

Angelicin induces apoptosis through intrinsic caspase-dependent pathway in human SH-SY5Y neuroblastoma cells

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Abstract Angelicin is structurally related to psoralens, a well-known chemical class of photosensitizers used for its antiproliferative activity in treatment of different skin diseases. To verify the activity of angelicin, we employed human SH-SY5Y neuroblastoma cells to investigate its cytotoxicity, although its mechanism of action has not yet been fully elucidated. Here, we examined the cellular cytotoxicity of angelicin by cell viability assay, DNA fragmentation by DNA ladder assay, and activation of caspases and Bcl-2 family proteins by western blot analyses. The results of our investigation suggest that angelicin increased cellular cytotoxicity in a dose- and time-dependent manner with IC_{50} of 49.56 μ M at 48 h of incubation. In addition, angelicin dose-dependently downregulated the expression of anti-apoptotic proteins including Bcl-2, Bcl-xL, and Mcl-1 suggesting the involvement of the intrinsic mitochondria-mediated apoptotic pathway which did not participate in Fas/FasL-induced caspase-8-mediated extrinsic, MAP kinases, and PI3K/AKT/GSK-3 β pathway. Furthermore, we clarified the dose-dependent upregulation of caspase-9 and caspase-3 which indicated that angelicin-induced apoptosis is mediated primarily through the intrinsic caspase-mediated pathway. In particular, the caspase-3 inhibitor, DEVD-fmk, induced a reduction in angelicin-induced cytotoxicity which confirmed that the intrinsic caspase-dependent pathway during this apoptosis which did not prevent cytotoxicity using MAP kinases and GSK-3 inhibitor. Taken together, our data shows that angelicin is an effective apoptosis-inducing natural compound of human SH-SY5Y neuroblastoma cells

which suggests that this compound may have a role in future therapies for human neuroblastoma cancer.

Keywords Angelicin · Neuroblastoma · Cytotoxicity · Bcl-2 family · Caspase · Anticancer

Introduction

Neuroblastoma, a tumor originating from the sympathetic nervous system, is the most frequently occurring extracranial malignancy in childhood [1]. In particular, in human neuroblastoma SH-SY5Y cells, a reliable model for studying the neurotoxicity of anticancer drugs involves the process of apoptosis [2]. Despite many advances in diagnosis and standard interventions in the past three decades, neuroblastoma has remained a formidable challenge to clinical and basic scientists [3]. In spite of intensive multi-agent therapy and bone marrow transplantation, most patients with high-risk neuroblastoma die due to its metastatic nature. Therefore, the need exists to develop novel agents to improve treatment outcomes in this high-risk group. For this reason, we searched for novel therapeutic strategies for neuroblastoma. Natural products have attracted interest in the development of new approaches for chemotherapy.

To address these questions, we have here reassessed angelicin—a well-known furocoumarin belonging to the class of photosensitizers used for their antiproliferative activity in the treatment of various skin diseases [4, 5]—could be used as a potential target for neuroblastoma therapies. However, angelicin derivatives are from either natural or synthetic compound for instance in the *Angelica archangelica* medicinal plant that exhibits interesting pharmacological activity when compared with the linear psoralens with low toxicity and different DNA-binding modality [6]. Moreover, photobiological

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effects considered were: skin phototoxicity, antiproliferative effects, genotoxicity, and the ability to induce hemolysis in erythrocytes, inactivation of prokaryotic and eukaryotic microorganisms and of viruses. Previous studies have reviewed the ability of some angelicins in inducing photocarcinogenesis as well as their activity as photochemotherapeutic agents [7]. Whereas, new psoralen derivatives have been synthesized, including angelicin, an angular psoralen. Thus, it allows only monofunctional photobinding thereby reducing undesirable side effects, especially long-term ones such as genotoxicity and skin cancer risk [7, 8].

Most of our knowledge about the molecular mechanism of angelicin-induced apoptosis in neuroblastoma cells has not been revealed by other investigators but has been characterized to a far less extent. However, apoptosis can be triggered by the death receptor-dependent extrinsic pathway and mitochondria-mediated intrinsic pathway [9]. Therefore, changes in expression of the Bcl-2 family members resulting in decreased anti-apoptotic (e.g., Bcl-2 and Bcl-xL) and increased pro-apoptotic (e.g., Bax and Bak) proteins may cause mitochondrial release of several pro-apoptotic molecules [10]. On the other hand, many forms of cellular stress that preferentially trigger the Bcl-2 family proteins play critical roles in the regulation of apoptosis via the control of mitochondrial membrane permeability and the release of cytochrome *c* and/or Smac/Diablo [11]. There is strong evidence that a sustained activation of Cytochrome *c* and Smac/Diablo after release from mitochondria promote cytosolic activation of intrinsic caspase cascade through formation of apoptosome and inhibition of the inhibitor-of-apoptosis proteins, respectively [12, 13]. It is noteworthy that in the present work, we have shown that the expression of caspases sequence specifically cleave various endogenous cellular substrates and induce morphological and biochemical features of apoptosis [10, 14]. Most types of apoptosis induced by cellular stress (notably anticancer treatment) involve caspase-3 as a major executioner in the final event of apoptosis, which upon activation cleaves the cytosolic inhibitor of caspase-activated DNase to release and translocate caspase-activated DNase (CAD) to the nucleus for nuclear DNA fragmentation [15].

