Abstract. Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a human oncoprotein that is overexpressed in multiple kinds of tumors including gastric cancer (GC). Mammalian target of rapamycin complex 1 (mTORC1) over-activation is detected in GC and many other cancers. Previous study found that CIP2A/mTORC1 controls cell growth and autophagy through direct association. CIP2A plays an ‘oncogenic nexus’ in several cancer types to participate in the tumorigenic transformation and chemoresistance. In the present study, we investigated whether Cucurbitacin B (CuB), a natural compound found in Cucurbitaceae, can be used in cisplatin (DDP)-resistant human GC cell line SGC7901/DDP. Results demonstrated that CuB treatment significantly suppressed SGC7901/DDP cell proliferation, induced caspase-dependent apoptosis, and autophagy. The activation of autophagy was mediated through CuB-induced inhibition of mTORC1. Furthermore, CuB inhibited mTORC1 via the activation of protein phosphatase 2A (PP2A) which is mediated by CIP2A inhibition. These findings indicated that CuB can inhibit the proliferation, induce caspase-dependent apoptosis, and autophagy of SGC7901/DDP cells by suppressing CIP2A/PP2A/mTORC1 signaling axis. Thus, CuB may be a novel effective candidate to treat DDP-resistant human GC cells.

Introduction

Gastric cancer (GC) is a type of common and fatal digestive malignant disease worldwide. Despite recent advances in diagnosis and treatment as well as declining incidence in some developed countries, it remains a major cause of cancer-related deaths in Eastern Asia (including China, Japan, and South Korea) (1,2). GC in early stages is curable by using endoscopic procedures. In China, approximately two-thirds of patients develop advanced or metastatic disease, and >50% have recurrent disease following curative surgery. Effective therapeutic approaches are still limited (3). Systematic chemotherapy is an extremely important therapeutic strategy for advanced GC. Among the patients, many are resistant to chemotherapy agents, including cisplatin (DDP), adriamycin, and 5-fluorouracil. Multidrug resistance (MDR) is largely responsible for ineffective chemotherapy (4). The mechanism of MDR remains unclear. Increased expression of MDR-associated protein (MRP), P-glycoprotein (P-gp), increased DNA damage repair, cell cycle arrest, and resistance of tumor cells to apoptosis are the mechanisms that might account for MDR (5). A large number of studies have shown that the dysfunction of signaling pathways in cancer cells is the major reason for tumorigenesis and metastasis (6). Therefore, finding novel therapeutic strategies and key target molecules for reversing resistance and diminishing the side effects of chemotherapy agents are the main goals of drug resistance cancer treatment protocol.

Mammalian target of rapamycin complex 1 (mTORC1), a complex composed of mTOR, Raptor and mSIN1, controls protein translation, cell growth, and metabolism (7). mTORC1 over-activation is detected in GC and many other cancers (7-11). mTORC1 activation could induce transcription of a number of key oncoproteins and MDR-associated proteins including cyclin D1 and hypoxia-inducible factor 1α (HIF-1α) (12). Activation of mTORC1 results in phosphorylation of important effectors [i.e., ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)] involved in cancer progression and...
apoptosis resistance. In addition, mTORC1 is also the major negative regulator of autophagy (7). Therefore, targeting mTORC1 may be an attractive therapeutic strategy for cancer drug resistance treatment.

The activation of mTORC1 is induced by numerous oncoproteins and mitogenic factors via the class I phosphoinositide3 kinase (PI3K)/Akt pathway (13). Cancers are induced by numerous oncoprotein that stabilizes c-Myc and activates Akt by inhibiting protein phosphatase 2A (PP2A)-mediated dephosphorylation of c-Myc and Akt (14). CIP2A can associate with mTORC1 directly and act as an allosteric inhibitor of mTORC1-associated PP2A, thereby enhancing mTORC1-dependent growth signaling and inhibiting autophagy (7). An increased expression of CIP2A protein was found in GC and several other cancers (14-18). In 2015, Zhang et al reported that CIP2A expression is associated with DDP resistance (19). Thus, effective and discerning CIP2A-mTORC1 axis inhibitors would be beneficial for therapy to DDP resistance and induce autophagy in GC.

