Cucurbitacin B reverses multidrug resistance by targeting CIP2A to reactivate protein phosphatase 2A in MCF-7/Adriamycin cells

FEN CAI[1,4*], LIANG ZHANG[1,3*], XIANGLING XIAO[1,4], CHAO DUAN[1,4], QIUYUE HUANG[1,4], CHUNSHENG FAN[1], JIAN LI[1,3], XUEWEN LIU[1,3], SHAN LI[1,2] and YING LIU[1,3]

[1]Department of Biochemistry, School of Basic Medical Sciences, [2]Department of Integrated Medicine, General Hospital of Dongfeng, [3]Institute of Basic Medical Sciences, [4]School of Biomedical Engineering, Hubei University of Medicine, Shiyan, Hubei 442000, P.R. China

Received February 26, 2016; Accepted June 2, 2016

DOI: 10.3892/or.2016.4892

Abstract. Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a human oncoprotein that is overexpressed in various tumors. A previous study found that CIP2A expression is associated with doxorubicin (Dox) resistance. In the present study, we investigated whether cucurbitacin B (CuB), a natural anticancer compound found in Cucurbitaceae, reversed multidrug resistance (MDR) and downregulated CIP2A expression in MCF-7/Adriamycin (MCF-7/Adr) cells, a human breast multidrug-resistant cancer cell line. CuB treatment significantly suppressed MCF-7/Adr cell proliferation, and reversed Dox resistance. CuB treatment also induced caspase-dependent apoptosis, decreased phosphorylation of Akt (pAkt). The suppression of pAkt was mediated through CuB-induced activation of protein phosphatase 2A (PP2A). Furthermore, CuB activated PP2A through the suppression of CIP2A. Silencing CIP2A enhanced CuB-induced growth inhibition, apoptosis and MDR inhibition in MCF-7/Adr cells. In conclusion, we found that enhancement of PP2A activity by inhibition of CIP2A promotes the reversal of MDR induced by CuB.

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide, with an estimated 1.7 million cases and 521,900 deaths in 2012 (1,2). One third of novel cancer diagnoses in females are breast cancer and one in eight women will be diagnosed with breast cancer, with a lifetime risk of mortality due to breast cancer of 3.4% (3). Systematic chemotherapy plays a critical role in treatment of breast cancer. Doxorubicin (Dox) treatment is one of the established clinically effective strategies for the treatment of breast cancer, and primarily functions by inhibiting topoisomerases and intercalating into the DNA double helix to interfere with DNA uncoiling, which induces cell death (4). However, the efficacy of Dox is limited by its drug resistance as well as side-effects. Increasing research indicates that multidrug resistance (MDR) contributes to the failure of chemotherapeutic drugs as treatment (5). There are at least two molecular pumps in tumor cell membranes, multidrug resistance-associated protein (MRP) and P-glycoprotein (P-gp), to expel the antitumor drugs out of the cancer cells and attenuate the drug effect. Some P-gp inhibitors used to reverse MDR were able to enhance chemosensitivity in resistant cells but caused cytotoxicity and a number of complications (6). Therefore, finding key target molecules and novel therapeutic strategies to overcome resistance and diminish the side-effects of chemotherapeutic agents are the main goals of any ideal cancer treatment protocol.

The protein phosphatase 2A (PP2A) is a key tumor suppressor that regulates signaling pathways with a high relevance in human cancer (7). Consistent with its role as a tumor suppressor, PP2A plays a critical role in the regulation of survival, cell cycle progression, and differentiation by negatively regulating the PI3K/Akt pathway and dephosphorylating and inactivating ERK and MEK1 family kinases (8). Aberrant expression, mutations, and somatic alterations of the PP2A scaffold and regulatory subunits are frequently found in human breast, lung, colon and skin cancers (9). Therefore, reactivation of PP2A activity based on its tumor suppressor properties is considered to be an attractive therapeutic strategy for human cancer treatment (10,11).

Cancerous inhibitor of protein phosphatase 2A (CIP2A), a human oncoprotein that stabilizes c-Myc by inhibiting PP2A-mediated dephosphorylation of Myc at serine 62 (8). CIP2A promotes the proliferation and aggressiveness of several cancer types including head and neck squamous cell carcinoma, oral squamous cell carcinoma, esophageal squamous cell carcinoma, colon, gastric, breast, prostate, tongue, lung,

Correspondence to: Professor Ying Liu or Professor Shan Li Department of Biochemistry, School of Basic Medical Sciences, Hubei University of Medicine, Shiyan, Hubei 442000, P.R. China
E-mail: ying_liu1002@163.com
E-mail: lishanhbmu@126.com

*Contributed equally

Key words: cucurbitacin B, breast cancer, CIP2A, PP2A, multidrug resistance, apoptosis

cervical cancer and acute myeloid leukemia (8,12-15). In 2011, Choi *et al* (16) reported that CIP2A expression is associated with Dox resistance. CIP2A has been found to involve in regulating MDR of cervical adenocarcinoma upon chemotherapy by enhancing MDR gene encoded P-gp expression through E2F1 (17). Thus, effective and discerning CIP2A inhibitors would be beneficial for adjuvant therapy to reduce the development of cancer resistance to Dox and reactivation of PP2A activity in breast cancer.