In this study, we extended our investigation to examine the activation of caspase cascade and mitochondrial activity, and provided evidence for the simultaneous activation of anti-apoptotic protein and the mitochondrial apoptotic pathway during angelicin-induced apoptosis in human malignant SH-SY5Y neuroblastoma cells. To fully understand the signaling mechanism that exerts this response, we investigated the therapeutic efficacies of angelicin in human neuroblastoma cells for induction of the intrinsic mitochondria-mediated apoptotic pathway. This study was undertaken to characterize and compare evidence that treatment of SH-SY5Y cells with angelicin causes down-

regulation of the anti-apoptotic Bcl-2 family proteins and results in activation of caspase cascade pathway rather than Fas/FasL, MAP kinase, and PI3K/AKT/GSK-3 β pathways which ultimately involves nuclear DNA breakdown for apoptosis. The present study was conducted to develop potential neuroblastoma cancer drugs.

Materials and methods

Materials

Angelicin [2-oxo-(2H)-furo-(2, 3-h)-1-benzopyran] was purchased from Sigma-Aldrich (St. Louis, MO). Angelicin was dissolved in 10 mM stock solution of dimethyl sulfoxide (DMSO) and then it was diluted with a medium to obtain the working concentration. Dimethyl sulfoxide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco/BRL (Grand Island, NY). Antibodies against Bcl-2, Bcl-xL, Mcl-1, Fas, and FasL were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-ERK1/2, phospho-p38, phospho-MEK1/2, cleaved caspase-3, caspase-9, caspase-8, β -actin, p-GSK-3 α ^{tyr279/tyr216}, GSK-3 α ^{Ser21}, GSK-3 β ^{Ser9}, p-AKT, AKT, and U0126 were obtained from cell signaling technology (Beverly, MA). DEVD-fmk was obtained from BD Biosciences. PD98059, SP600125, and SB203580 were purchased from Tocris (Bristol, UK). All other reagents were of analytical grade or of the highest purity available.

Cell culture

Human SH-SY5Y neuroblastoma cells were grown at 37 °C under a humidified atmosphere of 5 % CO₂. The cells were cultured in Dulbecco's DMEM with 10 % FBS, 50 U/ml penicillin, and 50 μ g/ml streptomycin. For the cell morphology experiment, the culture plates were examined under a bright-field microscope.

Cell viability assay

Cell viability was determined using a cytotoxicity assay kit, CCK-8 (Dojindo Lab, Japan) according to the manufacturer's protocol. The cells were plated into 96 wells to a density of 50–60 % confluence. After 24 h incubation in starvation media, the cells were treated with various concentrations of angelicin as described in the figure legends. After chemical treatment of 48 h, CCK-8 (10 μ l) was added to each well of the plates and incubated for 3 h. A 96-well microtiter plate reader (molecular devices) was used to determine the absorbance at 450 nm for CCK-8. The mean concentrations in each set of three wells were measured.

Detection of DNA fragmentation

For the detection of apoptotic DNA cleavage, the DNA fragmentation assay was performed using the ladder DNA fragmentation assay. In brief, cells were collected after treatment at various concentrations of angelicin as described in the figure legends and washed in PBS. The cells were then lysed with 500 μ l of genomic DNA extraction buffer (0.1 M NaCl, 10 mM EDTA, 0.3 M Tris-HCl, 0.2 M sucrose, pH 8.0). The lysate was incubated with 20 μ l of 10 % SDS solution and incubated at 65 °C for 30 min. 120 μ l of potassium acetate (pH 5.3) was added and stored on ice for 1 h after centrifugation for 10 min at 4 °C and 12,000 rpm. 2 μ l (10 mg/ml) of RNase was added to the supernatant, and incubated for 30 min at room temperature. The DNA was extracted by washing the resultant pellet in phenol/chloroform extraction and precipitation by ethanol, and then dissolved with distilled water. DNA fragmentation was visualized by electrophoresis in a 0.8 % agarose gel containing ethidium bromide.

Western blot analysis

Human SH-SY5Y neuroblastoma cells were starved on 60-mm culture dishes in DMEM with 0.5 % FBS for 24 h. Cells were pretreated with various concentrations of angelicin as indicated in each figure legend, and then washed twice with ice-cold PBS. Cells were lysed in lysis buffer (2 % SDS, Na₃VO₄, and protease inhibitor cocktail). After incubation on ice for 10 min and sonication for 10 s in 10 % amplitude, the lysates were centrifuged (13,000 rpm, 20 min). The supernatants were collected and protein concentrations were determined by Bradford assay (Bio-Rad, Richmond, CA). Equal amounts of proteins were separated by SDS-PAGE (8 %, 10 %, or 15 % reducing gels), transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and blocked with 5 % non-fat milk. Membranes were incubated overnight in primary antibody at 4 °C. Membranes were then washed in TBST (10 mM Tris, 140 mM NaCl, 0.1 % Tween-20, pH 7.6), incubated with appropriate secondary antibody, and washed again in TBST. Bands were visualized by enhanced chemiluminescence and exposed to X-ray film. The relative abundance of each band was quantified via the Bio-profile Bio-1 D application (Vilber-Lourmat, Marine la Vallee, France), and the expression levels were normalized to β -actin.

Statistical analysis

Results were expressed as mean \pm SEM. Statistical significance was analyzed by one-way ANOVA followed by Dunnett's test or paired *t* test using Prism 4 (GraphPad Software, La Jolla, CA, USA). *P* < 0.05 was considered significant.

