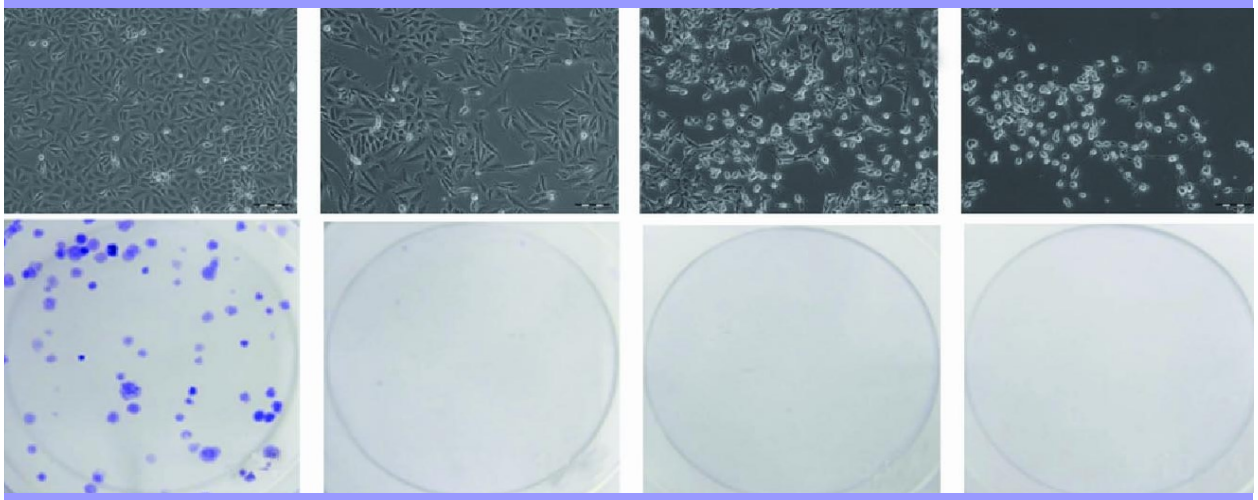

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Cucurbitacin B inhibits proliferation, induces G2/M cycle arrest and autophagy without affecting apoptosis but enhances MTT reduction in PC12 cells

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Abstract

In the present study, the effect of cucurbitacin B (a natural product with anti-cancer effect) was studied on PC12 cells. It significantly reduced the cell number, changed cell morphology and inhibited colony formation while MTT results showed increased cell viability. Cucurbitacin B treatment increased activity of succinode hydrogenase. No alteration in the integrity of membrane, the release of lactic dehydrogenase, the mitochondrial membrane potential, and the expression of apoptotic proteins suggested that cucurbitacin B did not induce apoptosis. The cell cycle was remarkably arrested at G2/M phase. Furthermore, cucurbitacin B induced autophagy as evidence by accumulation of autophagic vacuoles and the increase of LC3II. In addition, cucurbitacin B up-regulated the expression of *p*-beclin-1, *p*-ULK1, *p*-Wee1, p21 and down-regulated *p*-mTOR, *p*-p70S6K, CDC25C, CDK1, Cyclin B1. In conclusion, cucurbitacin B inhibited PC12 proliferation but caused MTT pitfall. Cucurbitacin B induced G2/M cell cycle arrest, autophagy, but not the apoptosis in PC12 cells.

Introduction

Cucurbitacin B is the most widely investigated triterpenoid of cucurbitacins. Accumulated data showed that cucurbitacins possess a number of biological properties, such as hepatoprotective, anti-inflammatory, and cardiovascular activities. In recent years, their anti-cancer effect has received much attention (Chen et al., 2005; Chen et al., 2012).

The *in vitro* cell viability analysis is one of the key indexes for the cytotoxic evaluation of drugs. Based on the reduction of the tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is widely applied to assess cell viability, proliferation and cytotoxicity (Mosmann, 1983). However, a panel of natural products such as genistein, ursolic acid, rottlerin, etc were reported to show MTT pitfall to the tested cells (Bernhard et al., 2003; Bruggisser et al., 2002; Es-Saady et al., 1996; Jayaprakasam et al., 2003; Pagliacci et al., 1993).

Cell viability is tightly controlled by cell proliferation and cell death. The former is a tightly controlled process consisting of multiple checkpoints responsible for the regulation of cell cycle progression. Sustained cell cycle arrest could cause apoptosis, an important programmed type of cell death. Autophagy, a conserved evolutionary lysosomal pathway for degrading cytoplasmic proteins, macromolecules, and organelles, has been implicated in cancer, cardiovascular, and neurodegenerative diseases (Vidal et al., 2014; Yang et al., 2011). Accumulated evidence suggested that autophagy often exert both beneficial and aggravating effects on diseases progression (Nilsson and Saido, 2014; Sridhar et al., 2012). Here, we investigated the

effect of cucurbitacin B on PC12 cells, a line cells derived from rat pheochromocytoma.

