one) after culturing for 12 h with another 6 wells for each concentration; MTT reagent (5 mg/mL, 10 μL for each well) was added 4 h before termination of culture; After incubation, supernatant in each well was disposed before adding 150 μL Dimethyl sulfoxide (DMSO). Cells underwent oscillation in the thermostatic shaker for 10 min before spectrophotometry (A value) was measured using enzyme standard analyzer, the detecting wavelength was 490 nm, inhibition rate of cell proliferation was calculated as follows: inhibition rate=(A value of the control group-A value of the experimental group)/(A value of the control group-A value of the background group)×100%. Half inhibition concentration (IC50) was calculated by mid-efficacy analysis (LOGIT method) with SPSS 11.0 for Windows (IBM, Armonk, NY, USA) in parent cells and drug resistance cells.

Two hours before adding different concentrations of VCR, bufalin of three low cytotoxic concentrations (0.0002, 0.001, 0.005 μmol/L) were added, the reversal index (RI) was calculated using the same MTT method described above according to the following formula: RI=IC50 in blank control group/IC50 in reversed group.

Cell uptake of adriamycin detection by flow cytometry (FCM)
Cells were seeded in six-well plates (2 mL per well) after adjusted to a concentration of 1×10^6/mL. Bufalin of different concentrations (0.001, 0.01 and 0.1 μmol/L) was then added for 2 h following conventional culture for 24 h. Adriamycin of a final concentration of 20 μg/mL was added and incubated for 2 h before cold PBS (4°C, 0.01 mol/L, pH 7.4) washing and centrifugation for two times, immediately thereafter, cells underwent flow cytometry analysis (receiving wavelength of 575 nm and excitation wavelength of 488 nm), the relative fluorescence intensity of adriamycin with gated cells were analyzed by CellQuest software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

FCM analysis of cell cycle and apoptosis rate
Cells treated with different concentrations of bufalin for 48 h were collected and underwent flow cytometry analysis of DNA content with methods same as literature reported, cell cycle and apoptosis rate of cells were measured using ModFit LT for Mac V3.0 software (Verity Software House, Topsham, ME, USA).

Cell morphology under inverted microscope
K562/VCR cells treated by 0.01 μmol/L of bufalin for 48 h were collected and living cells were observed under the inverted microscope while conventional smears of cells underwent Wright-Giemsa staining were observed under light microscope (BX53, Olympus, Tokyo, Japan).

Immunocytochemistry
K562/VCR cells treated with bufalin (0.01 μmol/L)
for 48 h underwent conventional smears. P-gp, MRP1, Bcl-xL and Bax were detected with immunocytochemistry following Envision two-step method. Cells with a pale-brown staining of the membrane or cytoplasm were defined as positive, quantitative analysis of the positive expression intensity was done using the Information Management System (IMS) computer cell image analysis system. Six vision fields were randomly selected under light microscope (× 100), the percentage of positive area and mean optical density value were calculated by default sampling procedures, the product of the above 2 figures was defined as the positive index (PI) of immunohistochemistry.

Statistical analysis
Statistical analysis was done with SPSS 11.0 for Windows (IBM, Armonk, NY, USA), mean differences between groups were compared by t test or variance analysis, data was presented as mean standard ± deviation (SD) and P<0.05 was significant level.

RESULTS

Reversal effect of bufalin
Cell proliferation of K562 and K562/VCR was significantly inhibited by bufalin for 72 h, with an IC50 of K562 parental cells and resistant K562/VCR cells 0.0943 μmol/L and 0.0401 μmol/L, respectively. In resistant cells, proliferation inhibition rates of bufalin with 0.0002, 0.001 and 0.005 μmol/L concentrations were 7.24%, 14.51% and 23.15%, respectively. These low-cytotoxic concentrations could partially reverse the resistance of the K562/VCR cells. The reversal indices were 4.85, 6.94 and 14.77 times (Figure 1).

Adriamycin uptake analysis
A concentration of 0.001 μmol/L enhanced the mean fluorescence intensity of adriamycin in K562/VCR cells approximately by 28.07% compared with the control group (P<0.05, Figure 2).

Figure 1 Effect of bufalin on enhancing the sensitivity of K562/VCR cell line to vincristine (VCR) in vitro (n=6)
Proliferation inhibition rates of bufalin to resistant cells were 7.24%, 14.51% and 23.15%, with concentrations of 0.0002 , 0.001 and 0.005 μmol/L respectively. These low-cytotoxic concentrations could partially reverse the resistance of the K562/VCR cells.
The s-phase fraction in all groups treated with different dose of bufalin for 48 h increased significantly. The G2/M ratio decreased with increasing drug concentration while the rate of apoptosis went proportionally with drug concentration. The G2/M ratio was negatively correlated to the cell apoptosis rate ($r = -0.98$, $P < 0.05$; Table 1).

