**Induction of apoptotic cell death by betulin in multidrug-resistant human renal carcinoma cells**

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**Abstract.** Betulin, a triterpene from the bark of various species of birch tree, has various biological effects, including antiviral, antifungal and anticancer activities. The aim of the present study was to elucidate the mechanisms underlying the apoptotic effect of betulin in RCC4 multidrug-resistant human renal carcinoma cells. To evaluate anticancer activity, we performed cell viability and caspase activity assays, a proteome profiler array and western blot analysis in RCC4 cells. Betulin significantly decreased RCC4 cell viability in a time- and concentration-dependent manner. Betulin activated caspase family proteins, including caspase-3, -7, -8 and -9, and increased the expression of apoptosis-related proteins, including PARP and Bcl-2 family members. In an apoptosis array, betulin activated the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors TRAIL R1/DR4 and R2/DR5, and tumour necrosis factor receptor 1 (TNFR1), suggesting that betulin treatment leads to induction of apoptosis through both intrinsic and extrinsic apoptosis pathways in RCC4 cells. Notably, betulin significantly enhanced cytotoxicity and PARP cleavage in etoposide-treated RCC4 cells, and downregulated the expression of multidrug resistance protein 1 (MDR1). Taken together, our findings suggest that the anticancer effects of betulin involve induction of apoptosis and sensitisation of RCC4 cells, providing potentially useful information applicable to the use of betulin in renal cancer treatment.

**Introduction**

Renal cell carcinoma (RCC) is the most common type of kidney cancer. RCC accounts for an estimated 3-5% of all malignant tumours, and this figure increases annually (1). Nearly 50% of patients suffer relapse and metastasis after curative surgical resection (2). RCC is highly resistant to radiotherapy and other formal chemotherapies (3). More recently, immunotherapy with cytokines, such as interleukin-2, interferon-α and interferon-γ, has become the preferred treatment for later disease phases; however, the response rate is less than 20% (4). Therefore, there is a need for an alternative systemic treatment for RCC that overcomes resistance to current anticancer drugs.

Resistance to multiple categories of chemotherapy agents, termed multidrug resistance (MDR), is a complex hurdle in cancer therapy (5). The mechanisms behind MDR are still under active investigation, yet members of the ATP binding cassette (ABC) transporter superfamily, which often act as cellular efflux pumps for a wide range of chemotherapeutic compounds are implicated (6). Over 49 MDR genes have been described, among which the ABCB1 gene, encoding the efflux transporter P-glycoprotein (P-gp) or MDR1, is probably the most well known (7). The prognostic significance of P-gp as an indicator for failure in chemotherapy has been demonstrated in a number of clinical studies (8).

Induction of apoptosis in cancer cells is desirable, and is a novel cancer therapeutic strategy (9). Apoptosis can be induced through two major pathways: i) the extrinsic pathway, which is initiated by specific ligands, such as the Fas-ligand (Fas-L), tumour necrosis factor (TNF) and the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), which activates caspase-8; and ii) the intrinsic pathway, which is activated by intracellular signals from the mitochondria to activate caspase-9 (10-12). Caspase-3, activated by the intrinsic and extrinsic pathways, induces the cleavage of poly(ADP-ribose) polymerase (PARP), a DNA repair enzyme, ultimately leading to programmed cell death (13).

Betulin [lup-20(29)-ene-3β,28diol] (Fig. 1A) is a triterpene compound found in plants. It is commonly isolated from the bark of various species of birch tree, including Betula pendula Roth., B. alba L. and B. pubescens Ehrh. (14), as well as from other plants, such as Pyrus pyrifolia (15) and Platycodon grandiflorum (16), by solvent extraction or purified chromatography. Betulin exhibits numerous biological effects, including antiviral activities against the human Epstein-Barr virus (EBV) (17), and antifungal activities against Epidermophyton floccosum and Microsporum spp. (18). Recently, betulin has been shown to exhibit activity against several cancer cell types, including DLD-1, PC-3, HeLa, SK-HEP-1, HepG2, A549 and...
Betulin decreases the viability of RCC4 multidrug-resistant human renal carcinoma cells in a time- and concentration-dependent manner. Four human cancer cell lines [RCC4 (kidney), AGS (stomach), HeLa (cervical) and SK-Hep-1 (liver)] were treated with 0, 6.25, 12.5, 25 or 50 μM betulin for 48 h, and cell viability was assessed using the MTT assay. Among the cell lines, the viability of RCC4 cells was potently inhibited by treatment with betulin (Fig. 1B). Betulin inhibited the viability of the RCC4 cells with IC50 values of 23.1 and 14.8 μM after 24 and 48 h, respectively (Fig. 1C). In addition, LDH release was observed after a 24-h betulin treatment, and this was significantly increased at betulin doses exceeding 12.5 μM (Fig. 1D). Accordingly, 10 and 25 μM betulin were used in further studies to identify the mechanisms underlying the effect on RCC4 cell viability.