Materials and Methods

Materials

Cucurbitacin B, D and E were purchased from the Shanghai Shunbo Biotech Co., Ltd (China). DMEM/F12 medium, heat-inactivated horse serum, fetal bovine serum, phosphate-buffered saline, 0.25% (w/v) trypsin/1 mM EDTA, penicillin and streptomycin were purchased from Gibco (USA). Propidium iodide, Hoechst 33342, and 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethyl-benzimidazolylcarbo-cyanine iodide (JC-1) were purchased from Life Technologies (USA). RIPA lysis buffer, PMSF, protease inhibitor cocktail and BCA protein assay kit were purchased from Pierce Biotechnology (USA). Monodansylcadaverine (MDC), trypan blue and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were purchased from Sigma Aldrich (USA). Cytotoxicity detection kit (lactate dehydrogenase) was purchased from Roche Diagnostics (Germany). All primary antibodies and secondary antibodies were purchased from Cell Signaling Technology (USA). Succinode hydrogennase kit was purchased from Nanjing Jiancheng Bioengineering Institute (China). ATP kit and crystal violet staining solution was purchased from Beyotime Institute of Biotechnology (China). PC12 was purchased from American Type Culture Collection (ATCC).

Cell culture

PC12 cells were cultured in medium DMEM/F12 supplemented with 15% (v/v) heat-inactivated horse serum, 2.5% (v/v) fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

MTT and lactate dehydrogenase assay

MTT assay was used to determine the cell viability. PC12 cells (1 × 10^4 cells/well) were cultured in 96-well plates. When cells reached approximately 60-70% confluence, cucurbitacin B was added and co-treatment with for 24 hours. Then, MTT solution (20 μL; 5 mg/mL) was added, and the cells were incubated at 37°C for an additional 4 hours. The insoluble formazan was dissolved in DMSO. The optical density was measured using a micro plate reader at 570 nm. The morphologic changes of PC12 after cucurbitacin B treatment were monitored with an AxioCam HRC CCD camera. To explore the effect of cucurbitacin B on membrane integrity, lactate dehyrogenase leakage after cucurbitacin B treatment was measured with a commercial kit following the manufacture's protocols. Meanwhile, the cell numbers were also counted to determine the effect of cucurbitacin B .

Trypan blue staining

PC12 cells (1 × 10^4 cells/well) were cultured in 96-well plates. When cells reached approximately 60-70% confluence, cucurbitacin B was added and co-treatment with PC12 cells. After 24 hours co-treatment, 0.4% trypan blue was added to each well followed by imaging with an AxioCam HRC CCD camera.

Colony formation assay

To investigate the effect of cucurbitacin B on PC12 proliferation, colony formation assay was performed. PC12 cells were seeded into 6-well plates at a density of 200 cells per well. After 72 hours, cells were treated with cucurbitacin B. After two weeks, cells were fixed using 4% paraformaldehyde and stained with crystal violet staining solution (Beyotime Institute of Biotechnology, China). The visible colonies (≥50 cells) were counted and the typical images were photographed by a common NIKON camera.

Measurement of ATP content and succinode hydrogenase activity

The cellular ATP content and succinode hydrogenase activity after cucurbitacin B treatment was measured with commercial kits following the manufacturer's protocols.

JC-1 and Hoechst 33342 staining

To investigate whether cucurbitacin B induced apoptosis in PC12 cells, JC-1 and Hoechst 33342 staining were performed. PC12 cells were cultured in black plates and treated with cucurbitacin B. The JC-1 and Hoechst 33342 staining for mitochondrial membrane potential ($\Delta\psi m$) and nuclear DNA was performed as our previous report (Chen et al., 2011).

Cell cycle assay

PC12 cells seeded into 6-well plates were treated with cucurbitacin B for the indicated time. Then cells were collected and then fixed in 70% ethanol and stored at -20°C overnight. Cells were stained with 100 μL propidium iodide staining solution (5 μg/mL RNase and 20 μg/mL propidium iodide) in the dark at room temperature for 30 min followed flow cytometry analysis. At least 10,000 events were counted for each sample. The percentages of cell distribution in G0/G1, G2/M, and S phases were analyzed by software FlowJo.

MDC staining

PC12 cells (1×10^4 cells/well) were cultured in 96-well plate and maintained in the incubator. After adhension, the cells were treated with different concentrations of cucurbitacin B for 24 hours. Then, cells were labeled with MDC (50 μM) for 10 min at 37℃, washed three times with PBS and imaged by the In Cell Analyzer 2000 System (GE healthcare, UK).

Western blotting

To explore the effect of cucurbitacin B on apoptotic related proteins and potential target protein expression, Western blotting was conducted. After 24 hours of cucurbitacin B (1.0-10 μM) treatment, cells were collected and incubated on ice with RIPA lysis buffer with 1% PMSF and 1% protease inhibitor cocktail for 30 min. Cell lysates were centrifuged at 12,500 ×g for 20 min at 4°C. The protein content was determined using a BCA protein assay kit. Protein samples (30 μg) were separated by 12% SDS-PAGE and then transferred to PVDF membranes. After blocked with 5% nonfat milk in PBST for 1 hour, primary antibodies (1:1000) including Bax, Bcl-2, caspase-3, caspase-7, caspase-8, caspase -9, PARP, cleaved-PARP, *p*-mTOR (Ser 2448), *p*-ULK1 (Ser 757), *p*-p70S6K (Thr421/Ser424), *p*-Beclin-1 (Ser 93/96), LC3B, and GAPDH were incubated overnight at 4°C. After three washes with PBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000) for 1 hour and the proteins bands were visualized with an ECL advanced Western blotting detection kit. Bands were obtained with molecular imager ChemiDoc XRS (Biorad). Quantitative assessment of protein bands was performed using Gel Doc™XR (Biorad) equipped with Quantity One software.