### Morphological changes

Before bufalin administration, K562/VCR cells had larger nuclear, prominent nucleoli with intact cell membranes and numerous mitotic figures. After treated by 0.01 μmol/L bufalin for 48 h, cell shrinkage, membrane blistering, nuclear condensation and other morphological changes were observed and became more noticeable with higher bufalin concentrations and longer duration of time. Some cells showed trypan blue stain resistance, suggesting the apoptotic cell characters (Figure 3).

### Immune cells chemical examination

P-gp and MRP1 protein expressions were positive in K562/VCR cell lines. Forty-eight hours after bufalin administration, no significant change was found in P-gp protein expression while positive index of MRP1 protein decreased significantly, whereas the Bax protein positive index was significantly elevated ($P < 0.05$, Table 2).

### DISCUSSION

Bufalin, one of the ligands extracted from Chansu (Venenum Bufonis), is a cardiac glycoside with anti-cancer effect. This regimen inhibits topoisomerase enzyme II (Topo II), protein kinase A and protein kinase C activity as well as down-regulating casein kinase-2 at low concentrations (0.001 mol/L), thereby inducing cell differentiation, at high concentrations (0.01 mol/L or higher) it plays selective role in leukemia cells not only by inhibition of DNA synthesis and regulation of casein kinase 2 shift, but also by adjusting Topo II activity after binding to Topo II-alpha and thereby decreasing its concentration and activity to induce apoptosis in leukemic cells. The present study discovered a powerful role of bufalin in destruction of K562 pro-cells and significant proliferative inhibition in human leukemia multidrug resistant K562/VCR cell line. On the other hand, IC50 in K562 parental cells showed no significant difference compared with resistant cells after 72 h of bufalin, suggesting that K562/VCR multi-drug resistant cell developed no cross-resistance to bufalin. Incubated for 2 h with low cytotoxic concentrations of bufalin enhanced the sensitivity of K562/VCR cells to VCR. The reverse index was concentration-dependent, indicating an in vitro reversal effect of bufalin on multidrug resistance.

MDR mediated by over-expression of ABC (ATP the protein ($P < 0.05$) and Bcl-xL protein ($P < 0.05$) both decreased significantly, whereas the Bax protein positive index was significantly elevated ($P < 0.05$, Table 2).
binding cassette)-type membrane carrier protein family can significantly reduce the effective intracellular concentration of chemotherapy drugs in leukemia cells, which serve as the predominant reason for drug resistance. Previous studies have indicated that MDR mechanisms of K562/VCR cell line were attributed mainly to over-expression of cell membrane P-glycoprotein (P-gp), which may lead to an increased intracellular anticancer drug efflux and elevated glutathione-S-transferase expression, therefore further increase the detoxification function of the cells to chemotherapeutic drugs, and expression of multidrug resistance associated protein (MRP). Some studies have found a reversal effect of bufalin on vinblastine resistant CEM/VLB cell line (100) and epirubicin resistant CEM/E1000 cell line, as well as the capacity to increase the accumulation of daunorubicin within both cell lines, but further studies are yet to be reported.

Our results showed that all concentrations of bufalin could increase the intracellular accumulation of ADM within K562/VCR cells, interestingly, this effect was negatively correlated with the concentration of bufalin, concentrations with low cytotoxicity could enhance intracellular adriamycin content of about 28.07% in K562/VCR cells. In addition, immunocytochemistry revealed a significantly lowered expression of MRP1 while no obvious effect on the expression of P-gp, suggesting that enhanced intracellular chemotherapeutic drug accumulation may be associated with lowered MRP1 expression after bufalin administration. Studies have shown that a variety of anticancer drugs with different chemical structures and distinct targets of can induce tumor cell apoptosis, while enhanced anti-apoptotic effect is a common phenomenon developed in tumor cells to evade chemotherapy. Recent researches have indicated the hindered apoptosis pathway might play an important role in tumor MDR development. The anti-apoptotic effects of tumor cells multidrug resistance by active components of materia medica cells multidrug resistance by active components of materia medica. Shanghai Zhong Yi Yao Za Zhi 2011; 45(6): 92-96.

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