Cucurbitacins are tetracyclic triterpene natural products that are mainly found in the members of family Cucurbitaceae. It has been used as a medicinal herb because it exhibits different biological activities such as anti-diabetic, anti-inflammatory, and anticancer activities against different cancer cell lines (18). Cucurbitacin B (CuB) is one of the most abundant forms of cucurbitacins which is shown to inhibit the growth of numerous human cancer cell lines such as breast, colon, leukemia, hepatic, pancreatic and glioblastoma, and xenografts (19-21). The effect of CuB on Dox-resistant breast cancer cells has not been previously evaluated. The aim of the present study was to investigate antitumor effects and possible mechanisms of CuB on Dox resistant human breast cancer cells.

Materials and methods

Reagents. Cucurbitacin B (CuB) with a purity of up to 98% was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). CuB was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at a stock solution of 40 mM and stored at -20˚C. Doxorubicin (Dox) was purchased from Sigma-Aldrich.

Cell culture. The human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Human Dox-resistant breast cancer cell line MCF-7/Adriamycin (MCF-7/Adr) was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT, USA) and antibiotics. MCF-7/Adr cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. Additionally, 2 μg/ml Adriamycin was added into MCF-7/Adr medium. All the cells were cultured in a humidified atmosphere with 5% CO_2 at 37˚C.

Cytotoxic assay and cell viability. Cells were seeded into a 96-well plate and pre-cultured for 24 h, then treated with CuB for 24 h. Cell cytotoxicity was determined by MTT assay. The absorbance was measured at 490 nm by automated microplated reader (BioTek Instruments, Inc., Winooski, VT, USA), and the inhibition rate was calculated as followed: Inhibition rate (%) = (average A_{490} of the control group - average A_{490} of the experimental group)/(average A_{490} of the control group - average A_{490} of the blank group) x 100%. Cell viability was estimated by trypan blue dye exclusion (22).

Soft-agar colony formation assay. Cells were suspended in 1 ml of RPMI-1640 containing 0.3% low-melting-point agarose (Amresco, Cleveland, OH, USA) and 10% FBS, and plated on a bottom layer containing 0.6% agarose and 10% FBS in 6-well plate in triplicate. After 2 weeks, plates were stained with 0.2% gentian violet and the colonies were counted under a light microscope (23).

Apoptosis determination by DAPI staining. Approximately $2x10^5$ cells/well of cells in a 12-well plate was treated with CuB for 24 h. Then cells in each treatment and control were stained by DAPI and examined and photographed by fluorescence microscopy as described (24).

Western blot analysis. Cell pellets were lysed in RIPA buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 1 mM DTT, 1 mM NaF, 1 mM sodium vanadate, 1 mM PMSF (Sigma-Aldrich) and 1% protease inhibitors cocktail (Merck). Protein extracts were quantitated and loaded on 8-12% sodium dodecyl sulfate polyacrylamide gel, electrophoresed and transferred to a PVDF membrane (Millipore, Kenilworth, NJ, USA). The membrane was incubated with primary antibody, washed, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce). Detection was performed by using a chemiluminescent western detection kit (Cell Signaling Technology, Danvers, MA, USA) (25). The antibodies used were anti-MRP1, anti-CIP2A, anti-Akt, anti-pAkt (Ser473) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-casp-9, anti-casp-3, anti-PARP, anti-PP2A, anti-ERK1/2, anti-pERK1/2 (Thr202/Tyr204), (Cell Signaling Technology), anti-P-gp (Abcam), and anti-GAPDH (AB10016; Sangon Biotech, Co., Ltd., Shanghai, China).

PP2A activity assay. PP2A immunoprecipitation phosphatase assay kit (Upstate-Millipore, Temecula, CA, USA) was used to measure phosphate release as an index of phosphatase activity according to the manufacturer's instructions. Briefly, 100 μg protein isolated from cells was incubated with 4 μg anti-PP2A monoclonal antibody overnight. A total of 40 μl of protein A agarose beads were added and the mixture was incubated at 4˚C for 2 h. Subsequently, the beads were collected and washed three times with 700 μl of ice-cold TBS and one time with 500 μl Ser/Thr assay buffer. The beads were further incubated with 750 mM phosphopeptide in assay buffer for 10 min at 30˚C with constant agitation. A total of 100 μl of Malachite Green Phosphate detection solution was added and the absorbance at 650 nm was measured on a microplate reader (26).

Transfection of siRNA. Two siRNAs targeting CIP2A were designed and synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China) referred to as siRNA1 and siRNA2. The siRNA sequences were as follows: 5'-CUGUGGUUGUGUUUGCACUTT-3' (CIP2A siRNA1), 5'-ACCAUUGAUAUCCUUAGAATT-3' (CIP2A siRNA2), 5'-UUCUCCGAACGUGUCACGUTT-3' [negative control (NC) siRNA].

Using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, MCF-7/Adr cells were transfected with 100 nM siRNA. In addition, 48 h after transfection, the cells were then harvested for western blot analysis and cell viability.

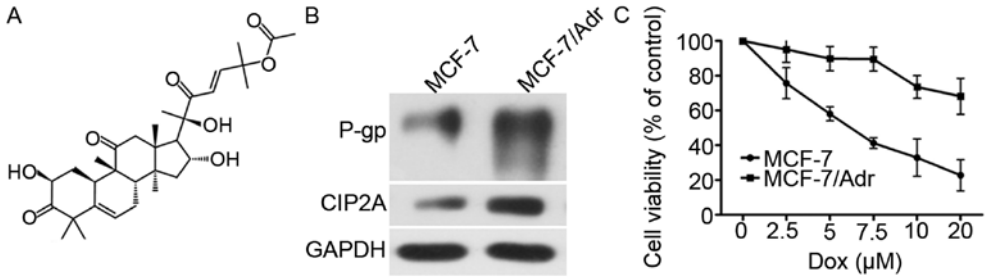

Figure 1. Chemical structure of CuB and characterization of MCF-7 and MCF-7/Adr cells. (A) Chemical structure of CuB. (B) Western blot analysis showing protein expression in MCF-7 and MCF-7/Adr cells using the indicated antibodies. (C) Cell viability was determined by MTT assay after Dox treatment in MCF-7 and MCF-7/Adr cells for 24 h.