Statistical analysis

The results presented are expressed as the mean ± standard deviation of at least three independent experiments (n ≥ 3). The data were submitted to One-way ANOVA and Tukey's multiple comparison test using the graph pad prism 5.0 software program. A value of $p<0.05$ was considered to be statistically significant for all the parameters evaluated.

Results

Cell numbers, changed morphology and proliferation of PC12 cells

Cucurbitacin B treatment for 24 hours dramatically reduced the cell numbers of PC12 cells by approximately 50% at three concentrations tested (Figure 1). It induced significant morphological changes as evidenced by the significant decrease of the long spindle-shaped cells, the increased round cells and the enhanced intercellular gaps (Figure 1). Moreover, the colony formation was completely inhibited by cucurbitacin B and no visible colony containing more than 50 cells were observed after cucurbitacin B treatment (Figure 1).

MTT reduction in PC12 cells

However, MTT results showed that compared with the control group, cucurbitacin B dramatically increased

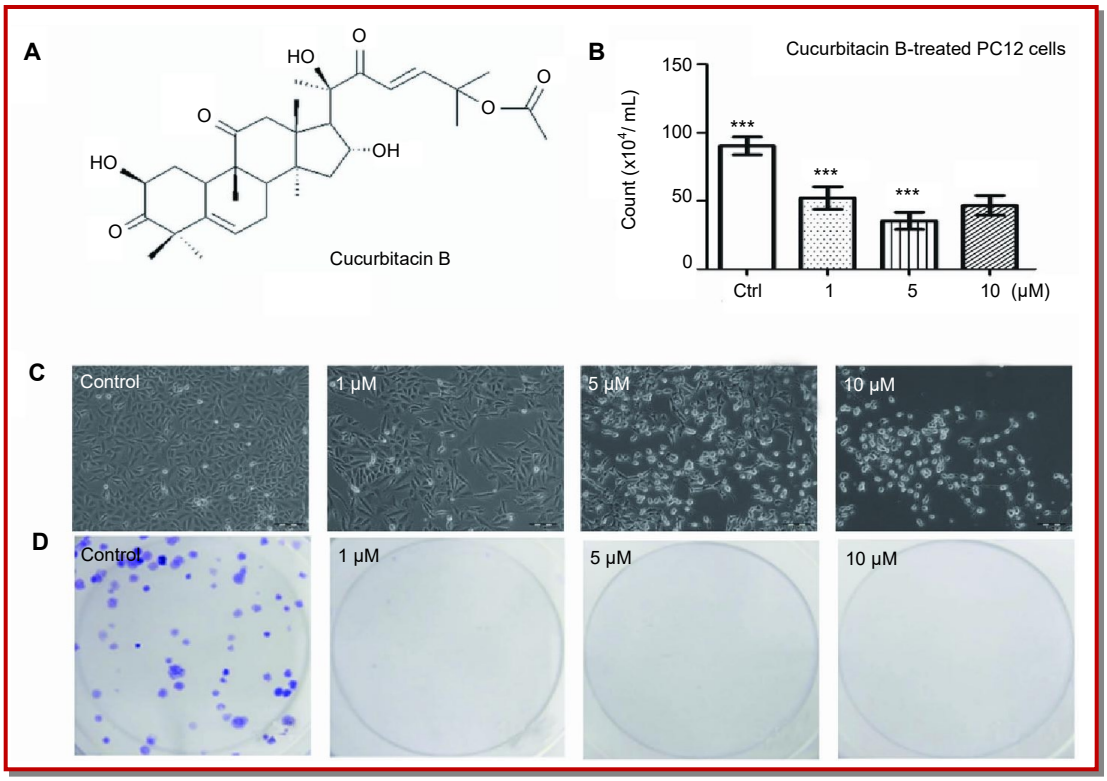

Figure 1: Effect of cucurbitacin B on the morphology, cell number and colony formation in PC12 cell line. Chemical structure of cucurbitacin B (A); Cell counts (B). Data were presented as the means ± SD of three independent experiments; ***$p<0.001$ versus the control group; Morphological observation (C); and Colony formation (D)