Results

Angelicin induces apoptosis in human SH-SY5Y neuroblastoma cells

To evaluate the effect of angelicin on cell viability, human SH-SY5Y neuroblastoma cells were exposed to different concentrations of angelicin for 48 h and the numbers of viable cells were determined by cell viability assay. We found that angelicin dose-dependently and significantly increased cancer cell death (Fig. 1a). However, the concentration of compounds required to cause 50 % (IC₅₀) cell death was determined from cell death versus concentration as 49.56 μ M at 48 h. In this test, incubation of cells with 10 μ M angelicin caused only about 14.35 % cell death, whereas 30 μ M caused about 38.52 % cell death. Moreover, concentration of angelicin from 50 to 100 μ M drastically increased cell death in SH-SY5Y cells, indicating dose-dependent cell death. Also, the number of dead cells by angelicin increased with time lapse. Consequently, we observed that angelicin significantly increased cellular death in response to 30 μ M at 24 h incubation. When the incubation period was increased up to 96 h, at the same concentration, a significantly higher amount of cell death was observed compared to the control (Fig. 1b). To further determine its inhibitory effects on SH-SY5Y cells, we were interested to know whether these cytotoxic effects might be caused by induction of apoptotic mechanisms. As a first indication, we analyzed the cleavage of DNA as well as accepted methods of apoptosis induction by DNA fragmentation assay. Moreover, genomic DNA was extracted from cells treated with 0 and 30 μ M of angelicin for 48 h and separated by agarose gel. We found that DNA fragments were visible at 30 μ M of angelicin-treated cells compared to untreated cells, indicating the extract-evoked cell apoptotic death (Fig. 1c). Taken together, these results indicate that angelicin increased cancer cell death in a dose- and time-dependent manner through the process of apoptosis.

Angelicin downregulates anti-apoptotic Bcl-2 family proteins in human SH-SY5Y neuroblastoma cells

To understand the molecular mechanisms involved in the activation of apoptosis induced by angelicin, we first evaluated whether this furanocoumarin regulates anti-apoptotic Bcl-2 family proteins expression in SH-SY5Y cells. Western blotting and densitometry analysis demonstrated that angelicin at 48 h dose-dependently changed the expression of the anti-apoptotic Bcl-2 family proteins in SH-SY5Y cells. Interestingly, anti-apoptotic proteins Bcl-2, Bcl-xL, and Mcl-1 expressions were dose-dependently decreased after prolonged treatment of angelicin (Fig. 2a). As measured by

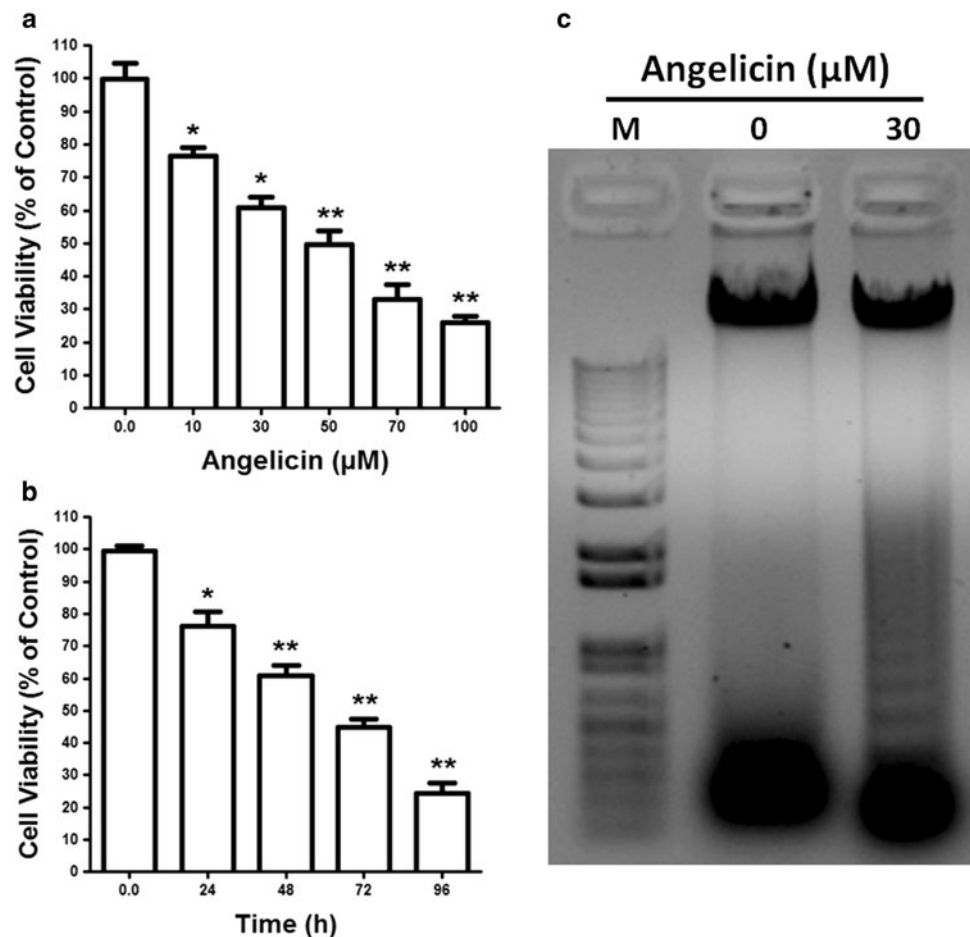


Fig. 1 Angelicin inhibited growth of human SH-SY5Y neuroblastoma cells. **a** Cells were cultured in 96-well dishes to near confluence 50–60 % and then starved in DMEM containing 0.5 % FBS for 24 h. Cells were exposed to angelicin in a different dose of 0–100 μM . Cell death was determined using the cytotoxicity assay kit (CCK-8, Dojindo Lab). Each point is mean \pm SEM of quintuple samples. Data are expressed as means from three independent experiments in which the activity in the absence of angelicin versus in the presence of angelicin was significantly different ($n = 3$, * $P < 0.05$, ** $P < 0.01$). **b** 30 μM of angelicin was used in a time-dependent manner. Results