Betulin induces cancer cell death by activating the caspase-mediated apoptosis pathway in RCC4 cells. To determine whether betulin-induced cytotoxicity is related to apoptosis, the expression of pro-apoptotic and anti-apoptotic proteins in RCC4 cells was assessed by western blotting. The expression levels of proteins associated with apoptosis
Figure 1. Inhibitory effect of betulin on the proliferation of human cancer cells. Cell viability was determined using the MTT assay. (A) Chemical structure of betulin. (B) Inhibition of viability by betulin in several human cancer cell lines. Cancer cells were treated with 0, 6.25, 12.5, 25 or 50 µM betulin for 48 h. (C) IC50 values of betulin in the RCC4 cells. Cells were treated with 0, 1.56, 3.12, 6.25, 12.5, 25 or 50 µM betulin for 24 or 48 h. (D) LDH release in response to betulin treatment of the RCC4 cells. Cells were treated with 0, 6.25, 12.5, 25 or 50 µM betulin for 24 h, and the release of LDH was determined using an LDH detection kit. Data are presented as the means ± SD of two independent triplicates. *P<0.05, **P<0.01 and ***P<0.001 indicate statistical significance, compared to an untreated control. LDH, lactate dehydrogenase.

Figure 2. Verification of caspase-dependent apoptosis induced by betulin in RCC4 cells. (A) Effect of betulin on caspase protein expression. Cells were exposed to 10 and 25 µM betulin for 12 and 24 h. Band intensity compared to untreated cells was calculated using ImageJ after neutralization relative to GAPDH expression. Data represent two independent experiments. (B) Relative luminescence indicating dose-dependent activation of caspase-3/7, -8 and -9 induced by a 24-h treatment with betulin. Data represent two independent triplicates. *P<0.05 and **P<0.01 indicate statistical significance, compared to an untreated control. (C) Assessment of the apoptotic effect of a 24-h betulin treatment using a caspase inhibitor. Cells were respectively exposed to Z-VAD-fmk, a general caspase inhibitor and Nec-1, a necroptosis inhibitor, for 30 min before addition of betulin (10 and 25 µM). After 24 h, cell viability was determined using the MTT assay. Data are presented as means ± SD. P-values indicate statistical significance, compared to betulin treatment (*P<0.05, Z-VAD-fmk; *P<0.05, Nec-1). Nec-1, necrostatin-1.
were clearly influenced by betulin treatment (Fig. 2A). Pro-caspase-3, -7, -8 and -9 were cleaved to their active forms after exposure to 10 and 25 µM betulin for 12 h in RCC4 cells. Betulin also induced truncation of Bid and cleavage of PARP in a time- and dose-dependent manner. Most caspases were strongly activated by a 24-h betulin treatment. Particularly at a 25 µM dose, all caspases were induced >6-fold, compared to the levels detected in the untreated RCC4 cells. In addition, levels of truncated Bid (t-Bid) and cleaved PARP increased by 12- and 6-fold, respectively. To confirm that apoptosis induced by betulin requires caspase activation, a caspase activity assay was performed in the RCC4 cells (Fig. 2B). Betulin at 10 and 25 µM doses significantly increased the activities of caspase-3/7, -8 and -9, ~10-, 5- and 7-fold compared to the non-treated cells, respectively. As shown in Fig. 3C, in the inhibitor study, pre-incubation with Z-VAD-fmk, a broad-spectrum caspase inhibitor, for 30 min significantly blocked betulin-induced cell death at 10 µM betulin. Pre-incubation with Nec-1, a necroptosis inhibitor, blocked betulin-induced cell death; however, the cell death was not completely blocked at 10 and 25 µM betulin compared to the effects of Z-VAD-fmk. These data support the hypothesis that the anticancer effects of betulin in RCC4 cells are related to apoptotic cell death resulting from activation of caspases.

**Betulin treatment leads to the expression of apoptosis-related proteins in RCC4 cells.** The betulin-induced expression of apoptosis-related proteins was calculated using proteome array panels (Fig. 3A). After a 24-h treatment of RCC4 cells with 10 µM betulin, the protein levels related to apoptosis, such as Bax, active caspase-3, TNF-related apoptosis-inducing ligand (TRAIL) receptor 1/DR4 and 2/DR5, and tumour necrosis factor receptor 1 (TNF R1) were upregulated. Bax and active caspase-3 levels were significantly upregulated, with active caspase-3 levels increasing >2-fold compared to the untreated cells. Betulin increased the levels of TRAIL R1/DR4 and R2/DR5 by 43 and 42%, respectively. Betulin also induced upregulation of TNFR1 protein expression by 72%. The NF-κB pathway is closely associated with the expression of TNFR1. Therefore, we determined the effect of betulin on the nuclear translocation of p65 in the RCC4 cells. As shown in Fig. 3B, the nuclear translocation of p65 was significantly suppressed by betulin in a concentration-dependent manner for 24 h. To confirm that betulin-induced apoptosis is associated with the mitochondrial-mediated pathway, the levels of Bcl-2 family proteins including Bcl-2, Bax, Bad and PUMA and XIAP, were estimated by western blot analysis (Fig. 3C). The results demonstrated that the betulin increased the expression of pro-apoptotic proteins. Specifically, a significant increase in Bax and PUMA expression was observed in response to a 10 µM dose of betulin for 24 h. Conversely,
the expression levels of anti-apoptotic proteins, such as Bcl-2 and XIAP, were decreased markedly following treatment with 10 µM betulin.