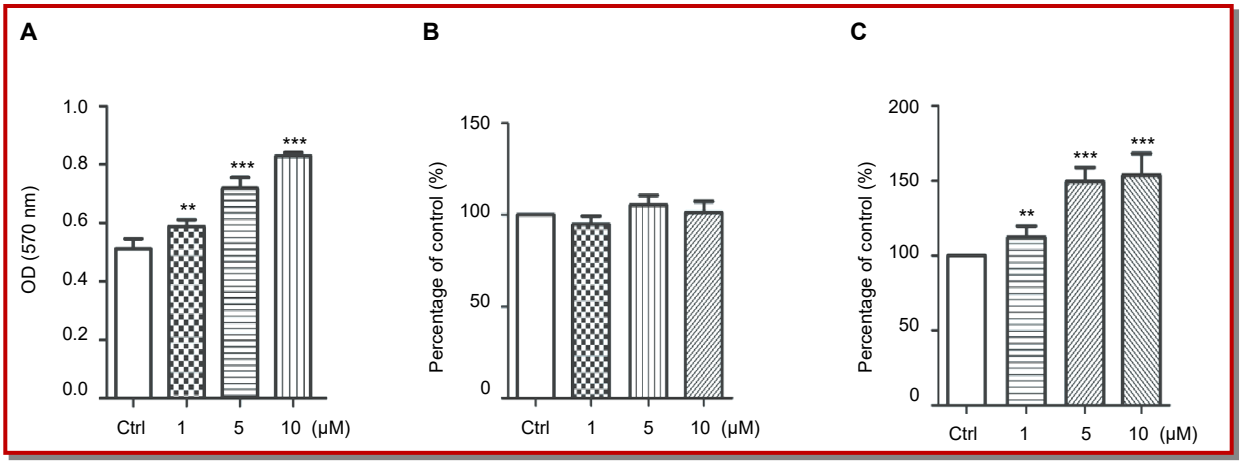

Figure 2: Effect of cucurbitacin B on PC12 cell viability using MTT assay (A); cellular ATP content (B); and activity of succinode hydrogenase (C). Data were presented as the means ± SD of three independent experiments. **p<0.01, ***p<0.001 versus the control (Ctrl) group

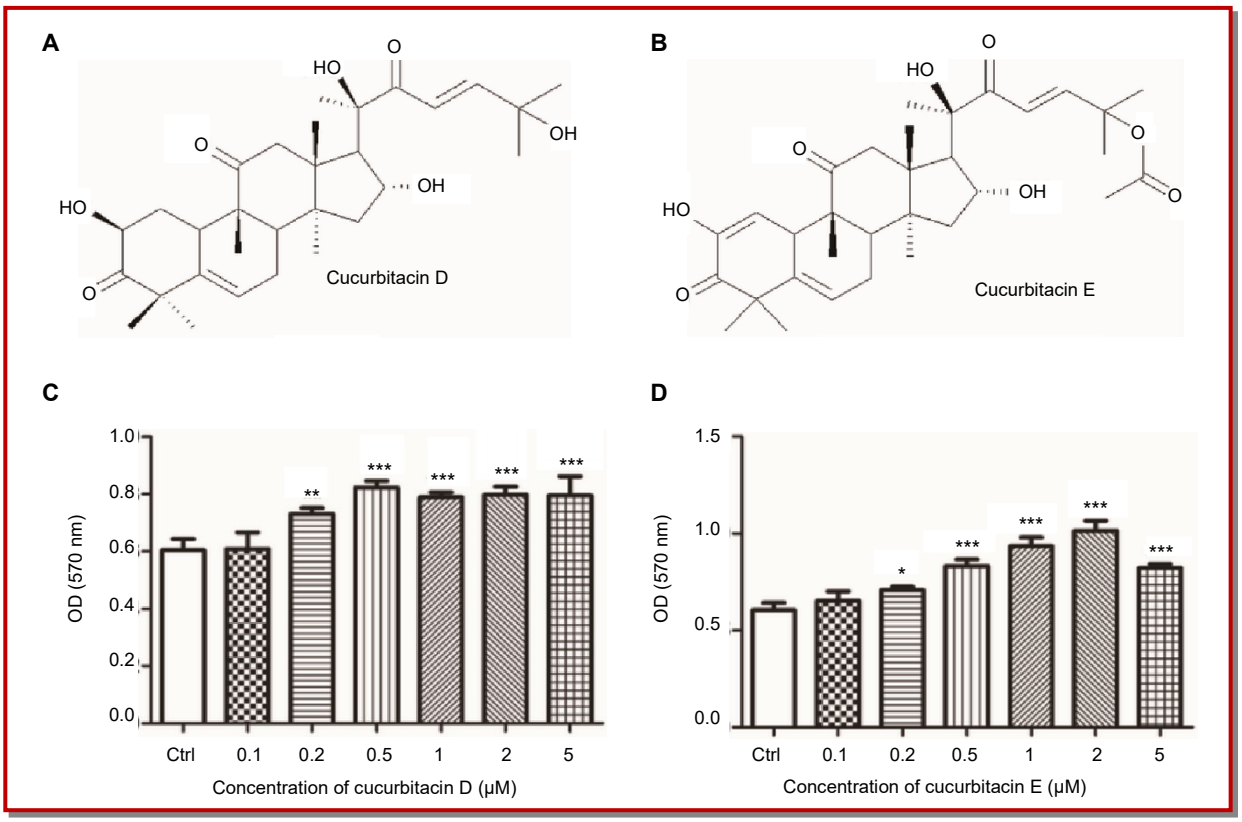

Figure 3: Effect of cucurbitacin D and E on cell viability. Chemical structure of cucurbitacin D and E (upper row); MTT assay (lower row). Data were presented as the means ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 versus the control (Ctrl) group

PC12 proliferation in a concentration-dependent manner (Figure 2). The cell viability was increased as much as about 50% after 24 hours of 10 μM cucurbitacin B treatment. However, in cell-free system, on direct reaction of cucurbitacin B with MTT was observed (data not shown). Meanwhile, cucurbitacin B treatment showed no obvious effect on the cellular ATP levels (Figure 2B) but increased intracellular succinode hydrogenase activity significantly (Figure 2C).

Cucurbitacin D and E, another two members of cucurbitacins also increased the cell viability (Figure 3). Furthermore, concentration-dependent manners were observed after both cucurbitacin D and E treatment from 0.2 μM to 5 μM.