are expressed as mean \pm SE and representatives of three independent experiments. Significant differences between 0 h treated and angelicin treated with different time period is indicated ($n = 3$, * $P < 0.05$, ** $P < 0.01$). **c** DNA fragmented cells were induced by angelicin. SH-SY5Y cells were grown in 100 mm culture dishes to near confluence 80 % and then starved in DMEM containing 0.5 % FBS for 24 h. The cells were then treated with 0 and 30 μM of angelicin. After 48 h, DNA was extracted and separated on 0.8 % agarose gel containing ethidium bromide. DNA fragments were visualized under UV light. *M* Marker

densitometry analysis, Bcl-2 protein was reduced 0.4-fold at 20 μM concentrations and between 30 and 50 μM angelicin significantly and dose-dependently decreased the expression of Bcl-2 protein (Fig. 2b). Concomitantly, levels of Bcl-xL and Mcl-1 proteins were significantly reduced after 30–50 μM of angelicin (Fig. 2c, d). This observation suggests that angelicin treatment can alter the protein levels of key members of the Bcl-2 family, which may contribute to the susceptibility of cancer cells to mitochondrial dysfunction.

Angelicin induces the activation of caspase-9 and caspase-3 on human SH-SY5Y neuroblastoma cells

To elucidate the molecular events required to involve death by the activation of apoptosis induced by angelicin in

SH-SY5Y cells, we next assessed the levels of some caspases including caspase-9 and caspase-3 by western blot analysis. Interestingly, we found that active forms of caspase-9 and caspase-3 were dose-dependently upregulated when induced by angelicin in SH-SY5Y cells; but remarkably, down-regulation of procaspase-9 was observed at 48 h incubation (Fig. 3a). As shown by densitometry analysis, angelicin dose-dependently downregulated procaspase-9 expression which produced at least a 0.7-fold decrease at 50 μM concentration compared to the control (Fig. 3b), while cleaved caspase-9 was dose-dependently and significantly upregulated above 4-fold increase at 50 μM during this apoptosis process in SH-SY5Y cells compared to untreated cells (Fig. 3c). Furthermore, the level of cleaved caspase-3 was dose-dependently

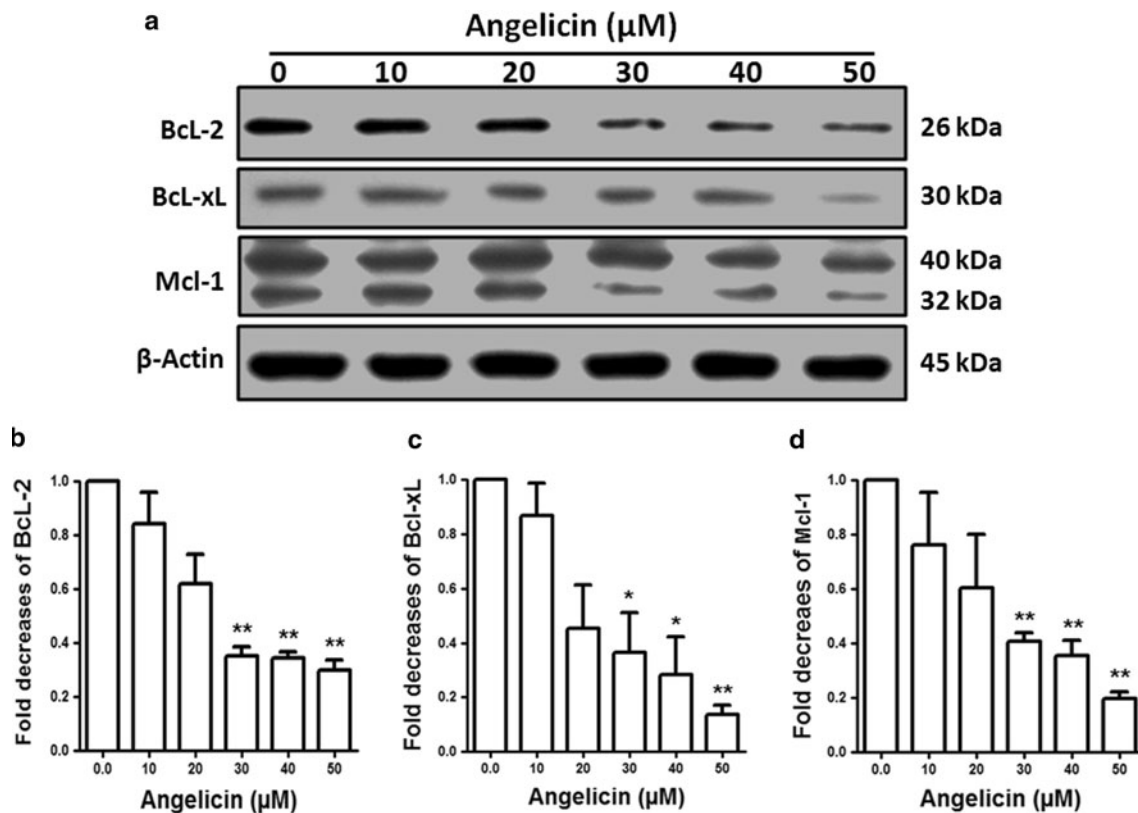


Fig. 2 Angelicin down-regulated anti-apoptotic Bcl-2 family protein expression. **a** SH-SY5Y cells were cultured in 60-mm culture dishes to near 80 % confluence and then starved in DMEM containing 0.5 % FBS for 24 h. After 24 h starvation, SH-SY5Y cells were treated with 0–50 μM angelicin. Whole cell lysates were subjected to 10 % SDS-PAGE and the levels of Bcl-2, Bcl-xL, and Mcl-1 proteins were detected by western blotting as described in materials and methods.