Cucurbitacin B (CuB) is a natural product that is extensively distributed in medicinal plants Cucurbitaceae family is shown to inhibit the growth of numerous human cancer cell lines such as colon, breast, leukemia, pancreatic hepatic, and glioblastoma (20-22). The effect of CuB on DDP-resistant GC cells previously has not been evaluated. The aim of this work was to investigate antitumor effects and possible mechanisms of CuB on DDP resistant human GC cells.

**Materials and methods**

**Reagents.** CuB with a purity of up to 98% was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. 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. DDP and 3-methyladenine (3-MA, #M9281) were purchased from Sigma-Aldrich.

**Cell culture.** Human DDP-resistant gastric cancer cell line SGC7901/DDP and human GC cell line SGC7901 were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). SGC7901/DDP cells were grown in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Chalfont, UK), 500 ng/ml DDP, and antibiotics and incubated in a humidified atmosphere without CO₂ at 37˚C. SGC7901 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) with FBS and antibiotics and incubated in a humidified atmosphere with CO₂ at 37˚C.

**Cytotoxic assay and cell viability.** Cells were seeded into 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 A490 of the control group - average A490 of the experimental group) / (average A490 of the control group) x100%. Cell viability was estimated by trypan blue dye exclusion (23,24).

**Western blotting.** 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; Merck Millipore, Darmstadt, Germany), and 1% protease inhibitors cocktail (Merck Millipore). Lysates were normalized for total protein (25 µg) and loaded on 8-12% sodium dodecyl sulfate polyacrylamide gel, electrophoresed, and transferred to a PVDF membrane (Merck & Co., Inc., Kenilworth, NJ, USA), followed by blocking with 5% skimmed milk at room temperature for 1 h. The membrane was incubated with primary antibodies overnight at 4˚C and rinsed with Tris-buffered saline with Tween-20.

The primary antibodies used were anti-MRP1 (ratio of 1:500 dilution; catalog no. sc-13960), anti-CIP2A (1:500; catalog no. sc-80662), anti-phospho-Akt (S473) (1:500; catalog no. sc-7985), anti-Akt (1:500; catalog no. sc-8312), (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-caspase-9 (1:1000; catalog no. 9508), anti-caspase-3 (1:1000; catalog no. 9662), anti-PARP (1:1000; catalog no. 9542), anti-PP2A (1:1000; catalog no. 2038), anti-LC3B (1:1000; catalog no. 2775), anti-HIFα (1:1000; catalog no. 5537), anti-p4E-BP1 (Thr37/46) (1:1000; catalog no. 2855), anti-mTOR (1:1000; catalog no. 2983), anti-pmTOR (Ser2448) (1:1000; catalog no. 5536), anti-P70S6K (1:1000; catalog no. 9202), anti-pP70S6K (1:1000; catalog no. 92775) (Cell Signaling Technology, Danvers, MA, USA), anti-Bcl-1 (1:2000; catalog no. 11306-1-AP), anti-4E-BP1 (1:2000; catalog no. 60624-1-IG) (Proteintech Group, Inc., Rosemont, USA), anti-P-gp (1:2000; catalog no. ab170904) (Abcam, Cambridge, UK), and anti-GAPDH (1:5000; catalog no. M20006; Abmart, Shanghai, China).

The blots were then washed, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; catalog no. E030120-01 and E030110-01; EarthOx, LLC, San Francisco, CA, USA) at room temperature for 1.5 h. Detection was performed by using a SuperSignal® West Pico Trial kit (catalog no. QA210131; Pierce Biotechnology, Inc., Rockford, IL, USA) (25). The defined sections of the film were scanned for image capture and quantification using Adobe Photoshop software (CS4, Adobe Systems Inc., San Jose, CA, USA) and ImageJ software (National Institutes of Health, Bethesda, MD, USA).
Autophagy assays. The cells were transfected with pQCXIP-GFP-LC3 plasmid using the Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the recommended protocol by the manufacturer and then fixed in 4% paraformaldehyde. The percentage of cells with fluorescent dots representing GFP-LC3 translocation was counted by Olympus confocal laser scanning microscope.