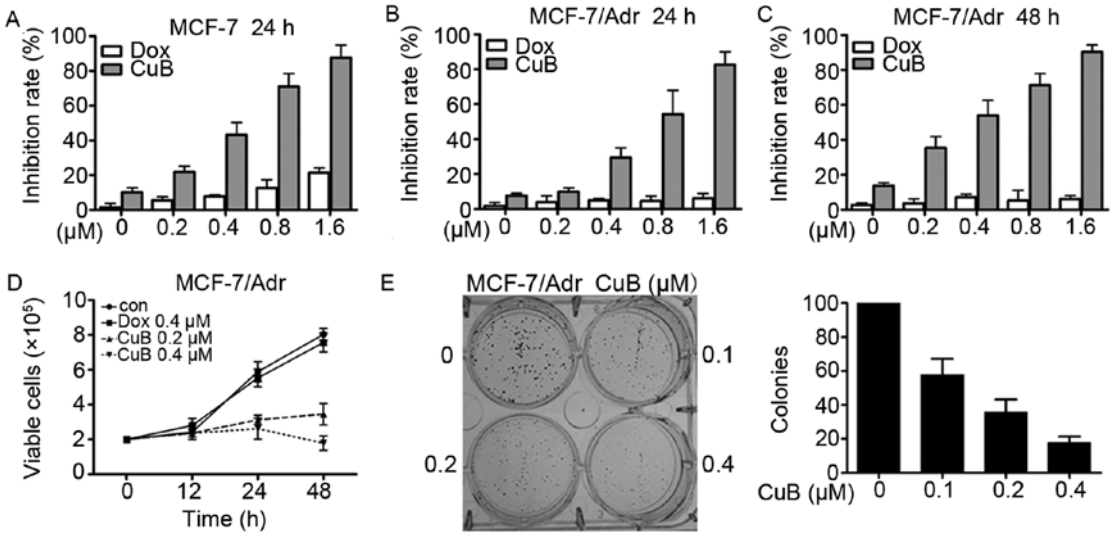

Figure 2. Effect of CuB on MCF-7 and MCF-7/Adr breast cancer cells. (A-C) The inhibitory effects of CuB on MCF-7 and MCF-7/Adr cells analyzed by MTT assay. CuB, cucurbitacin B; Dox, doxorubicin. (D) Inhibitory effects of CuB and Dox on cell viability of MCF-7/Adr cells assayed by trypan blue exclusion assay. (E) The colony formation assays of MCF-7/Adr cells treated with CuB and Dox at indicated concentration.

Statistical analysis. All experiments were repeated at least three times and the data are presented as the mean ± SD unless noted otherwise. Differences between data groups were evaluated for significance using Student's t-test of unpaired data or one way analysis of variance and Bonferroni post-test. P-values <0.05 indicate statistical significance.

Results

Chemical structure of CuB and characterization of MCF-7 and MCF-7/Adr cells. The chemical structure of CuB is shown in Fig. 1A. First, the P-gp and CIP2A expression level was compared between MCF-7/Adr and MCF-7 cells by western blot analysis, which confirmed P-gp and CIP2A overexpression in MCF-7/Adr cells (Fig. 1B). MCF-7 and MCF-7/Adr cells were exposed to various concentrations of Dox (2.5-80 μM) for 24 h. The half-maximal inhibitory concentration (IC_{50}) of Dox against MCF-7 cells is 6.2 μM, while IC_{50} of Dox against MCF-7/Adr cells is 37.78 μM. As shown in Fig. 1C, the Dox cytotoxicity was higher in MCF-7 cells than in MCF-7/Adr cells.

Effects of CuB on MCF-7 and MCF-7/Adr breast cancer cells. MCF-7 and MCF-7/Adr cells were seeded in 96-well plates for 24 and 48 h and then treated with different concentrations of CuB and Dox (Fig. 2A-C). After 24 or 48 h, the cell viability was evaluated by the MTT assay according to the manual. Absorbance at 490 nm was measured on an automated microplate reader. We found that CuB had moderate cytotoxicity to MCF-7 and MCF-7/Adr cells with an IC_{50} of 0.45 and 0.66 μM (Table I). By trypan blue exclusion assay, we found that CuB rapidly reduced viable MCF-7/Adr (Fig. 2D) in a dose- and time-dependent manner. We investigated CuB's effect on cell colony formation activity, and the results showed that CuB significantly inhibited the clonogenic ability of MCF-7/Adr (Fig. 2E). These results suggested that CuB inhibited the anchorage-dependent (cell proliferation) and anchorage-independent (colony formation) growth of MCF-7/Adr cells.

