

Original Research Article

Involvement of protein kinase C- δ activation in betulin-induced apoptosis of neuroblastoma

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Abstract

Purpose: To investigate the clinical benefits and underlying mechanisms of action of betulin in the treatment of cancer using a neuroblastoma (NB) cell model.

Method: Cell viability assay (MTT assay) was applied to investigate the effects of betulin on proliferation and apoptosis of SK-N-SH cell. The expression or translocation of apoptosis-related biomarkers, which include protein kinase C (PKC) family members, were analyzed and quantified by Western blotting, caspase activity assay or enzyme-linked immunosorbent assay (ELISA).

Results: Betulin treatment significantly inhibited the growth of SK-N-SH cells ($p < 0.001$), with half-maximal inhibition concentration (IC₅₀) of 8 $\mu\text{mol/mL}$. Furthermore, betulin treatment increased the activity of PKC- δ , which subsequently activated caspases 3, 8 and 9, thus initiating mitochondria-mediated endogenous apoptotic pathways in SK-N-SH cells

Conclusion: Data generated in this study suggest that betulin inhibits cell proliferation and promotes apoptosis via PKC- δ activation, which may provide new insights into NB treatment from the perspective of adjuvant chemotherapy and prevention of tumor recurrence.

Keywords: Betulin, Neuroblastoma, Apoptosis, protein kinase C- δ , Adjuvant chemotherapy, Tumor recurrence, Caspase

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INTRODUCTION

Neuroblastoma (NB) is a type of pediatric extra-cranial solid cancer, which occurs during the development of sympathetic nerves [1]. It is highly prevalent in infants and children, with an average death rate of 15 % globally, accounting for 7 % of all malignant tumors diagnosed in children (under 14 years old) [2-4]. The international NB staging system categorized NB into five stages: stages 1 to 4, and a special stage 4+ [5]. Stage 3 and stage 4/4+ are identified as the late stage, during which the

disease progression deteriorates rapidly [6]. In the late stage, NB cells migrate to multiple tissues, including bone marrow, bone, lymph nodes, liver and intracranial region [2,7]. The long-term survival rate in the late-stage patients is less than 40 % even with increasing doses of drug [8]. Therefore, there is the urgent need to find natural drug candidates that can inhibit the growth of NB without adverse effect on healthy tissues. Such agents should qualify for use as adjuvant agents during chemotherapy and prognosis.

Betulin was first discovered and purified from the bark of *Betula platyphlla* [9]. It is a triterpenoid compound which exerts remarkable anti-bacterial, anti-fungal, anti-viral, anti-inflammatory and especially anti-cancer activities [10-12]. Available evidence indicate that betulin could induce apoptosis and inhibit the growth of tumor cells, and yet exhibit little toxic effects on normal cells [13-18]. Evidently, betulin because of its unique ability to discriminate tumor cells from healthy cells, is a natural therapeutic agent which should be further explored *in vitro* as an anti-cancer drug in pre-clinical and clinical trials.

Protein kinase C (PKC) belongs to a class of serine/threonine protein kinases that regulate differentiation, migration, proliferation, and apoptosis by catalyzing the phosphorylation of serine or threonine residue [19]. According to their second messenger requirements, PKC can be divided into three sub-families: classic PKC (c PKC), new PKC (n PKC) and non-classical PKC (α PKC). PKC-δ belongs to nPKC sub-family which is composed of an N-terminal regulatory region and a C-terminal catalytic domain. Activation of PKC-δ requires diacylglycerol (DAG) and phorbol ester (PMA), but not calcium ions. Activated PKC-δ regulates cell death and cell survival via phosphorylation/de-phosphorylation [23,24]. Known as type I programmed cell death, apoptosis is highly controlled process, functioning as one of the major anti-cancer mechanisms.

This study was aimed at investigating if betulin inhibits the proliferation of neuroblastoma SK-N-SH cells by activating endogenous apoptosis. In addition, the effect of botulin on the upstream of apoptotic signaling was investigated.

EXPERIMENTAL

Cell culture

Human neuroblastoma SK-N-SH cells were gifts from Department of Oncology, First Hospital of Jilin University (Jilin, China). SK-N-SH cells were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5 % CO₂ humidified atmosphere.

Cell transfection

Transfection was performed via LipofectAmine 2000 (Invitrogen). LipofectAmine 2000 was mixed with serum-free RPMI-1640 at 22 °C for 5 min, and then co-incubated with plasmid DNA or shRNA for 25 min. Lipid-DNA complex was added to SK-N-SH cells and incubated for 24 h for further transfection.