Damage on the integrity of cell membrane

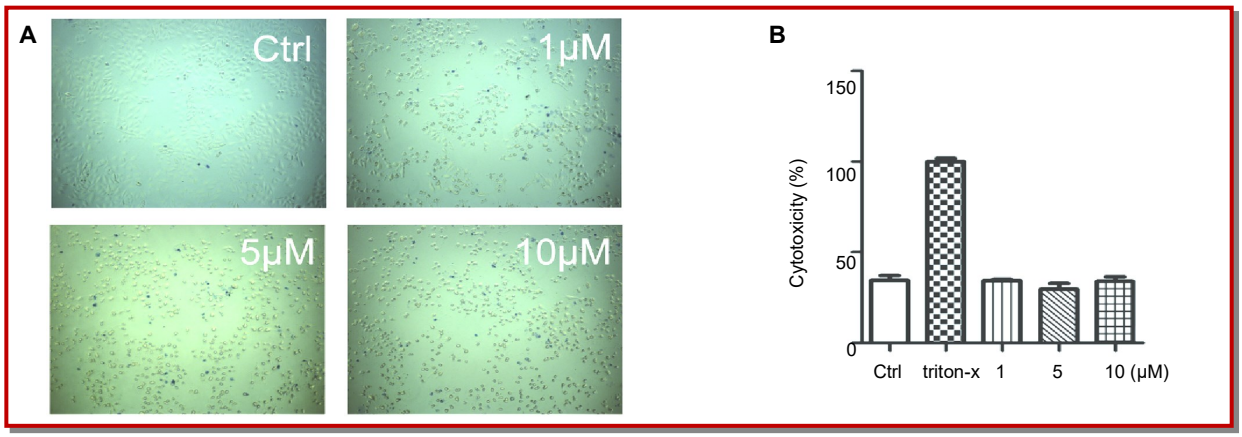

Figure 4: Effect of cucurbitacin B on membrane integrity. Trypan blue staining (A); Cytotoxicity using detection of lactate dehydrogenase level (B). Ctrl, Control

Trypan blue staining showed that there were only a few cells stained with blue (Figure 4A). Consistently, cucurbitacin B showed no effect on lactate dehydrogenase leakage (Figure 4B).

Effect on apoptosis in PC12 cells

JC-1 staining demonstrated that there was intense red fluorescence in untreated PC12 cells suggesting the high mitochondria membrane potential (Figure 5). No significant changes of red fluorescence after CuB treatment was observed. Though Hoechst 33342 staining showed that some cells nuclei became smaller and condensed, no typical apoptotic body was observed (Figure 5). Western blotting assay revealed that cucurbitacin B showed no effect on apoptotic related protein expressions, such as Bax, Bcl-2 protein expressions and PARP, caspase-3, caspase-7, caspase-8, caspase-9 proteolytic cleavage (Figure 5).

G2/M cell phase arrest in PC12 cells

The cell cycle distribution analysis revealed that cucurbitacin B treatment caused significant accumulation of cells in G2/M phase in PC12 cells (Figure 6). Western blotting assay showed that CuB increased the expression of p-Wee1 (Ser 642) and p21, decrease the expression of cyclin B1, CDK1, CDC25C (Figure 6).

Autophagy in PC12 cells

MDC, an auto-fluorescent dye, which can accumulate in the acidic vesicular organelles, was used to detect