CuB reverses the resistance of MCF-7/Adr cells to Dox. MTT assay revealed a significant difference between the growth-inhibiting effect of Dox on normal MCF-7 cells and on Dox resistant MCF-7/Adr cells (Fig. 1C). The IC_{50} of MCF-7 cells was 6.2 μM, vs. an IC_{50} of 37.78 μM for the MCF-7/Adr cells. However, the Dox IC_{50} of MCF-7/Adr cell was 18.09 μM (0.05 μM CuB treatment) and 12.94 μM (0.1 μM CuB treatment) (Table II). The resistance index (RI) of MCF-7/Adr parent group was 6.09, the MCF-7/Adr CuB 0.05 μM group

Table I. IC_{50s} of CuB on breast cancer cell lines.

Cell lines	MCF-7	MCF-7/Adr
IC_{50} (μM)	0.45±0.03	0.66±0.05

The cells were treated with CuB at various concentrations for 24 h, the cell cytotoxicity was analyzed by MTT assay, and the IC_{50} was calculated using CalcuSyn (version 2.0; Biosoft, Cambridge, UK). Values shown are means plus or minus SD of quadruplicate determination.

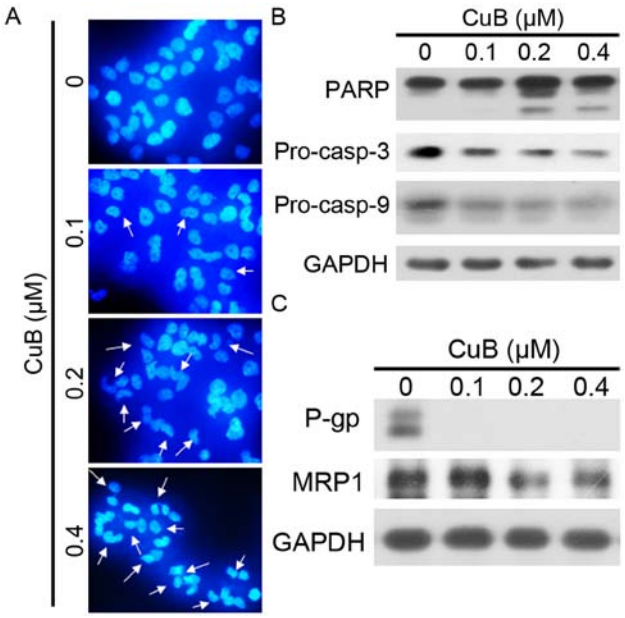

Figure 3. CuB induces apoptosis and influences the expression of P-gp, MRP1 in MCF-7/Adr cells. (A) MCF-7/Adr cells were incubated with various doses of CuB for 24 h. Cells were examined by DAPI staining. (B and C) MCF-7/Adr cells were treated with increasing concentrations of CuB for 24 h. Western blot analysis was conducted using the indicated antibodies.

2.91 and the MCF-7/Adr CuB 0.1 μM group 2.09. The RI calculation formula was used to find the IC_{50} of resistant cells/IC_{50} of sensitive cells. Following the treatment with CuB (0.05 and 0.1 μM), the IC_{50} of Dox to the MCF-7/Adr cells was reduced from 37.78 to 18.09 and 12.94 μM by reversion fold (RF) 2.09- and 2.92-fold. The RF calculation formula was used to find the IC_{50} of Dox on MCF-7/Adr cells/IC_{50} of Dox (with 0.05 or 0.10 μM CuB) on MCF-7/Adr cells. Thus, low doses of CuB (0.05 and 0.1 μM) can reverse Dox resistance.

CuB induces apoptosis and influences the expression of P-gp, MRP1 in MCF-7/Adr cells. We next tested whether or not CuB induces apoptosis of MCF-7/Adr cells. By an optical light microscope, we found some dead MCF-7/Adr cells floating in the medium treated with CuB. The cell death is reminiscent of the phenomena induced by apoptosis. We investigated the nucleus morphological changes by DAPI staining. As shown in Fig. 3A, we observed the nuclear condensation and fragmentation with CuB treatment which are typical changes in cell apoptosis. Furthermore, a western blot analysis was used to detect the activation of the caspase-9 (casp-9) initiator caspase,

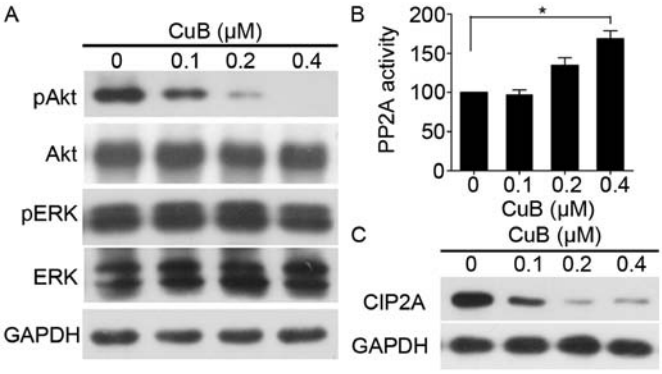

Figure 4. CuB targets CIP2A to reactivate protein phosphatase 2A. (A) MCF-7/Adr cells were treated with increasing concentrations of CuB for 24 h. Western blot analysis was conducted using the indicated antibodies. (B) MCF-7/Adr cells were treated with increasing concentrations of CuB for 24 h. PP2A activity was measured by PP2A immunoprecipitation phosphatase assay. *P<0.05. (C) MCF-7/Adr cells were treated with increasing concentrations of CuB for 24 h. Western blot analysis was conducted using the indicated antibodies.

caspase-3 (casp-3) effector caspase and its substrate, poly(ADP-ribose) polymerase (PARP) (Fig. 3B). CuB was demonstrated to induce a significant dose-dependent decrease in pro-casp-9, pro-casp-3 and the cleavage of PARP, in MCF-7/Adr cells, indicating that CuB induced caspase-dependent apoptosis. We detected expression levels of MDR related factors P-gp and MRP1 by western blot analysis (Fig. 3C). The results indicated that P-gp, and MRP1 expression of MCF-7/Adr cells were downregulated by treatment with increasing concentration of CuB. In addition, the decreased expression of P-gp and MRP1 in MCF-7/Adr cells may in part contribute to the reversal of MDR.