Reagents and materials

Betulin, PMA, MTT, HRP labeled goat anti-rabbit antibody, rabbit anti-mouse and rabbit anti-goat antibodies were purchased from Sigma (St. Louis, USA). Z-VAD-FMK, GF109203X were purchased from Selleck (Houston, USA); RPMI-1640 medium and Gibco fetal bovine serum were purchased from Invitrogen (Carlsbad, USA); Caspase assay reagent was obtained from Calbiochem (La Jolla, USA), while mitochondrial extraction kit, BCA protein assay kit, and ultra-sensitive ECL chemiluminescence kit were products of Beyotime (Shanghai, China). PKC activity test kit was purchased from Abcam (Cambridge, England).

Primary rabbit anti-PARP, mouse anti-cytochrome c, rabbit anti-SMAC, rabbit anti-COX IV, rabbit anti-CD95 and mouse anti-β-Actin were products of Abcam (Cambridge, England). Rabbit anti-Bax, rabbit anti-TNF-R2 and rabbit anti-Bak were purchased from CST (Danfoss, USA), while primary rabbit anti-DR4, rabbit anti-DR5, rabbit anti-PKC-α, mouse anti-PKC-ζ, and rabbit anti-PKC-δ were supplied by Santa Cruz (California, USA). pGPU6 -Neo-PKCδ plasmid was obtained from GenePharma (Jiangsu China). pcDNA3.1 (+)-PKC-δ-CF plasmid was a gift from First Hospital of Jilin University. Full-featured microplate reader (Synergy H1) was purchased from BioTek (Vermont, USA).

MTT assay

Cells were seeded in 96-well plates at 10⁴ cells per well. Following 20-h seeding, the cells were treated with betulin at different concentrations (0, 2.5, 5, 7.5, 10, 12.5 and 15 μM) for 46 h, and 20 μL of 5 mg/mL MTT was subsequently added to each medium, and left to incubate for 2 h at 37 °C. Then, the medium was discarded and 150 μL DMSO was added into each well. The absorbance of the samples was measured at 550 nm using BioTek Synergy H1.

Western blot analysis

Protein concentrations of samples were measured by BCA assay. Samples were resolved by SDS-polyacrylamide gels. Proteins from SDS-PAGE were transferred onto nitrocellulose membranes. Membranes were incubated in blocking buffer (5 % milk in PBST with 0.1 % Tween-20) for 1 h; After PBS-Tween buffer wash, membranes were incubated with primary antibody (1:1000 dilution) in blocking buffer (3 % milk in PBST with 1 % of sodium azide) for 3 h at room temperature or at 4 °C overnight .followed by 1 h incubation with

appropriate secondary antibody-HRP at room temperature. Detection was carried out with enhanced chemiluminescence using ECL reagent kit.

Caspase activity assay

Total cell lysate (100 µg) and 200 µL reaction buffer were co-incubated at 37 °C for 1 h. The fluorescent intensity was measured at 405nm (excitation) and 505 nm (emission). Two hundred microliters of reaction buffer and 100 µL lysis buffer were used as negative control. The reaction buffer (20 mM HEPES, pH 7.4) contained 100 mM NaCl, 10 mM DTT, 0.1 % CHAPS, 10 % sucrose and 25 µM caspase substrates.

Isolation of mitochondria

Mitochondria extraction was performed following the extraction kit protocol. SK-N-SH cells in logarithmic growth phase were trypsinised and centrifuged. Cell pellets were washed and re-suspended in mito-separation buffer on ice. Cell lysates were subsequently homogenized and centrifuged at 600 x g for 10 min. The supernatant was transferred in appropriate centrifuge tubes and further centrifuged at 11000 x g for 10 min. The pellet (mitochondria) was separated from the supernatant and then re-suspended in storage buffer.

Assay of PKC activity

PKC activity was assayed by ELISA. SK-N-SH cell pellets were re-suspended in lysis buffer [(20 mM propanesulfonic acid (MOPS), Ethylene glycol-bis (oxyethylenetriolo) tetraacetic acid (EGTA, 5 mM) and 2 mM ethylene diamine tetraacetic acid (EDTA), 1 % Nonidet P40 (NP40), 50 mM β-glycerophosphate, 50 mM sodium fluoride, 1mM sodium vanadate, 1 mM dithiothreitol and 1mM Benzamidine, 1 mM PMSF and 10 µg/mL leupeptin and aprotinin)] on ice for 10 min, followed by centrifugation at 12000 rpm for 20 min at 4 °C.