β -Actin was used as a loading control. The intensities of the Bcl-2 bands (**b**), the Bcl-xL bands (**c**), and the Mcl-1 bands (**d**) were determined by densitometric scanning and analyzed by Bio-Profil software and the expression levels were normalized to β -actin. Results are expressed as mean \pm SE and representatives of three independent experiments ($n = 3$, * $P < 0.05$, ** $P < 0.01$)

upregulated by the increased concentrations of angelicin when compared with the cells which were not treated with angelicin wherein significantly above 6-fold increases at 50 μM (Fig. 3a, d) were observed. Altogether our results demonstrate that the caspase cascade signaling pathway is involved in the angelicin-induced apoptosis of SH-SY5Y cells.

Angelicin induces apoptosis is caspase-dependent rather than MAP kinase, PI3K/AKT/GSK-3 β and Fas receptor-mediated signaling pathways

To ensure whether the cytotoxic effects of angelicin-induced apoptosis was the result of direct activation of caspase, MAP kinase, PI3K/AKT/GSK-3 β , and Fas/FasL receptor-mediated pathways, we tested the effect of angelicin in different inhibitors and also their protein expressions with different specificity. First, we examined whether angelicin induced the activation of MAP kinases during the apoptosis. We found that angelicin did not activate the phospho-p38 mitogen-activated protein kinase (MAPK),

phospho-ERK1/2, and phospho-JNK expressions in SH-SY5Y cells at 48 h (Fig. 4a). In addition, to further confirm the involvement of MAPK in angelicin-induced apoptosis, the p38 MAPK inhibitor—SB203580, the ERK1/2 inhibitor—PD98059, and the JNK inhibitor—SP600125 were used. The result obtained by cellular viability suggests that all MAP kinase inhibitors did not prevent cellular toxicity induced by angelicin in SH-SY5Y cells at 48 h incubation (Fig. 4b). Second, to characterize the role of PI3K/AKT/GSK-3 β -induced apoptosis, we investigated the phosphorylation of AKT, GSK-3 α^{Ser21} , GSK-3 β^{Ser9} , and GSK-3 $\alpha^{\text{tyr279}}/\beta^{\text{tyr216}}$ and also their inhibitors in SH-SY5Y cells. As shown in Fig. 4a, AKT was not phosphorylated; however, angelicin had no effect on the level of expression of the AKT protein itself. We then investigated the effect of angelicin on the proteins downstream (GSK-3) of the AKT signaling pathway. We found that angelicin did not change the phosphorylation of GSK-3 α^{Ser21} , GSK-3 β^{Ser9} , and GSK-3 $\alpha^{\text{tyr279}}/\beta^{\text{tyr216}}$, as shown in Fig. 4a, without having any affect on the expression of the nonphosphorylated GSK-3 β protein. As can be noted in Fig. 4a, SH-SY5Y cells