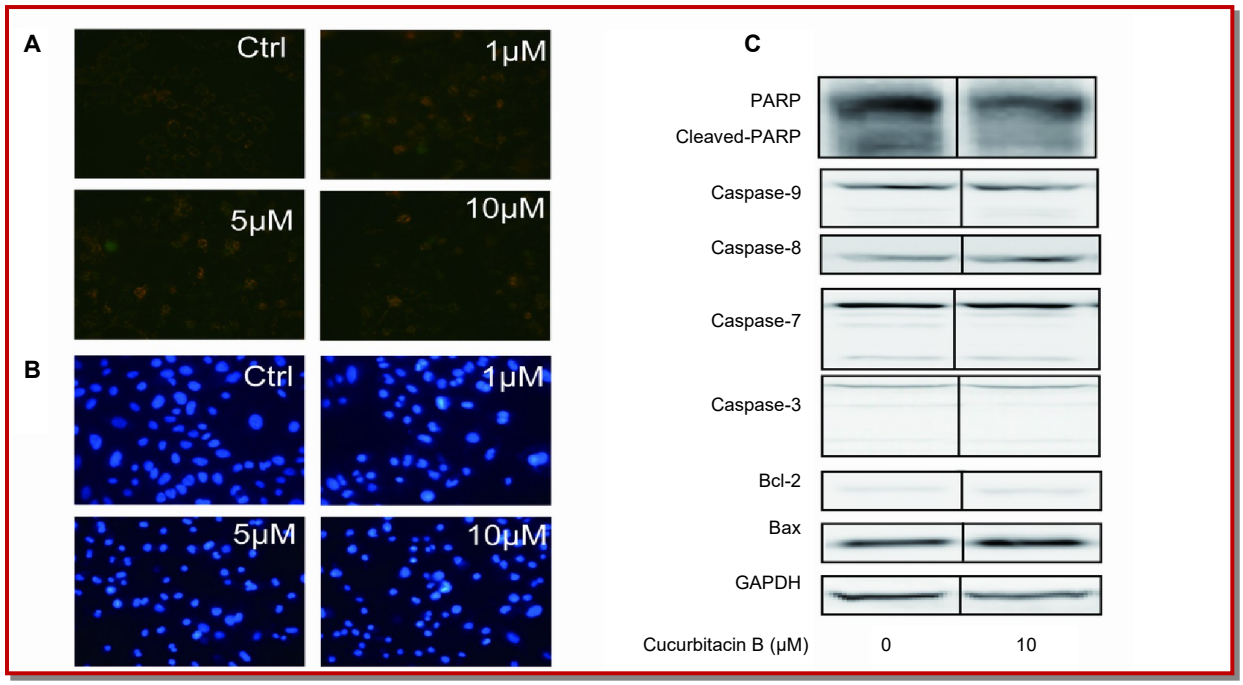

Figure 5: Effect of cucurbitacin B on JC-1 staining, Hoechst 33342 staining and the expression of apoptotic related proteins. JC-1 staining (A); Hoechst 33342 staining (B); Expression of Bax, Bcl-2, caspase-3, caspase-7, caspase-8, caspase-9, PARP and cleaved-PARP (C). Ctrl, Control

autophagic activity. As shown in Figure 7A, compared with the control group, cucurbitacin B increased the green fluorescence in a dose-dependent manner. Western blotting results revealed that cucurbitacin B increased the expression of LC3II, *p*-ULK1 (Ser 757), *p*-Beclin-1 (Ser 93/96), decreased the expression of *p*-mTOR (Ser 2448) and *p*-p70S6K (Thr421/ Ser424) (Figure 7). Blocking autophagy by chloroquine, the lysosomal inhibitor, further enhanced the expression of LC3B in cucurbitacin B-treated PC12 cells, decreased the expression of *p*-mTOR (Ser 2448) (Figure 7). Similar effect was observed after treatment of bafilomycin A1, another autophagy inhibitor.

Discussion

Accumulated data revealed that cucurbitacins demonstrated multiple bioactivities such as anti-inflammatory (Jayaprakasam et al., 2003; Recio et al., 2004), antioxidant (Tannin-Spitz et al., 2007), especially the anticancer effect (Lee et al., 2010; Miró, 1995). Cucurbitacin B inhibited the proliferation of a series of tumor cell lines *in vitro* by induce cell cycle arrest and apoptosis (Chan et al., 2010b; Yasuda et al., 2010a). Here, we investigated the effect of cucurbitacin B on PC12 cells.

The MTT assay is a widely used screening method to measure cell viability and proliferation (Mosmann, 1983). However, previous studies showed that a series of natural products such as ursolic acid, genistein (Pagliacci et al., 1993), rottlerin, FCCP (Maioli et al., 2009), and kaempferol (Bruggisser et al., 2002) could result in MTT pitfalls. In this study, MTT assay showed that CuB treatment significantly promoted PC12 cell proliferation as determined by OD value. However, the cell number was dramatically decreased after cucurbi-