Fifteen micrograms of protein from each sample was used for ELISA. Cell lysate was first incubated with 40 µL primary antibody for 1h and subsequently with an HRP-conjugated secondary antibody (1: 1000) for 20 min. Lysate-antibody mixtures were incubated with 60 µL TMB substrates for 45 min and then mixed with the stop buffer. The absorbance of each sample was measured at 450 nm.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using unpaired two-sample t-test, with SPSS. $P < 0.05$ was deemed statistically significant.

RESULTS

Effect of betulin on SK-N-SH cell viability

To explore the effects of betulin treatments on SK-N-SH cells, changes in viability and the apoptotic cell death-related biomarkers in cells were monitored following betulin treatments. With increasing betulin concentration, SK-N-SH cell viability gradually decreased in a dose-dependent manner, with an IC_{50} at approximately 8PP µmol /mL (Figure 1A). At 10 µmol/mL betulin, cell viability was significantly reduced to only 19.11 % ($p < 0.005$) when compared to untreated controls.

To investigate the basis of betulin-induced loss of cell viability, the levels of apoptotic biomarkers were analyzed after betulin treatments. The cells exhibited increased caspase-3 activities after betulin treatment (Figure 1B), indicating that apoptotic signaling was induced. In addition, Western blot showed that one of the apoptotic biomarkers, poly (ADP-ribose) polymerase (PARP), was cleaved 8 h after betulin treatment (Figure 1E), suggesting apoptosis was fully activated. These results show that betulin inhibited SK-N-SH cells survival by inducing apoptosis. Furthermore, to verify the specific apoptosis pathways that were activated, the activities of caspase sub-types were assayed. Eight hours post-drug-treatment, caspase-9 exhibited significantly higher caspase activity ($p < 0.005$, Figure 1C) when compared to caspase-8 ($p < 0.05$, Figure 1D). This suggests that betulin induced apoptosis via the caspase-9 pathway.

Effect of butelin on apoptosis pathways

In order to understand the mechanism by which betuliin induced apoptosis, the downstream pathways of caspase-8 and caspase-9 were examined. The results presented in Figure 2 showed that betulin treatment had no effect on extrinsic apoptotic biomarkers (Figure 2D), which is consistent with the changes in caspase-8 activity (Figure 1C). These results show that betulin does not activate the extrinsic pathway of apoptosis.