PP2A activity assay. PP2A immunoprecipitation phosphatase assay kit (Upstate, 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. Protein A agarose beads (40 µl) 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. 100 µl of Malachite Green Phosphate Detection Solution was added and the absorbance at 650 nm was measured on a microplate reader (27).

Transfection of DNA and siRNA. The pOTENT-1-CIP2A expression plasmid was purchased from Youbio Co. (Changcha, China). Transfection of the pOTENT-1-CIP2A plasmid into GC cells were carried out using Lipofectamine 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer’s protocol. Two siRNAs targeting CIP2A were designed and synthesized by Shanghai GenePharma Co., referred to as siRNA1 and siRNA2. The siRNA sequences were as follows: 5’-CUGUGGUUGUUGUUUGCACUTT-3’ (CIP2A siRNA1), 5’-ACUAAUGAUCUAGAATT-3’ (CIP2A siRNA2), 5’-UCUCCGAACGUGUCACUGUUTT-3’ [negative control (NC) siRNA].

Using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol, SGC7901/DDP cells were transfected with 100 nM siRNA. At 48 h after transfection, the cells were then harvested for western blotting, and cell viability (28).

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 hoc test. P-values <0.05 indicate statistical significance.

Results

Chemical structure of CuB and characterization of SGC7901 and SGC7901/DDP GC cells. The chemical structure of CuB is shown in Fig. 1A. Firstly, the P-gp, HIF-1α, and CIP2A expression was compared between SGC7901 and SGC7901/DDP cells, which confirmed P-gp, HIF-1α, and CIP2A were overexpressed in SGC7901/DDP cells (Fig. 1B). SGC7901 and SGC7901/DDP cells were exposed to various concentrations of DDP (2.5-80 µM) for 24 h. The half-maximal inhibitory concentration (IC50) of DDP against SGC7901 cells is 6.2 µM. while IC50 of DDP against SGC7901/DDP cells is 37.78 µM. As shown in Fig. 1C, the DDP cytotoxicity was higher in SGC7901 cells than in SGC7901/DDP cells.

Effects of CuB on SGC7901 and SGC7901/DDP GC cells. SGC7901 and SGC7901/DDP cells were seeded in 96-well plates for 24 h, 48 h and then treated with different concentrations of CuB and DDP (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 SGC7901 and SGC7901/DDP cells with an IC50 of 216.70 and 170.25 nM (Table I). By trypan blue exclusion assay, we found that CuB rapidly reduced viable SGC7901/DDP cells (Fig. 2D) in a dose- and time-dependent manner. We
investigated the CuB effect on cell colony formation activity, and the results showed that CuB significantly inhibited the clonogenic ability of SGC7901/DDP (Fig. 2E). These results suggested that CuB inhibited the anchorage-dependent (cell proliferation) and anchorage-independent (colony formation) growth of SGC7901/DPP cells.

**CuB induces apoptosis and autophagic death of SGC7901/DDP GC cells.** To investigate whether CuB induced apoptosis in SGC7901/DDP GC cells, microscopy with DAPI staining was carried out. As shown in Fig. 3A, cytoplasmic shrinkage occurred after treatment with 100 nM CuB, supporting that apoptosis was induced. To determine which cell death pathways was induced by CuB, SGC7901/DDP cells were treated with increasing concentrations of CuB for 24 h. Western blotting results showed that PARP cleavage, as well as a significant dose-dependent decrease in pro-caspases-3 and -9 (pro-casp-3 and pro-casp-9), were detected in CuB-treated cells (Fig. 3A and B). These results indicate that CuB induces cell death via caspase-dependent apoptosis.