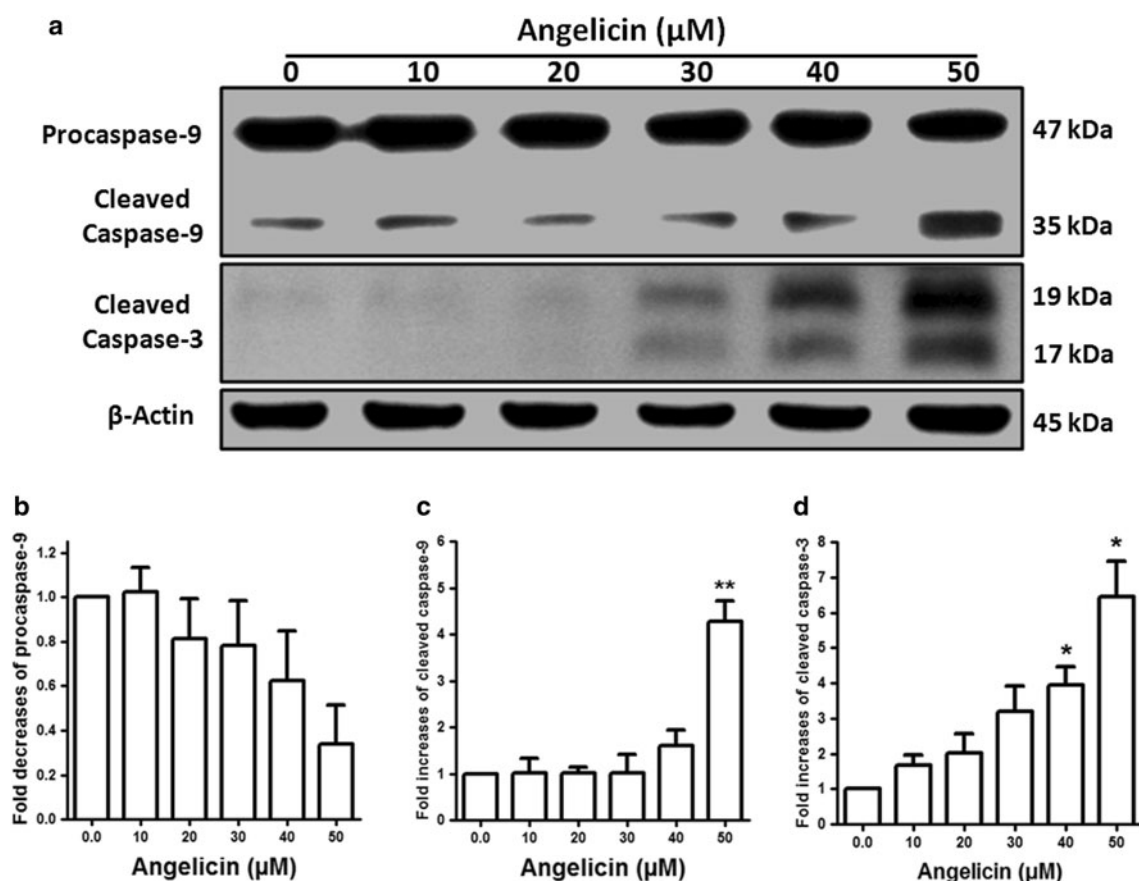


Fig. 3 Angelicin increased the levels of cleaved caspases. **a** SH-SY5Y cells were cultured in 60-mm culture dishes to near 80 % confluence and then starved in DMEM containing 0.5 % FBS for 24 h. After starvation, SH-SY5Y cells were treated with 0–50 μ M angelicin. Whole cell lysates were subjected to 10 and 15 % SDS-PAGE and the levels of caspase-3 and caspase-9 were detected by western blotting as described in materials and methods. β -Actin was

used as a loading control. The intensities of the procaspase-9 bands (**b**), the cleaved caspase-9 bands (**c**), and the cleaved caspase-3 bands (**d**) were determined by densitometric scanning and analyzed by Bio-Profil software and the expression levels were normalized to β -actin. Results are expressed as mean \pm SE and representatives of three independent experiments ($n = 3$, * $P < 0.05$, ** $P < 0.01$)

did not express constitutively active GSK-3 β , and angelicin treatment had no effect on the expression of phosphorylation at Ser 9 of GSK-3 β . Next, we employed inhibitors of GSK-3 (Bio, which inhibit GSK-3 α and GSK-3 β) and PI3K (Wattmannin) by cell viability assay. Indeed, we observed that all inhibitors did not prevent cellular cytotoxicity induced by 30 μ M angelicin at 48 h (Fig. 4b) which suggests that the PI3K/AKT/GSK-3 β pathway did not decrease Mcl-1 levels during this apoptosis. Finally, we examined the activity of the death receptor-mediated apoptotic pathway, specifically the Fas receptor, Fas ligand, and caspase-8 by western blot and also caspase-8 inhibitor by cell viability. No major changes were observed in the expression of either Fas receptor or Fas ligand and caspase-8 proteins at 30 μ M of angelicin in SH-SY5Y cells at 48 h (Fig. 4c). Consequently, we found that angelicin-induced cellular apoptosis was not prevented by the caspase-8 inhibitor, Z-IETD-FMK (Fig. 4d). This explanation implies that

angelicin-induced apoptosis does not involve Fas receptor or Fas ligand mediator of caspase-8 activation in SH-SY5Y cells. To ensure that this effect was a direct activation of the effector caspase-3 by the intrinsic caspase pathway via caspase-9 activation, we examined caspase-3 inhibitor by cell viability assay. As shown in Fig. 3d, the level of caspase-3 activity reached the maximum level at 50 μ M angelicin treatment at 48 h. Interestingly, we found that angelicin-induced cellular apoptosis was significantly decreased by the caspase-3 inhibitor, DEVD-fmk (Fig. 4d). Therefore, it is clear that angelicin selectively induces apoptosis through the caspase-3-mediated pathway in human SH-SY5Y neuroblastoma cells. Taken together, our observations suggest that angelicin-induced apoptosis is directly mediated by the intrinsic caspase signaling but is not involved in the MAPK, PI3K/AKT/GSK-3 β , and Fas/FasL signaling pathway in human SH-SY5Y neuroblastoma cells.