CuB targets CIP2A to reactivate protein phosphatase 2A. Phosphorylated Akt (pAkt) is a cancer MDR locus (27). We next investigated the role of Akt in CuB-induced apoptosis in MCF-7/Adr cells. As shown in Fig. 4A, CuB decreased Akt phosphorylation in a dose-dependent manner. PP2A, one of the main serine-threonine phosphatases, plays a critical role in the regulation of cell cycle progression, survival, and differentiation by negatively regulating the PI3K/Akt pathway and dephosphorylating and inactivating MEK1 and ERK family kinases (26). We tested effects of CuB on PP2A activity, and found that CuB upregulated PP2A activity (Fig. 4B). CIP2A is an oncogenic PP2A inhibitor protein that is highly expressed in malignant cancers (28). Furthermore, we tested effects of CuB on CIP2A expression and found that the protein level of CIP2A decreased (Fig. 4C), indicating that CuB targeted CIP2A, at least in part, to reactivate PP2A. Taken together, these data indicate that the CIP2A/PP2A/Akt pathway may mediate the sensitizing effect of CuB.

Silencing CIP2A enhances CuB-induced growth inhibition and apoptosis in MCF-7/Adr. We next examined whether CIP2A knockdown would alter cellular sensitivity to CuB. Two siRNAs targeting CIP2A were synthesized and used. As shown in Fig. 5A, knockdown by both siRNA1 and siRNA2 markedly decreased CIP2A protein in MCF-7/Adr cells. These data showed that CIP2A siRNA1 and 2 were specific and efficient

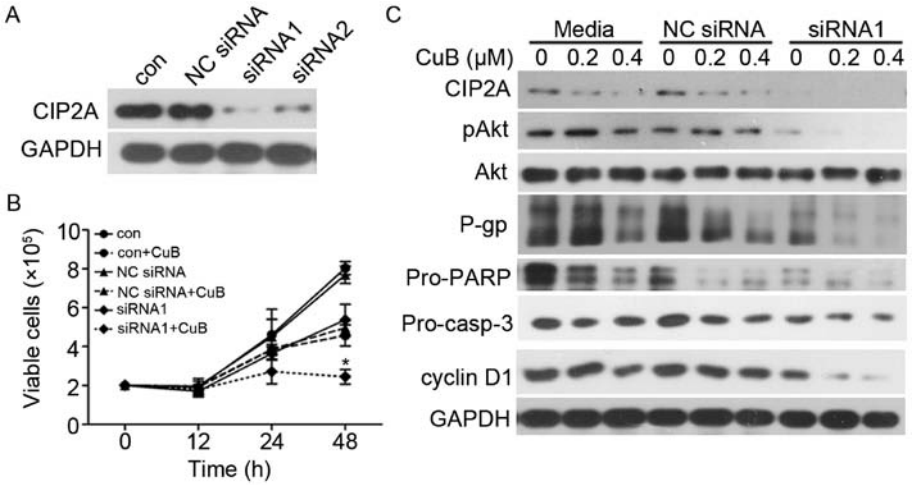

Figure 5. Silencing CIP2A enhances CuB-induced growth inhibition and apoptosis in MCF-7/Adr. (A) MCF-7/Adr cells were transfected with 100 nM CIP2A-specific siRNA or NC siRNA for 48 h. Cells were harvested for western blot analyses. (B) MCF-7/Adr cells were transfected with CIP2A-specific siRNA or NC siRNA, followed by treatment with CuB (0.4 μM) for indicated times, and analyzed by trypan blue exclusion assay. *P<0.05. (C) MCF-7/Adr cells were transfected with CIP2A-specific siRNA or NC siRNA, followed by treatment with CuB (0, 0.2 and 0.4 μM) for 24 h. Western blot analysis was conducted using the indicated antibodies.

Table II. Reversing effect of CuB on MCF-7/Adr cells.

Groups, CuB (μM)+Dox (μM)	Inhibition rate (%)	IC_{50} (μM)	Resistance index	Reversion fold
0+5	9.51±0.53	37.78	6.09	1
0+10	14.84±2.94			
0+20	23.75±3.61			
0+30	52.03±5.36			
0+50	71.64±8.34			
0+80	83.35±3.89			
0.05+5	15.35±2.74	18.09	2.91	2.09
0.05+10	25.62±3.88			
0.05+20	36.48±4.92			
0.05+30	63.65±5.12			
0.05+50	86.46±3.71			
0.05+80	89.61±7.15			
0.1+5	20.17±3.58	12.94	2.09	2.92
0.1+10	35.72±5.46			
0.1+20	48.69±5.77			
0.1+30	75.47±4.83			
0.1+50	89.43±6.72			
0.1+80	98.51±6.02			

The Dox IC_{50} of MCF-7 cell was 6.20 μM. Data are presented as the mean ± SD; n=4. The RI calculation formula was used to find the IC_{50} of resistant cell/IC_{50} of sensitive cells. The RF calculation formula was used to find the IC_{50} of Dox on MCF-7/Adr cells/ IC_{50} of Dox (with 0.05 or 0.10 μM CuB) on MCF-7/Adr cells.

in reducing CIP2A expression. To evaluate the role of CIP2A in CuB-induced proliferation inhibition, MCF-7/Adr cells were transfected with siRNA1 or siRNA2 targeting CIP2A, followed by CuB treatment. Cell viability and western blot analysis were used to detect cell growth and protein expression. CIP2A silencing enhanced CuB-induced growth inhibition (Fig. 5B) and promoted CuB-induced apoptotic effect (Fig. 5C). Notably, CIP2A depletion also enhanced CuB-induced MDR inhibition. These data demonstrate that CIP2A plays a critical role in CuB-reversed MCF-7/Adr multidrug resistance.