Autophagy is a lysosomal degradation process for cytoplasmic constituents during the stress condition. To assess whether autophagy is also involved in CuB-induced cell death, we subsequently evaluated the expression level of LC3 II and Beclin1 of cells treated with CuB. A marked increase of LC3 II and Beclin1 was observed in CuB treated cells (Fig. 3C). When autophagy is initiated, LC3 is cut on the C-terminal and produces LC3 II protein, which is then transferred on the autophagosomes (LC3-positive vesicle) (29). To detect the autophagosome formation, the fluorescent autophagy marker GFP-LC3 plasmid was transfected into SGC7901/DDP cells which were then treated with CuB for 24 h, followed by confocal microscopy assessment. We showed that while control cells displayed a diffuse staining, SGC7901/DDP cells upon CuB exhibited a speckled fluorescent staining pattern, indicating the redistribution of LC3 to autophagosomes (Fig. 3D). Autophagy has been reported to play contradictory roles in tumor progression or suppression (30). To demonstrate CuB induced autophagic cell death, CuB and autophagy inhibitor 3-MA was combined to treat SGC7901/DDP cells (Fig. 3E). Our results showed that 3-MA significantly reversed the cell proliferation inhibited by CuB. Taken together, these findings suggest that CuB induces caspase-dependent apoptosis and autophagic death of SGC7901/DPP cells.

**CuB influences the expression of P-gp, HIF-1α and inhibits CIP2A/PP2A/mTORC1 signaling axis in SGC7901/DDP cells.** We detected expression levels of MDR related factors...
P-gp, and HIF-1α by western blotting analysis (Fig. 4A). The results indicated that P-gp, and HIF-1α expression of SGC7901/DDP cells were downregulated by treatment with increasing concentration of CuB. The decreased expression...
of P-gp and HIF-1α in SGC7901/DDP cells may in part contribute to the reversal of MDR. Considering that mTORC1 is a major negative regulator of autophagy (7), and CuB activates autophagy in SGC7901/DDP cells, we next determined whether CuB inhibited the activation of mTORC1. As shown in Fig. 4B, CuB decreased the phosphorylation of mTORC1 effectors (mTOR, p70S6K and 4E-BP1). Akt is an upstream regulator of mTORC1 and a downstream substrate of serine-threonine phosphatase PP2A (9). We tested effects of CuB on Akt phosphorylation (pAkt) and PP2A activity, and found that CuB downregulated pAkt and upregulated PP2A activity (Fig. 4C and D). These results suggested that CuB-induced mTORC1 inactivation may be mediated by PP2A/Akt. CIP2A is an oncogenic PP2A inhibitor protein that is highly expressed in malignant cancers including GC (31). Furthermore, we tested effects of CuB on CIP2A expression and found that the protein level of CIP2A decreased (Fig. 4E), indicating that CuB may target CIP2A to reactivate PP2A, then inhibits Akt/mTORC1 signal pathway. CIP2A is essential for CuB-induced proliferation, autophagy, and apoptosis. We next examined whether CIP2A depletion would alter cellular sensitivity to CuB. Two siRNAs targeting CIP2A were synthesized and used. As shown in Fig. 5A, knockdown by siRNA markedly decreased CIP2A protein in SGC7901/DDP cells. These data showed that CIP2A siRNA was specific and efficient in reducing CIP2A expression. To evaluate the role CIP2A plays in CuB-induced proliferation inhibition, SGC7901/DDP cells were transfected with a CIP2A expression plasmid (CIP2AOE), and then western blotting was used to detect CIP2A expression 48 h after transfection. (E) SGC7901/DDP cells were transfected with a CIP2A expression plasmid (CIP2A OE), and then trypan blue exclusion assay were used to detect proliferation, 48 h after transfection. (F) SGC7901/DDP cells were transfected with a CIP2A expression plasmid (CIP2A OE) for 48 h.
overexpression antagonized CuB-induced growth inhibition (Fig. 5E) and inhibited CuB-induced autophagy and apoptosis effects by upregulating pAkt and pmTOR (Fig. 5F).

These data demonstrate that CIP2A plays a critical role in CuB-induced SGC7901/DDP autophagy and apoptosis.

Discussion

DDP, as a first-line chemotherapeutic drug for GC patients, especially for advanced stage, activated apoptosis by inducing DNA damage through crosslinking of the DNA (32). However, cancer cells often develop several mechanisms to overcome DDP-induced apoptosis and DNA damage, leading to DDP resistance (33-35). Therefore, investigation of the molecular mechanism conferring DDP resistance is urgently needed.