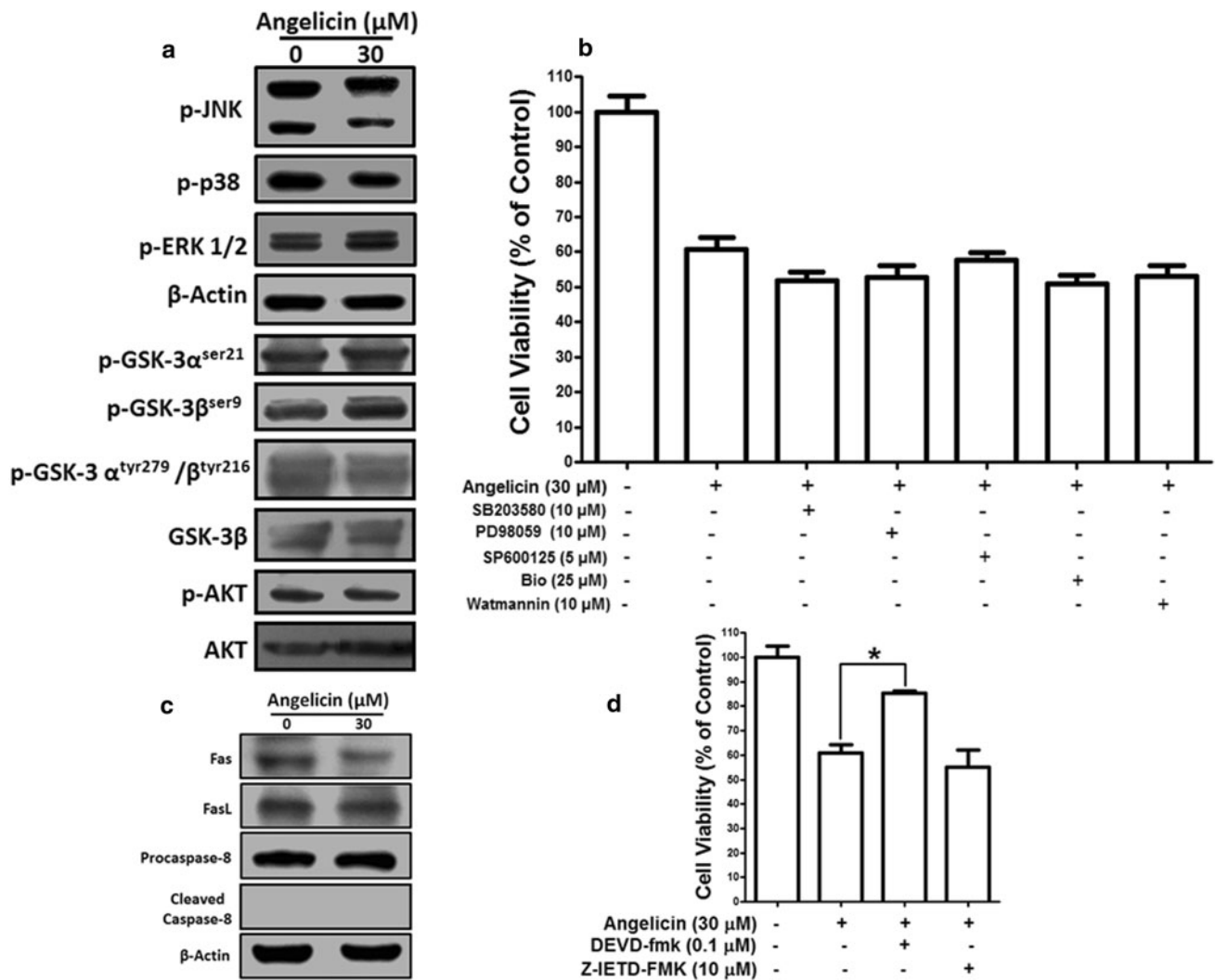


Fig. 4 Effect of angelicin on different signalings and inhibitors. **a**, **c** SH-SY5Y cells were cultured in 60-mm culture dishes to near 80 % confluence and then starved in DMEM containing 0.5 % FBS for 24 h. After starvation, SH-SY5Y cells were treated with 30 μM angelicin. Whole cell lysates were subjected to 10 % SDS–PAGE and the levels of p-JNK, p-p38, p-ERK1/2, p-GSK-3α^{Ser21}, p-GSK-3β^{Ser9}, p-GSK-3α^{Tyr279}/β^{Tyr216}, p-AKT, AKT, Fas, FasL, and caspase-8 were detected by western blotting as described in materials and methods. β-Actin was used as a loading control. **b** Cells were cultured in 96-well dishes to near confluence 50–60 % and then starved in DMEM containing 0.5 % FBS. After 24 h starvation, SH-SY5Y cells

were pretreated with 10 μM SB203580, 10 μM PD98059, 5 μM SP600125, 25 μM Bio and 10 μM Watmannin for 1 h before exposure to 30 μM angelicin for 48 h. Cell number was quantified by CCK-8 kit. **d** SH-SY5Y cells were cultured in 96-well dishes to near confluence 50–60 % and then starved in DMEM containing 0.5 % FBS for 24 h. After starvation, cells were pretreated with 0.1 μM DEVD-fmk and 10 μM Z-IETD-FMK for 1 h just before exposure to 30 μM angelicin for 48 h. Cell number was quantified by CCK-8 kit. Results are expressed as mean ± SE and representatives of three independent experiments ($n = 3$, $*P < 0.05$)

Discussion

The question arised in the present study was how angelicin is described as an apoptosis-inducing agent in neuroblastoma cancer cell line. However, little is known regarding the mechanisms involved in the induction of prograded cell death by angelicin. Here, we investigated the influence of angelicin on the cell viability of cultured neuroblastoma cancer cells and focused our mechanistic investigations on activation of caspases as well as Bcl-2 family protein

expression. In addition, it will also be necessary to determine why the degree of response to angelicin varies among different types of molecular mechanisms in neuroblastoma cancer cells.

Apoptosis is one of the most prevalent pathways through which chemotherapeutic agents can inhibit the overall growth of cancer cells. Our main finding was that half-maximal inhibition of cell viability was observed in the above 50 μM range, and IC₅₀ values decreased with increasing incubation doses and times (Fig. 1a, b).

However, concentrations that caused cytotoxic effects and induced apoptosis detected by DNA fragments were observed to be higher than that of the untreated cells (Fig. 1c). This might be due to an initial inhibition of DNA synthesis and arrest of cells in the S-phase, similar to the effects observed in angelicin plus UVA light which has been shown previously to induce apoptosis in a human tumor T-cell line (Jurkat) [16], human keratinocytes cell line NCTC-2544 [17], and human leukemia K562 cells [18]. In this study, we noted that different concentrations of angelicin (10–50 μM) induced apoptosis of human SH-SY5Y neuroblastoma cells in a dose-dependent and time-dependent manner without UVA light (Fig. 1). This observation suggests that the potent *in vitro* efficacy of angelicin in neuroblastoma cancer cells indicates that angelicin may potentially prove to be useful in the prevention of neuroblastoma carcinoma. Moreover, it remains to be determined whether angelicin suppresses the development of neuroblastoma cancer in both animal and human cancer models.

A number of studies have shown that the balance between pro-apoptotic and anti-apoptotic Bcl-2 protein family members controls the mitochondrial apoptotic pathway [19–21]. A potential concern with the permeability of the mitochondrial membrane is precisely regulated by the Bcl-2 family proteins [22, 23]. It has been proposed that angelicin was shown to affect the levels of the Bcl-2 family proteins. Angelicin treatment was found to reduce the levels of the anti-apoptotic Bcl-2, Bcl-xL, and Mcl-1 proteins determined by western blotting analysis (Fig. 2). The finding of this study demonstrates that the alteration in Bcl-2 family proteins contributed to an increase in mitochondrial membrane permeability and subsequent caspase-9 activation in angelicin-treated human SH-SY5Y neuroblastoma cells. Therefore, the result of this study shows that angelicin induces apoptosis through the activation of caspases via the mitochondria-mediated apoptotic pathway.