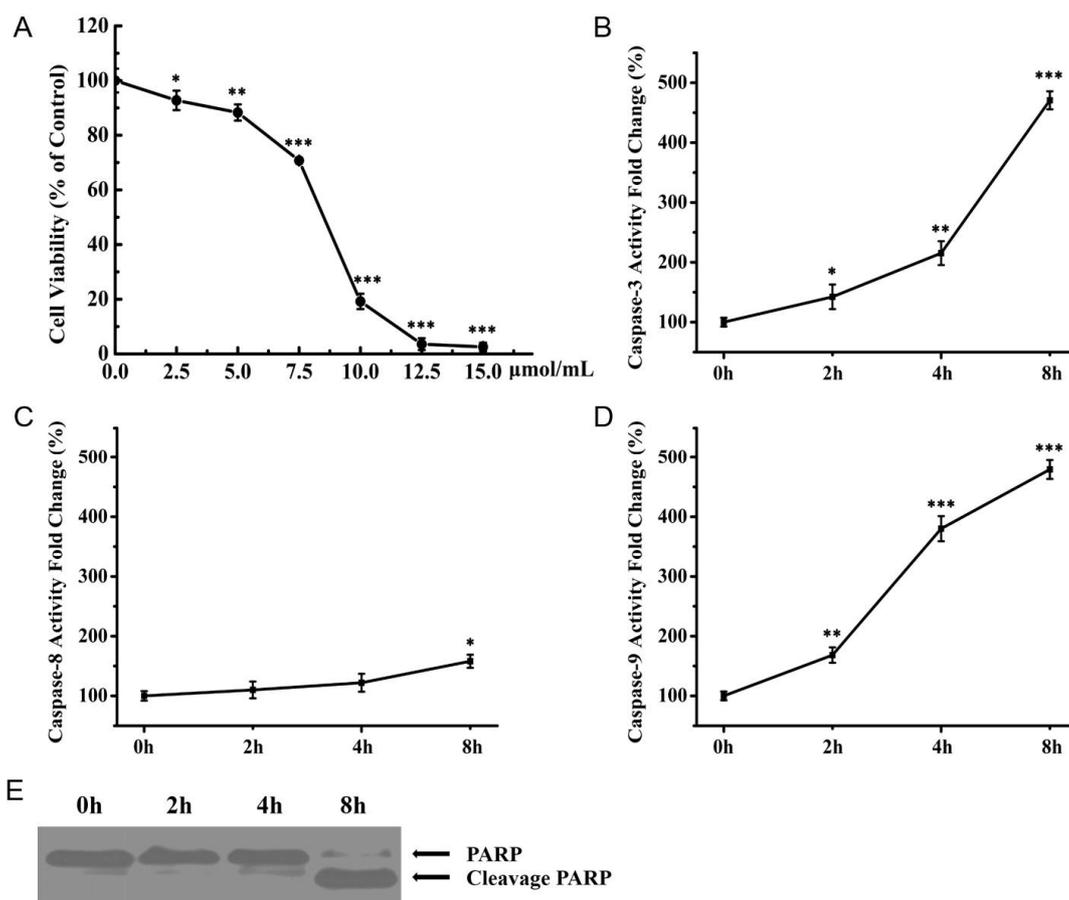


Figure 1: Effect of betulin on cell viability and apoptotic biomarkers. (A) SK-N-SH cells were treated with different concentrations of betulin (0, 2.5, 5, 7.5, 10, 12.5 and 15μM) for 48 h, and cell viability was quantified using MTT assay. (B-D) Time course analysis of caspase-3 (B), 8 (C) and 9 (D) activity following 15μM betulin treatment for indicated periods (0, 2, 4, 8 h). Protein sample (50 μg) was used to verify the changes in caspase activities. (E) Western blot analysis of PARP cleavage. SK-N-SH cells were treated with 15μM betulin for indicated periods, and 50 μg protein of cell lysates were resolved by SDS-PAGE. The cleaved PARP was detected using rabbit anti-PARP (1:5000) and anti-rabbit HRP (1:3000); (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$)

In contrast to the extrinsic pathway, caspase-9 activity increased dramatically following betulin treatments (Figures 2A, 2B). However, when apoptosis was inhibited, caspase-9 activity was decreased significantly, suggesting that caspase-9 was activated after betulin treatment. Betulin treatment showed no significant effects on Bax, Bak, Smac and cytochrome C expression levels (Figure 2C), yet it promoted the translocation of these biomarkers. Results showed that betulin treatment led to the release of Smac and cytochrome C from mitochondria into the cytosol (Figure 2B), while Bax and Bak were accumulated in mitochondria (Figure 2A).

Effect of PKC in betulin-induced apoptosis

PKC activity was changed in cells treated with betulin (Figure 3A). To further confirm the relationship between activation of apoptotic signaling and PKC activation, caspase inhibitor Z-VAD-FMK was used. Combined treatment with Z-VAD-FMK and betulin significantly inhibited

caspase-3 activity (Figure 3B), indicating that betulin treatment activated apoptotic signaling in a caspase-dependent manner. Interestingly, in this study, it was found that Z-VAD-FMK had little effects on PKC activation (Figure 3C), which indicates that PKC might be one of the upstream factors involved in betulin-induced apoptosis.

To investigate if PKC was essential for betulin-induced apoptosis, caspase-3 and caspase-9 activities were assayed with and without GF109203X treatment. Co-treatment with GF109203X and betulin significantly decreased caspase-3 (Figure 3D) and caspase-9 (Figure 3E) ($p < 0.005$) activities in SK-N-SH cells, resulting in the total inhibition of apoptosis.

In order to ascertain if PKC could inhibit the release of apoptotic factors, the extent to which the proteins involved in caspase-9 pathways were expressed was analyzed. Immunoblot results showed that in cells treated with betulin and GF109203X, signal factors such as Smac