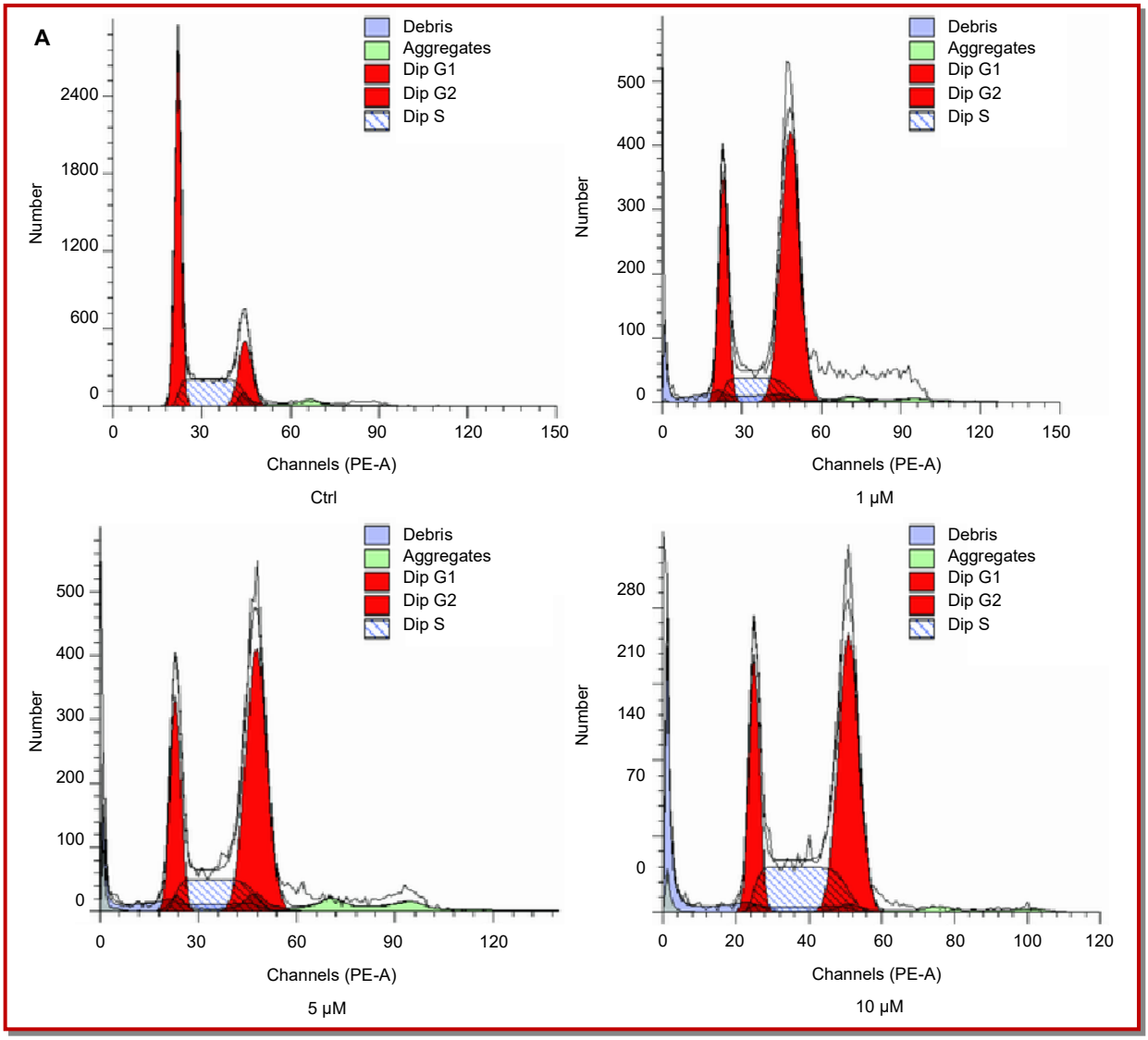

Figure 6A: Effect of Cucurbitacin B on G2/M cell cycle arrest. Expression of p-Wee1, cyclin B1, CDK1, CDC25C and p21. Ctrl, Control

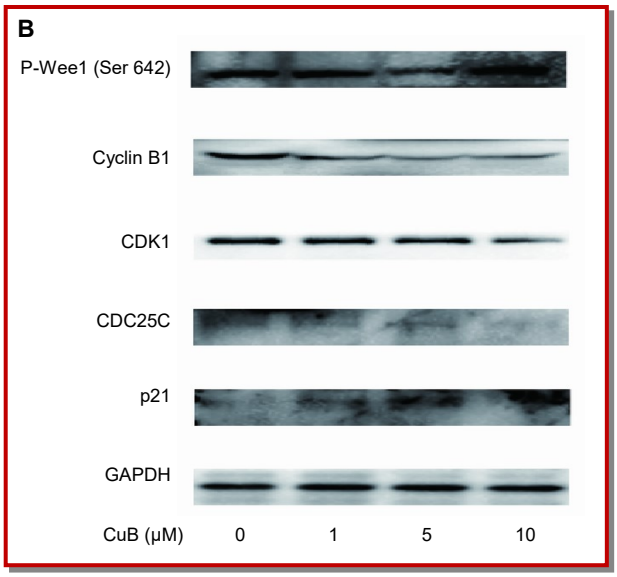

Figure 6B: Flow cytometer to detect G2/M cell cycle arrest. CuB, Cucurbitacin B; Ctrl, Control

tacin B treatment suggesting that this was a MTT pitfall. This was further confirmed by the morphological changes, trypan blue staining, and colony formation. The release of LDH in culture medium was considered as a symbol for membrane integrity injury. In present study, the LDH release was not affect by cucurbitacin B treatment suggesting that cucurbitacin B might not induce cell membrane injury. In SH-SY5Y cells, another commonly used human neuroblastoma cell line, no such phenomenon was observed (data not shown). Furthermore, both Cucurbitacin D and E displayed similar MTT pitfalls in PC12 cells suggesting that this might be a common phenomenon to cucurbitacins. To the best of our knowledge, this is the first report about the MTT pitfall on cucurbitacins. The principle of MTT assay is that the mitochondrial SDH in living cells can reduce exogenous MTT to water-insoluble blue-purple crystalline formazan and deposited in the cells, but not in dead cells. Some natural products and extract with reductive potentials could directly react with MTT in a cell-free system resulting in blue formazan formation in the absence of cells (Bruggisser et al., 2002). Genistein induced MTT pitfall in cancer cells could be attributed to increase in cell volume, mitochondrial mass and G2/M phase arrest (Pagliacci et al., 1993).

In the present study, co-incubation of cucurbitacin B with MTT showed no colorimetric changes in cell-free system precluded the possibility of direct reductive effect of cucurbitacin B on MTT. Morphological observation showed no increase but decrease in the cell volume. The activity of SDH was significantly increased by cucurbitacin B should responsible for the increased reduction of MTT. Taken together, the MTT assay was not suitable for the cell viability detection for the cucurbitacins in PC12 cells.

Cucurbitacin B showed no pro-apoptotic effect on PC12 cells, which unlike its effect on other cells, such as MCF-7 (Dakeng et al., 2012), GBM (Yin et al., 2008), K562 (Chan et al., 2010b). JC-1 staining showed that CuB has no effect of $\Delta\Psi m$, whose decrease was an early event of