Discussion

Tumorigenesis and chemoresistance is tightly related to malfunction of the cellular signaling pathways that control cell proliferation, survival, or death. The malfunction contains

increased expression of ATP-dependent drug efflux pumps and decreased influx, increased drug metabolism enzymes, impairment of cell death pathways, enhancement of cell survival pathways, alternation of drug metabolism, mutations in cell cycle pathways and superior DNA repair (29). The aims of the present study were to identify an effective MDR reversing agent with fewer side-effects and to gain insight regarding its molecular mechanism. Compounds from natural source constitute an indispensable candidate drug library for pharmacotherapy. CuB is a representative therapeutic agent for anticancer activities. Recently, CuB was reported to enhance the anticancer effects of cisplatin, gemcitabine, methotrexate, docetaxel, and gemcitabine in laryngeal squamous, pancreatic, and breast cancers and osteosarcoma (30). However, the relationship between CuB and Dox-resistance in MCF-7/Adr cells has yet to be firmly established. Inhibition of cell proliferation is an efficient strategy in cancer therapy. In the present study, we first showed that CuB inhibited MCF-7/Adr cell proliferation (Fig. 2B and C), cell viability (Fig. 2D) and soft-agar colony formation (Fig. 2E).

We investigated the effect of the combination of CuB and Dox on the MCF-7/Adr cells and identified that CuB in combination with Dox had an improved effect compared with Dox or CuB alone. Following the treatment with CuB (0.05 and 0.1 μM), the IC_{50} of Dox to the MCF-7/Adr cells was significantly reduced from 37.78 to 18.09 and 12.94 μM by 2.09- and 2.92-fold (Table II). The RI of of MCF-7/Adr parent group was 6.09, the MCF-7/Adr CuB 0.05 μM group 2.91 and the MCF-7/Adr CuB 0.1 μM group 2.09, respectively. Furthermore, the present study explored the mechanisms of CuB in reversing Dox resistance.

Evading apoptosis is one of the hallmarks of drug resistance, and targeting apoptosis has become a cancer therapeutic strategy (31). Apoptosis is accompanied by various morphological changes, including nuclear condensation, apoptotic bodies, DNA fragmentation and cell surface changes. Nuclear morphology in MCF-7/Adr cells was analyzed using DAPI staining; we found changes in nucleus condensation, which are typical characteristics of apoptosis (Fig. 3A). Therefore, CuB might have the ability of induction of cell apoptosis. The mechanisms of apoptosis involve two signaling pathways: the mitochondrial pathway (intrinsic apoptotic pathway) and the cell death receptor pathway (extrinsic apoptotic pathway) (32). The extrinsic and intrinsic apoptotic pathways that ultimately lead to activation of effector caspases (casp-3, -2 and -7) have been characterized (33,34). The decrease of pro-casp-9, pro-casp-3, as well as the proteolysis of PARP (Fig. 3B), indicate that casp-3 is activated. Thus, CuB may trigger apoptosis by activating the intrinsic apoptosis pathway which results in activation of effector casp-9. Drug resistance is largely mediated through overexpression of MDR, MRP, drug resistance protein, and proteasome subunits, increases in antioxidant defenses, and TOP2 activity; these results have been widely verified (35-37). The present study identified that the treatment of CuB was able to reverse the MDR of the MCF-7/Adr cells via the downregulation of P-gp and MRP1 (Fig. 3C).

PP2A is a serine/threonine phosphatase that has a critical role in regulating various cellular processes, including signaling transduction, protein synthesis, cell cycle determination, metabolism, apoptosis and stress response (38). Because loss of PP2A function has been identified in various malignant diseases such as cancer of the colon, liver, lung and breast, it has been suggested that PP2A functions as a tumor suppressor and enhancing PP2A activity could be an effective approach for anticancer treatment (39). In this study, we showed that by inhibiting CIP2A, activity of PP2A was significantly enhanced and expression of pAkt was downregulated in MCF-7/Adr cells (Fig. 4). Several cellular inhibitors of PP2A have been identified, including SET (26) and CIP2A. CIP2A, originally named KIAA1524 or P90, has been cloned from patients with HCC (8). CIP2A is associated with clinical aggressiveness in human breast cancer and promotes the malignant growth and metastasis of breast cancer cells. Induction of CIP2A is often associated with chemoresistance in cancer cells, and the inhibition of CIP2A in combination with chemotherapy may enhance the efficacy of cancer treatment (40). We knocked down CIP2A expression in MCF-7/Adr cells and found that CIP2A depletion significantly promoted CuB induced apoptosis and reversed MDR (Fig. 5). Our data validated the mechanism by which CuB-reversed MDR and induced cancer cell apoptosis in MCF-7/Adr cells, that is, reversed MDR and induction of cancer cell apoptosis by inhibiting CIP2A to reactivate PP2A and enhance PP2A-dependent pAkt downregulation.