CuB is a representative natural compound for anticancer activities. Recently, CuB was reported to enhance the anticancer effects of DDP in lung, ovarian, cutaneous squamous cell carcinoma, and laryngeal squamous cell carcinoma (20,36-38). However, the relationship between CuB and DDP-resistance in GC cells has yet to be firmly established. In the present study, CuB alone showed an inhibitory effect on both DDP-sensitive and DDP-resistant GC cell lines (Fig. 2).

Apoptosis, which consists of extrinsic and intrinsic pathways, is the main cell death response to chemotherapy. Furthermore, evading apoptosis is one of the hallmarks of chemoresistance, and targeting apoptosis has become a cancer therapeutic strategy (39). Apoptosis is accompanied by various morphological changes, including nuclear condensation, DNA fragmentation, and apoptotic bodies. Nuclear morphology of SGC7901/DDP cells was analyzed using DAPI staining; significant nucleus condensation change in CuB treated SGC7901/DDP was observed, which are typical characteristics of apoptosis (Fig. 3A). The extrinsic and intrinsic apoptotic pathways that ultimately lead to activation of effector casp-3 have been characterized (40,41). In SGC7901/DDP cells, treatment with CuB for 24 h caused downregulation of pro-casp-9 with generation of activated casp-9 (Fig. 3B). The intrinsic apoptotic signal led to activation of casp-3, reflected by a decrease of pro-casp-3 and increase of active casp-3 with cleavage of its substrate PARP (Fig. 3B). Thus, CuB may trigger apoptosis by activating the intrinsic apoptosis pathway which results in activation of effector casp-9. Apoptosis is a programmed cell death that plays an important role in tumor progression and chemoresistance (42). In autophagy, LC3 II plays indispensable roles in autophagosome formation. Beclin1 functions as the key factor in the formation of autophagosomes (10). Our present results illustrated that CuB activated autophagy by the changes of LC3 II and Beclin 1 levels (Fig. 3C). In our further study, drug resistance is largely mediated through overexpression of MDR, HIF-1α, drug resistance protein, and proteasome subunits, increases in antioxidant defenses, and TOP2 activity; these results have been widely verified (33-35). The present study identified that the treatment of CuB was able to reverse the MDR of the SGC7901/DDP cells via the downregulation of P-gp, and HIF-1α (Fig. 4A).

CIP2A, originally named KIAA1524 or P90, was cloned from patients with HCC (14). 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 (43). The functions of CIP2A in autophagy are not fully understood yet. Puustinen et al reported that the CIP2A/PP2A/mTORC1 signal axis is responsible for promoting cell growth and autophagy inhibition (7). They found that CIP2A can associate with mTORC1 and act as an allosteric inhibitor of mTORC1-associated PP2A, thereby enhancing mTORC1-dependent growth signaling and inhibiting autophagy. Our present study demonstrated that CuB promoted the formation of autophagysia via the inhibition of mTORC1 through dephosphorylating p70S6K and 4E-BP1 (Fig. 4B). Next, we detected CIP2A expression and PP2A activation and found that CuB significantly downregulated CIP2A expression and upregulated PP2A activity (Fig. 4C-E). Furthermore, we knocked down CIP2A expression in SGC7901/DDP cells and found that CIP2A depletion significantly promotes CuB induced apoptosis, autophagy, and reversed MDR (Fig. 5A-C). Moreover, our results showed that CIP2A overexpression significantly antagonized CuB induced cell proliferation, apoptosis and autophagy (Fig. 5D-F). Our data validated the mechanism by which CuB-induced cancer cell apoptosis and autophagy in SGC7901/DDP cells, that is, induction of cancer cell apoptosis and autophagy by inhibiting CIP2A to reactivates PP2A and enhance PP2A-dependent mTORC1 inactivation.

In conclusion, our results suggest that further studies on the detailed molecular modification of the CIP2A/PP2A/mTORC1 signaling axis by CuB and exploring its possible application in other malignant diseases are warranted.

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