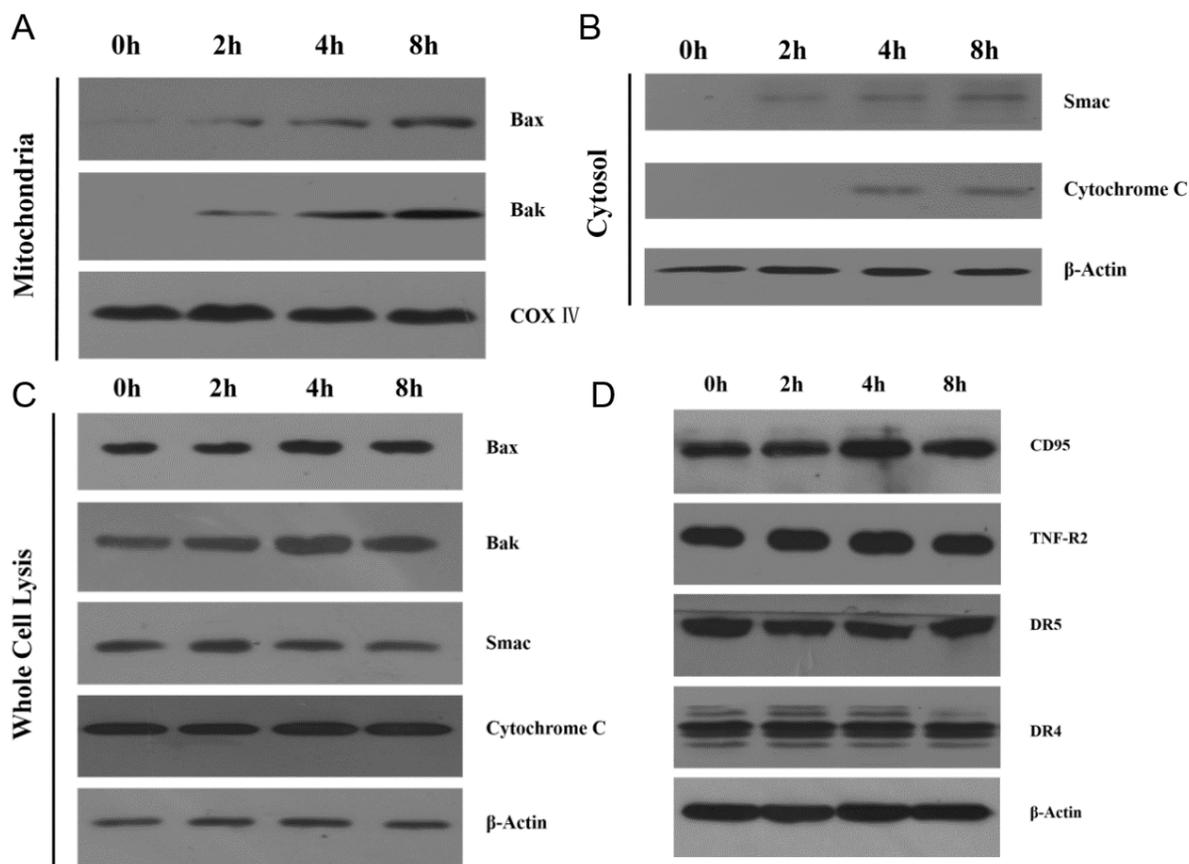


Figure 2: Effect of betulin on activation of intrinsic or extrinsic apoptotic pathways. SK-N-SH cells were treated with 15 μ M betulin for the time period indicated. (A) mitochondrial fraction; (B) cytosol fraction; (C) total cell lysates; (D) caspase-8 down-stream proteins (CD95, TNF-R2, DR5 and DR4) were resolved by SDS-PAGE to verify protein expressions, using rabbit anti-Bax (1 :1000), rabbit anti-Bak (1:1000), rabbit anti-Smac (1:8000), mouse anti-Cyto C (1:2000), rabbit anti-COX IV (1:2000), and mouse anti β -actin (1:5000) with secondary anti-rabbit HRP (1:3000) and anti-mouse HRP (1:4000)

and Cyto C were still released into the cytosol fraction (Figure 3F) and translocations of Bax and Bak were also detectable. However, caspase-3/9 and PARP activities were not activated (Figure 3G). Evidently PKC had no effects on translocation and release of apoptotic factors but could inhibit the activation of caspase-9.

Role of PKC- δ in butelin-mediated apoptosis

Neither PMA nor GF109203X exhibited perceptible impacts on cell viability. However, in the combined treatment with betulin, cell viability significantly increased in the presence of GF109203X (Figure 4A; $p < 0.05$). In contrast to GF109203X, cell viability was significantly reduced ($p < 0.01$) in the co-treatment cases involving PMA and betulin, indicating that PKC activation was critical for SK-N-SH survival.