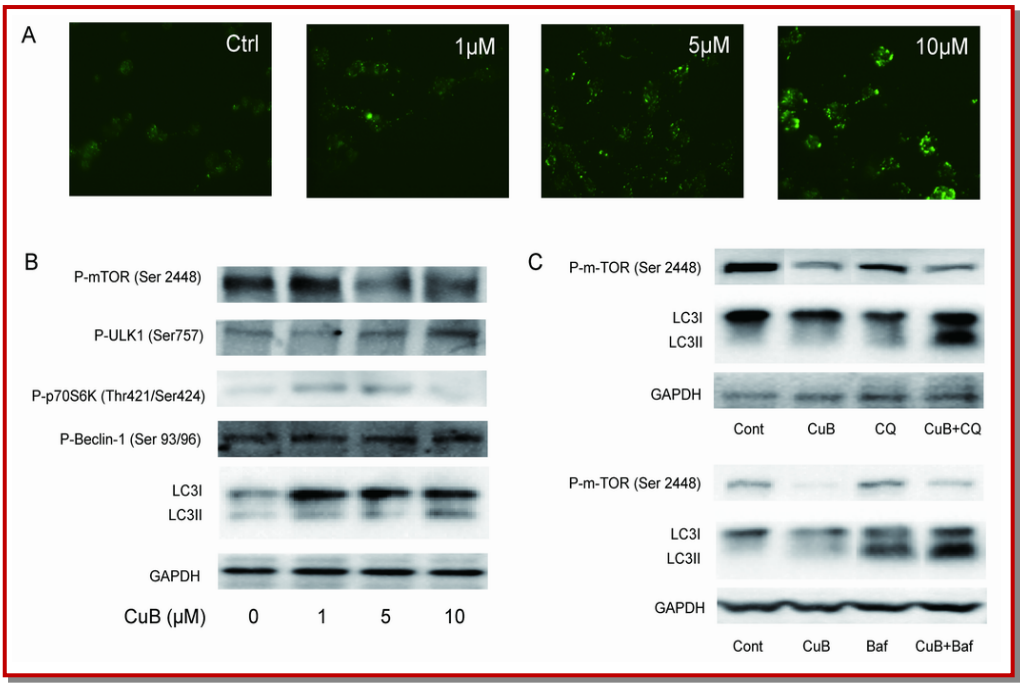

Figure 7: Autophagic inducible effect of cucurbitacin B on PC12. (A) MDC staining. (B) Expression of *p*-mTOR (Ser 2448), *p*-ULK1 (Ser 757), *p*-p70S6K (Thr421/Ser424), *p*-Beclin-1 (Ser 93/96), LC3B. (C) Expression of p-mTOR (Ser 2448) and LC3B, when co-treatment with autophagy inhibitor CQ and Baf. Ctrl, Control; CQ, Chloroquine; Baf，Bafilomycin A1

apoptosis. Hoechst 33342 results suggested that the nuclear DNA was intact. Furthermore, PARP, caspase-3, caspase-7, caspase-8, caspase-9, the apoptotic markers and executors didn't affected by CuB. In addition, expressions of Bcl-2 and Bax were not affected. These results indicated that cucurbitacin B didn't induce apoptosis in PC12 cells.

In PC12 cells, cucurbitacin B induced G2/M phase arrest, which was consist with previous studies in a series of cancer cells such as SKBR-3 and MCF-7 cells (Dakeng et al., 2012), PANC-1 cells (Zhang et al., 2010), W480 cells (Yasuda et al., 2010b), K562 cells (Chan et al., 2010a), GBM cells (Yin et al., 2008). Cyclin B is synthesized in late S phase and G2 phase, which could combined with CDK1 in G2/S to promote cells into the M phase and formed M-CDK. Two events occur in G2/M phase: CDC25C is activated by M-CDK and further dephosphorylated CDK; the expression of phosphorylated Wee 1 is increased. p21 is a powerful CDKs inhibitor, it combines with the N-terminal of CDKs and down-regulated the activity of CDKs. In the cell phase of G2/M, p21 acts as an important CDKs inhibitor (Evan and Vousden, 2001). Here we found that CuB treatment increased the expression of *p*-Wee1 (Ser 642) and p21 but decreased the expression of Cyclin B1, CDK1, CDC25C, which contributing to its G2/M arrest.

Recent studies showed that cucurbitacin B induced autophagy in Jurkat, Hela cells (Zhang et al., 2012; Zhu et al., 2012). MDC is a specific autophagolysosome marker for staining autophagic vacuoles (Munafo and Colombo, 2001). LC3, detected as two distinct bands during autophagy, is commonly used as a biochemical marker of autophagosomal formation (Barth et al., 2010; Mizushima and Yoshimori, 2007). We found that cucurbitacin B induced accumulation of autophagic vacuoles and increased LC3B expression suggesting that cucurbitacin B induced autophagy in PC12 cells. This was further confirmed by the enhanced expression of LC3II by co-treatment cucurbitacin B with chloroquine or Baf, two autophagic inhibitors. Autophagy initiation is closely linked to mTOR, a pleiotropic protein in cell growth, followed by downstream P70S6K. Activation of ULK1, a mammalian serine/threonine protein kinase, recruits a series of signals to trigger the autophagosome formation (Chan et al., 2007). Beclin-1, the mammalian ortholog of yeast ATG6, may act as a nexus point between autophagy, endosomal, and perhaps also cell death pathways (Funderburk et al., 2010). In this study, cucurbitacin B inhibited the phosphorylation of mTOR and P70S6K but increased phosphorylation of ULK1 and Beclin-1 suggesting that cucurbitacin B might induce autophagy in a *p*-mTOR dependent manner.

Conclusion

Cucurbitacin B inhibited PC12 cell proliferation, induced G2/M cell cycle arrest and autophagy without affecting apoptosis. It caused MTT pitfall and was not suitable for the cell viability detection in PC12 cells.

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Conflict of Interest

Authors declare no conflict of interest

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