In conclusion, our results suggest that further studies investigating the detailed molecular modification of the PP2A/CIP2A signaling pathway by CuB and exploring its possible application in other malignant diseases are warranted.

Acknowledgements

The present study was supported by grants from the National Natural Sciences Foundation of China (grant no. 81400157); the Natural Science Foundation of Hubei Provincial Department of Education (grant no. Q20152106); the Faculty Development Grants from Hubei University of Medicine (grant nos. 2014QDJZR08, 2015QDJZR16); the Foundation for Innovative Research Team of Hubei University of Medicine (grant no. 2014CXX05); the Key Discipline Project of Hubei University of Medicine and the National Training Program of Innovation and Entrepreneurship for Undergraduates (grant no. 201610929001).

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2015. CA Cancer J Clin 65: 5-29, 2015.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. CA Cancer J Clin 65: 87-108, 2015.
3. Zhu XF, Li W, Ma JY, Shao N, Zhang YJ, Liu RM, Wu WB, Lin Y and Wang SM: Knockdown of heme oxygenase-1 promotes apoptosis and autophagy and enhances the cytotoxicity of doxorubicin in breast cancer cells. Oncol Lett 10: 2974-2980, 2015.
4. Zheng Y, Lv X, Wang X, Wang B, Shao X, Huang Y, Shi L, Chen Z, Huang J and Huang P: MiR-181b promotes chemoresistance in breast cancer by regulating Bim expression. Oncol Rep 35: 683-690, 2016.
5. Faneyte IF, Kristel PM, Maliepaard M, Scheffer GL, Scheper RJ, Schellens JH and van de Vijver MJ: Expression of the breast cancer resistance protein in breast cancer. Clin Cancer Res 8: 1068-1074, 2002.
6. Jeon YJ, Kim JH, Shin JI, Jeong M, Cho J and Lee K: Salubrinal-mediated upregulation of eIF2α phosphorylation increases doxorubicin sensitivity in MCF-7/ADR cells. Mol Cells 39: 129-135, 2016.