Having established that PKC activity was critical for SK-N-SH survival, attempt was made to find out the PKC sub-types involved in betulin-induced apoptosis. For this purpose, western

blotting was performed using specific antibodies (Figure 4B). The results showed that there were no changes in the levels of PKC- α and PKC- ζ expressions after betulin treatment, but PKC- δ expression was increased, which was consistent with the finding that PKC activity was reduced when GF109203X was introduced. To confirm if PKC- δ played an important role in cell survival, cells were transfected with the activated form of PKC- δ (PKC- δ -CF). SK-N-SH cells over expressing PKC- δ -CF, on treatment with betulin, had significantly reduced viability when compared to betulin-treated untransfected cells ($p < 0.01$). Treatment with PKC inhibitors diminished this effect.

PKC- δ knockdown significantly decreased the apoptosis-inducing effect of betulin in SK-N-SH cells (figure 4C; $p < 0.01$). PMA treatments showed little effects on cell viability in the PKC- δ knockdown cells.

However, in combined treatments with PKC- δ -CF, it was obvious that overexpression restored betulin-mediated damages. This is an indication

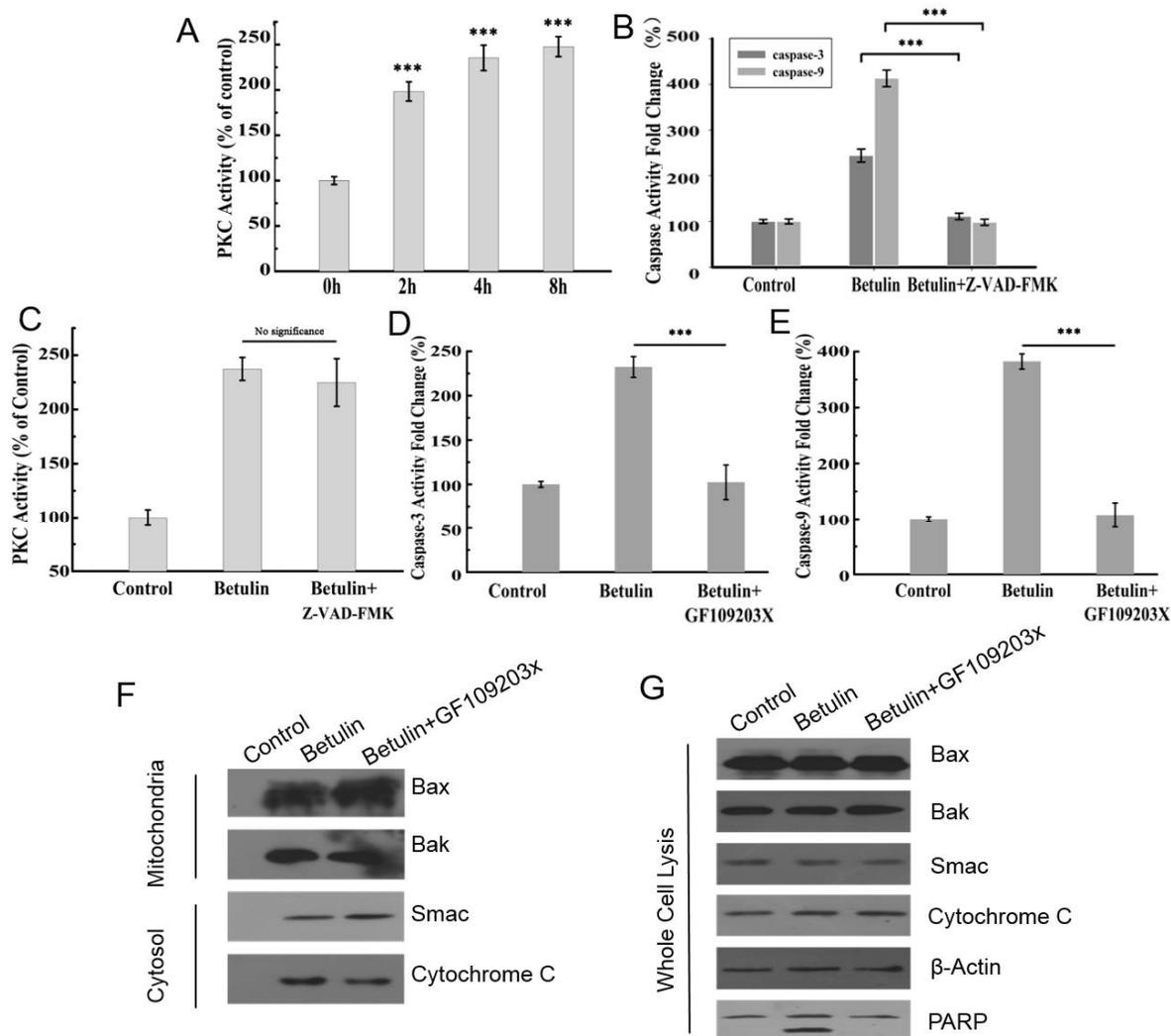


Figure 3: Effects of combined betulin and Z-VAD-FMK or GF109203XC treatments on apoptosis. (A) PKC activities in cells treated with 15 μM betulin for the periods indicated were quantified using ELISA. (B-C) SK-N-SH cells were treated with 15 μM betulin and/or 10 μM Z-VAD-FMK for 4 h. Caspase-3/9 activities (B) and PKC activities (C) of the treated and untreated cells were assayed. (D-G) SK-N-SH cells were treated with 15 μM betulin and/or 4 μM GF109203XC for 4 h. Caspase-3 (D) and caspase-9 (E) activities of treated or untreated cells were quantified. Cell lysates were further resolved