In the mitochondria-mediated pathway, apoptotic stimuli enhances the permeability of the outer mitochondrial membranes and releases pro-apoptotic factors into the cytoplasm which subsequently bind to apoptosis protease-activating factor 1 (Apaf-1) and inactive procaspase-9 to form an apoptosome, thereby resulting in caspase-9 activation which triggers the subsequent cleavage of caspases-3 [24, 25]. Based on the other author's observation, caspases perform critically important roles in the induction of apoptosis and caspases are classified based on their mode of activation as either initiator (caspase-9) or effector caspase (caspase-3). These caspases cleave a variety of cellular substrates, most notably PARP, and repair single-strand DNA nicks in which cleaved DNA is a useful marker for apoptosis [26, 27]. Therefore, we determined

the effects of angelicin on the activation of caspase-9 and caspase-3. In the present study, we observed that treatment of SH-SY5Y cells with angelicin for 48 h resulted in a dose-dependent increase in the cleavage of caspase-9 and caspase-3 compared with the cells which were not treated with angelicin (Fig. 3). Jurkat cells and normal lymphocytes have shown that psoralen and UVA (PUVA) treatment induced apoptosis by activation of caspase-3, -8, and -9 [28]. These results show that the activation of these caspases is one of the principle mechanisms by which angelicin induces apoptosis.

Most previous studies have shown that caspase activation is triggered primarily via two distinct interconnected pathways, namely the death receptor and the mitochondria-mediated pathways [29]. In the death receptor-mediated pathway, the binding of death receptor ligands (e.g., FasL and TRAIL) to their specific death receptors (e.g., Fas, DR4, and DR5) located on the plasma membrane induces the activation of caspase-8 which directly triggers the activation of downstream caspase-3 and/or cleaves Bid, a BH3-only pro-apoptotic Bcl-2 family protein [26]. Thereafter, we determined that angelicin treatment did not induce an increase in the levels of Fas/FasL activation and caspase-8, and found that caspase-8 inhibitor did not prevent cellular viability (Fig. 4c, d). This finding demonstrates that Fas/FasL-mediated caspase-8 did not contribute to the activation of caspase-3 via direct activation of this enzyme in angelicin-treated SH-SY5Y neuroblastoma cells. Moreover, inhibition of the interaction of FasL with the Fas receptor leads to a reduction in apoptosis in response to genotoxic stress and growth factor withdrawal [30–33]. Consistent with the idea that FasL is a target in the MAP kinases signaling pathway that induces apoptosis is the presence of AP-1-binding sites in the human FasL promoter region, which presumably contribute to the dependence of Fas/FasL interactions on MAP kinases phosphorylation [30–33]. However, in this study, p38, ERK1/2, and JNK MAP kinases in SH-SY5Y cells were not involved by induction of FasL and apoptosis (Fig. 4a, b). According to this observation, we presume that angelicin-induced apoptosis were not involved in the Fas/FasL interactions in MAPK signaling pathway.

In addition, one of the direct downstream targets for AKT is GSK-3 β . Phosphorylation of GSK-3 β by AKT inhibits its own enzymatic activity as a serine/threonine kinase [34, 35]. As a consequence of the activation of PI-3-K signaling, GSK-3 β activity is attenuated by activated AKT. When active, GSK-3 β can phosphorylate a number of protein targets. One of the primary targets for GSK-3 β is β -catenin, in which phosphorylation by GSK-3 β leads to its proteosome degradation [36]. Similarly, one of the Bcl-2 family members of anti-apoptotic factor Mcl-1 is phosphorylated by GSK-3 β and is subsequently degraded [37].

Activity of AKT blocks the ability of GSK-3 β to phosphorylate and degrade Mcl-1. Through this regulation on Mcl-1 by inhibiting GSK-3 β , AKT also indirectly affects the balance of Bcl-2 family of pro- and anti-apoptotic factors. In this study, we found that angelicin did not affect the phosphorylation of AKT, which is closely linked with cell survival. Phosphorylation of the upstream kinase PI3K was also not affected by angelicin (Fig. 4a). However, inhibition of AKT (Wattmannin) and GSK-3 (Bio) signaling did not prevent cellular viability of angelicin-induced SH-SY5Y cells (Fig. 4b). Thus, it is suggested that cytotoxicity of tumor cell viability and induction of apoptosis by angelicin did not take part through activation of the PI3K/AKT/GSK-3 β pathway. Based on all our studies, we suggest that angelicin-induced apoptosis is mediated through the mitochondria-mediated caspase cascade pathway, rather than the Fas death receptor and the MAP kinases pathway. A later report by Cappellini et al. [38] demonstrated that PUVA treatment in Jurkat cells induced a rapid activation of p38 MAPK but not other members of the MAPK family. Collectively, our findings suggest that angular psoralen angelicin activates the apoptotic pathway that is not involved in the activation of the death receptor-mediated pathway in SH-SY5Y cells, but via indirect activation of caspase-3.

Conclusion

This study shows that angelicin increases cytotoxicity and induces apoptosis in human SH-SY5Y neuroblastoma cells, and this effect is mediated by the activation of the intrinsic apoptotic pathway. However, the findings of the present investigation also indicate that angelicin activates the caspase-3-dependent apoptosis via mitochondria-mediated apoptotic pathway. This observation provides a molecular basis for using angelicin as a potential apoptosis-inducing agent. Thus, more studies should be conducted in the future to evaluate the potential use of angelicin as a human neuroblastoma cancer preventive agent in animal and human experimental models.

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