7. Perrotti D and Neviani P: Protein phosphatase 2A: A target for anticancer therapy. Lancet Oncol 14: e229-e238, 2013.
8. Junttila MR, Puustinen P, Niemelä M, Ahola R, Arnold H, Böttzauw T, Ala-aho R, Nielsen C, Ivaska J, Taya Y, *et al*: CIP2A inhibits PP2A in human malignancies. Cell 130: 51-62, 2007.
9. Seshacharyulu P, Pandey P, Datta K and Batra SK: Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer. Cancer Lett 335: 9-18, 2013.
10. Schönthal AH: Role of serine/threonine protein phosphatase 2A in cancer. Cancer Lett 170: 1-13, 2001.
11. Lv P, Wang Y, Ma J, Wang Z, Li JL, Hong CS, Zhuang Z and Zeng YX: Inhibition of protein phosphatase 2A with a small molecule LB100 radiosensitizes nasopharyngeal carcinoma xenografts by inducing mitotic catastrophe and blocking DNA damage repair. Oncotarget 5: 7512-7524, 2014.
12. Li W, Ge Z, Liu C, Liu Z, Björkholm M, Jia J and Xu D: CIP2A is overexpressed in gastric cancer and its depletion leads to impaired clonogenicity, senescence, or differentiation of tumor cells. Clin Cancer Res 14: 3722-3728, 2008.
13. Liu Z, Ma L, Wen ZS, Hu Z, Wu FQ, Li W, Liu J and Zhou GB: Cancerous inhibitor of PP2A is targeted by natural compound celastrol for degradation in non-small-cell lung cancer. Carcinogenesis 35: 905-914, 2014.
14. Ren J, Li W, Yan L, Jiao W, Tian S, Li D, Tang Y, Gu G, Liu H and Xu Z: Expression of CIP2A in renal cell carcinomas correlates with tumour invasion, metastasis and patients' survival. Br J Cancer 105: 1905-1911, 2011.
15. Liu CY, Shiau CW, Kuo HY, Huang HP, Chen MH, Tzeng CH and Chen KF: Cancerous inhibitor of protein phosphatase 2A determines bortezomib-induced apoptosis in leukemia cells. Haematologica 98: 729-738, 2013.
16. Choi YA, Park JS, Park MY, Oh KS, Lee MS, Lim JS, Kim KI, Kim KY, Kwon J, Yoon Y, *et al*: Increase in CIP2A expression is associated with doxorubicin resistance. FEBS Lett 585: 755-760, 2011.
17. Liu J, Wang M, Zhang X, Wang Q, Qi M, Hu J, Zhou Z, Zhang C, Zhang W, Zhao W, *et al*: CIP2A is associated with multidrug resistance in cervical adenocarcinoma by a P-glycoprotein pathway. Tumour Biol 7: 2673-2682, 2015.
18. Lai GM, Ozols RF, Young RC and Hamilton TC: Effect of glutathione on DNA repair in cisplatin-resistant human ovarian cancer cell lines. J Natl Cancer Inst 81: 535-539, 1989.
19. El-Senduny FF, Badria FA, El-Waseef AM, Chauhan SC and Halaweish F: Approach for chemosensitization of cisplatin-resistant ovarian cancer by cucurbitacin B. Tumour Biol 37: 685-698, 2015.
20. Chan KT, Meng FY, Li Q, Ho CY, Lam TS, To Y, Lee WH, Li M, Chu KH and Toh M: Cucurbitacin B induces apoptosis and S phase cell cycle arrest in BEL-7402 human hepatocellular carcinoma cells and is effective via oral administration. Cancer Lett 294: 118-124, 2010.
21. Chan KT, Li K, Liu SL, Chu KH, Toh M and Xie WD: Cucurbitacin B inhibits STAT3 and the Raf/MEK/ERK pathway in leukemia cell line K562. Cancer Lett 289: 46-52, 2010.
22. Liu Y, Cao W, Zhang B, Liu YQ, Wang ZY, Wu YP, Yu XJ, Zhang XD, Ming PH, Zhou GB, *et al*: The natural compound magnolol inhibits invasion and exhibits potential in human breast cancer therapy. Sci Rep 3: 3098, 2013.
23. Cao W, Liu Y, Zhang R, Zhang B, Wang T, Zhu X, Mei L, Chen H, Zhang H, Ming P, *et al*: Homoharringtonine induces apoptosis and inhibits STAT3 via IL-6/JAK1/STAT3 signal pathway in Gefitinib-resistant lung cancer cells. Sci Rep 5: 8477, 2015.
24. Chou CC, Yang JS, Lu HF, Ip SW, Lo C, Wu CC, Lin JP, Tang NY, Chung JG, Chou MJ, *et al*: Quercetin-mediated cell cycle arrest and apoptosis involving activation of a caspase cascade through the mitochondrial pathway in human breast cancer MCF-7 cells. Arch Pharm Res 33: 1181-1191, 2010.
25. Liu Y, Dong Y, Zhang B and Cheng YX: Small compound 6-*O*-angeloylplenolin induces caspase-dependent apoptosis in human multiple myeloma cells. Oncol Lett 6: 556-558, 2013.
26. Liu H, Gu Y, Wang H, Yin J, Zheng G, Zhang Z, Lu M, Wang C and He Z: Overexpression of PP2A inhibitor SET oncoprotein is associated with tumor progression and poor prognosis in human non-small cell lung cancer. Oncotarget 6: 14913-14925, 2015.
27. Radisavljevic Z: AKT as locus of cancer multidrug resistance and fragility. J Cell Physiol 228: 671-674, 2013.
28. Rincón R, Cristóbal I, Zazo S, Arpí O, Menéndez S, Manso R, Lluch A, Eroles P, Rovira A, Albanell J, *et al*: PP2A inhibition determines poor outcome and doxorubicin resistance in early breast cancer and its activation shows promising therapeutic effects. Oncotarget 6: 4299-4314, 2015.
29. Holohan C, Van Schaeybroeck S, Longley DB and Johnston PG: Cancer drug resistance: An evolving paradigm. Nat Rev Cancer 13: 714-726, 2013.
30. Guo J, Zhao W, Hao W, Ren G, Lu J and Chen X: Cucurbitacin B induces DNA damage, G2/M phase arrest, and apoptosis mediated by reactive oxygen species (ROS) in leukemia K562 cells. Anticancer Agents Med Chem 14: 1146-1153, 2014.
31. Hanahan D and Weinberg RA: Hallmarks of cancer: The next generation. Cell 144: 646-674, 2011.
32. Marsden VS, O'Connor L, O'Reilly LA, Silke J, Metcalf D, Ekert PG, Huang DC, Cecconi F, Kuida K, Tomaselli KJ, *et al*: Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. Nature 419: 634-637, 2002.
33. Nicholson DW: Caspase structure, proteolytic substrates, and function during apoptotic cell death. Cell Death Differ 6: 1028-1042, 1999.
34. Johnstone RW, Ruefli AA and Lowe SW: Apoptosis: A link between cancer genetics and chemotherapy. Cell 108: 153-164, 2002.
35. Alcantara LM, Kim J, Moraes CB, Franco CH, Franzoi KD, Lee S, Freitas-Junior LH and Ayong LS: Chemosensitization potential of P-glycoprotein inhibitors in malaria parasites. Exp Parasitol 134: 235-243, 2013.
36. Luo L, Sun YJ, Yang L, Huang S and Wu YJ: Avermectin induces P-glycoprotein expression in S2 cells via the calcium/calmodulin/NF-κB pathway. Chem Biol Interact 203: 430-439, 2013.
37. Ying L, Zu-An Z, Qing-Hua L, Qing-Yan K, Lei L, Tao C and Yong-Ping W: RAD001 can reverse drug resistance of SGC7901/DDP cells. Tumour Biol 35: 9171-9177, 2014.
38. Cristóbal I, Rincón R, Manso R, Caramés C, Zazo S, Madoz-Gúrpide J, Rojo F and García-Foncillas J: Deregulation of the PP2A inhibitor SET shows promising therapeutic implications and determines poor clinical outcome in patients with metastatic colorectal cancer. Clin Cancer Res 21: 347-356, 2015.
39. Yu HC, Hung MH, Chen YL, Chu PY, Wang CY, Chao TT, Liu CY, Shiau CW and Chen KF: Erlotinib derivative inhibits hepatocellular carcinoma by targeting CIP2A to reactivate protein phosphatase 2A. Cell Death Dis 5: e1359, 2014.
40. Côme C, Laine A, Chanrion M, Edgren H, Mattila E, Liu X, Jonkers J, Ivaska J, Isola J, Darbon JM, *et al*: CIP2A is associated with human breast cancer aggressivity. Clin Cancer Res 15: 5092-5100, 2009.