by SDS-PAGE to verify the changes in protein expression in mitochondrial and cytosol fraction (F) or in total cell lysates (G) using rabbit anti-Bax (1 :1000), rabbit anti-Bak (1:1000), rabbit anti-Smac (1:8000), mouse anti-Cyto C (1:2000), rabbit anti-PARP (1:5000) secondary anti-rabbit HRP (1:3000) and anti-mouse HRP (1:4000). (***)*p* < 0.005

that PKC-δ played a significant role in betulin-induced apoptosis. These results revealed that betulin treatment activated PKC-δ which subsequently activated intrinsic apoptosis pathways, leading to the programmed death of SK-N-SH cells.

DISCUSSION

Neuroblastoma is a type of nerve tissue cancer which commonly occurs in infants and young children (0-14 years old), and accounts for 15% of childhood death due to tumor. It is difficult to diagnose at early stages. Neuroblastoma is highly heterogeneous because of its complicated

genetic backgrounds, clinical symptoms and pathogenic stages [2,4,6]. For high-risk patients or patients at late stage, it is difficult to arrest the disease progression even with increased doses of the drugs in extant use for its management and/ or treatment. Currently, chemotherapy is still the primary approach for treating neuroblastoma although drug resistance might result in the failure of chemotherapy. Drug resistance is an adopted defense system by which tumor cells survive drug treatment.

The hypothesis on which this study was based was that changes in certain biological characteristics of NB in high-risk patients have

allowed the tumor to evade synthetic drugs, thus making it difficult to treat. Drug resistance occurs due to PKC activity and its subcellular translocation, as well as abnormal inhibition of apoptosis [25]. The present study shows that betulin regulates the activity of PKC, the enzyme which initiates apoptosis in SK-N-SH cells

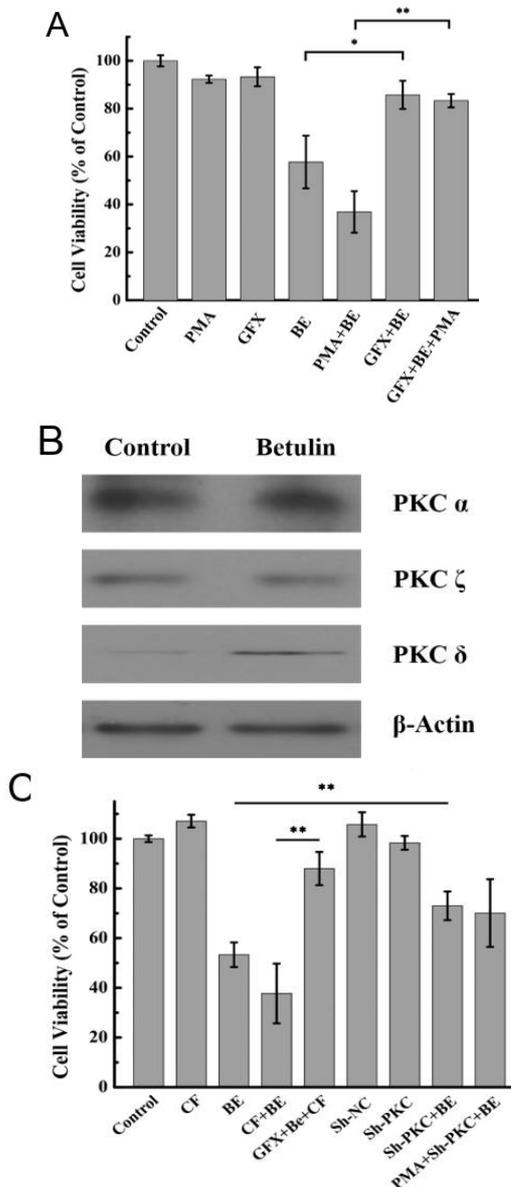


Figure 4: Effect of betulin on PKC-mediated cell death. (A) SK-N-SH cells were treated with 8 μ M betulin, 100nM PMA and/or 2 μ M GF109203XC for 48h as indicated, and cell viability was quantified using MTT assay. (B) Immuno-blot analysis of PKC. Cells treated with/without betulin were lysed and resolved by SDS-PAGE to analyze the expression of PKC α , PKC δ and PKC ζ using rabbit anti-PKC- α (1:1000), mouse anti-PKC- ζ (1:800), rabbit anti-PKC- δ (1:1000) and relevant secondary HRP antibodies. (C) 24 h post-transfection, SK-N-SH cells transiently over-expressing PKC δ and PKC δ knockdown cells were treated with 8 μ M betulin, 100nM PMA and 2 μ M GF109203XC for 48 h, followed by MTT assay to verify cell viability in the presence of the drug; (* p < 0.05, ** p < 0.01)

This study has demonstrated that betulin-induced apoptosis was mediated by mitochondria-linked endogenous pathways. During the caspase-9-dependent apoptotic process, Smac and cytochrome C were released into cytoplasm, while Bax and Bak accumulated in mitochondria. Yet, there were no significant changes in the expression of the apoptotic proteins. Bax and Bak translocation promoted the permeability of mitochondrial membrane to released pro-apoptotic factors, and activated caspase-3 [26], which initiated apoptosis. This suggests that betulin possesses anti-tumor potential, which should be further investigated in animal experiments and clinic trials.

In view of the apoptosis-inducing ability of betulin, the mechanism underlying its apoptotic activity was investigated. It was discovered that betulin treatment significantly increased PKC activity. When PKC was inhibited, cell viability was significantly increased, indicating that PKC participated in betulin-induced apoptosis. PKC is a class of phosphokinases that plays prominent roles in cell differentiation, migration, proliferation, and apoptosis [27]. Cells treated with both PKC inhibitor and betulin had lower caspase-9 activity, and PARP cleavage was also effectively inhibited. Thus, PKC plays an important role in the regulation of SK-N-SH cells apoptosis induced by Betulin.

Although PKC has been recognized as one of the important targets for tumor drugs as early as 2010, the clinical effects of PKC manipulation was not promising. Here, evidence is provided that PKC plays a key role in betulin-induced apoptosis, and that simply activating or inhibiting the activity PKC has no impact on tumor viability. This finding might partially explain why drugs targeting PKC have so far produced no anti-cancer effects. In this betulin-induced apoptosis system, it was observed that due to butelin treatment PKC and caspase-9 were thereby triggering on apoptosis in SK-N-SH cells. Furthermore, it was discovered that PKC- δ was the activated member of the PKC sub-family during the apoptotic process. Therefore, it was analyzed in order to understand its role in betulin-induced apoptosis of SK-N-SH cells. Using a PKC- δ -CF construct, the effect of PKC- δ over-expression on apoptosis in the presence of the drug was analyzed. MTT assay showed that the sensitivity of SK-N-SH cells to betulin was up-regulated when PKC- δ was over-expressed, while knockdown of PKC- δ in SK-N-SH cells significantly increased the cell survival, even under the treatment of PMA. This suggests that

PKC- δ is one of the main subtypes of PKC involved in betulin-mediated apoptosis.

In this study, the hypothesis was that in betulin-induced apoptosis, signaling factors such as Bax and Bak were translocated to mitochondria, increasing the permeability of the mitochondrial membrane, thus enhancing the release of apoptotic related factors into the cytoplasm. These pro-apoptotic factors activated caspase-9, and subsequently caspase-3, thereby initiating apoptosis. For cancer therapy, researchers aim to overcome drug resistance. Extensive studies have been carried out, yet the drugs continue to exhibit limited efficacy against cancer cells. As a natural drug, betulin has been reported to regulate signal transduction, and promote apoptosis with little side effects in a cell model [13]. Based on the findings in this study it can be speculated that betulin can be utilized as a complementary drug to induce programmed death of tumor cells.

CONCLUSION

This study provides bases for better understanding of the mechanism of NB proliferation as well as focused strategy for meaningful management and ultimate treatment of neuroblastoma. Betulin could be a promising natural agent against cancer that should be considered for future pre-clinical and clinical trials from the perspective of adjuvant chemotherapy and prevention of tumor recurrence.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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