**Betulin enhances the sensitivity of RCC4 cells to anticancer drugs.** We hypothesised that betulin sensitises renal cancer cells to anticancer drugs. To test this, we treated cells with betulin in combination with anticancer drugs, including 5-FU, etoposide and temozolomide. 5-FU, etoposide and temozolomide weakly inhibited the growth of the RCC4 cells after 24 h by 13.54, 24.38 and 2.43%, respectively, compared to the control condition, whereas the addition of 25 µM betulin resulted in inhibition of cell growth by up to 56.12% (Fig. 4A).

In addition, by co-treating cells with etoposide in the presence of betulin, the cytotoxicity of the RCC4 cells was significantly increased. The cytotoxicity induced by co-treatment with 5 µM etoposide and 5 µM betulin was almost equal to that of 10 µM betulin alone (Fig. 4B). To confirm the synergistic effect of betulin and etoposide on cancer cell death, the levels of cleaved PARP and MDR1 were assessed in RCC4 cells using western blot analysis. As shown in Fig. 4C, co-treatment with 10 µM etoposide and 10 µM betulin increased the level of cleaved PARP >2- and 3-fold, compared to betulin and etoposide treatment alone, respectively. In the case of MDR1, elevated expression of MDR1 was observed in the RCC4 cells, and this was decreased ~2-fold by co-treatment with 10 µM betulin and 10 µM etoposide. However, treatment with betulin or etoposide alone did not affect MDR1 expression.

**Discussion**

The induction of apoptosis in cancer cells by natural compounds is a key goal for cancer prevention and therapy. Betulin is a pharmacologically active triterpene that is naturally occurring in birch bark. It is used in its extracted form or as a base for chemical modification to compounds such as betulinic acid (22). Previous studies have reported that betulin has an anticancer effect in human lung, cervical and liver cancer cells (23). Betulin has also been shown to protect hepatoma cells from damage by ethanol-induced liver stellate or cadmium-induced apoptosis, via a mechanism involving inhibition of reactive oxygen species (ROS) production or apoptosis induction (24,25). In HepG2 cells, betulin was found to induce apoptotic cell death through the intrinsic apoptotic pathway that includes activation of caspase-9 and -3 (23). Caspase is a key mediator of apoptosis that is activated via endoplasmic reticulum stress, extracellular stimuli and mitochondrial damage (26). The activation of caspase-8 is initiated by the stimulation of cell-surface death receptors, such as Fas, TNF and TRAIL. This leads to activation of caspase-3 or -7, or modulation of the mitochondrial pathway via truncation of Bid (27). Conversely, caspase-9 is activated
by cytochrome c released from the mitochondria, which in turn, leads to the activation of effector caspases, such as caspase-3, -6 and -7. Activated caspase-3 induces cleavage of PARP, a DNA repair enzyme, and ultimately, apoptosis (28). The present study revealed that betulin treatment inhibited the viability of human renal cancer cells and increased the activity of caspases, including caspase-3, -7, -8 and -9. Furthermore, our data indicated that betulin regulates mitochondrial signalling pathways associated with apoptosis, including the Bcl-2 family and XIAP pathways, which suggests that sensitisation of cancer cells by betulin to apoptosis is caspase-9-dependent. Notably, in the present study, betulin significantly increased the levels of TRAIL R1/DR4 and R2/DR5, and TNFR1 in an apoptosis array. TRAIL induces apoptosis in a variety of transformed or tumour cells, but not in normal cells; however, many cancer cells are resistant to TRAIL (27). Binding of TRAIL to the TRAIL R1/DR4 and R2/DR5 receptors results in trimerisation to form a death-inducing signalling complex (DISC). DISC recruits the adapter protein FADD and caspase-8, leading to activation of intact caspases and cleavage of PARP (29). Therefore, increased expression of the TRAIL death receptors could play an important role in the sensitisation of cancer cells to TRAIL-associated apoptosis following proteasome inhibition. TNFR1 induces the activation of NF-κB and plays a role in caspase-8 activation by controlling the receptor interacting protein-1 (RIP1) (30). NF-κB is an apoptosis regulator, and its inhibition results in apoptosis in a variety of cell types found to be initially resistant to TNF-α-induced apoptosis (31,32). Furthermore, in cancer therapy, suppression of NF-κB translocation can increase the effectiveness of cancer therapy (33). Hence, our data demonstrated that stimulation of TRAIL receptors and TNFR1 by betulin results in activation of caspase-8 and inhibition of NF-κB signalling. Therefore, we suggest that betulin treatment leads to induction of apoptosis through both the intrinsic and extrinsic pathways in RCC4 cells.

Additionally, we demonstrated that betulin inhibits cell viability by inducing caspase-mediated apoptosis in RCC4 human renal carcinoma cells shown to be resistant to anticancer drugs, such as 5-FU, temozolomide and etoposide. Among them, etoposide is a topoisomerase inhibitor that disrupts the repair of DNA damage. DNA-damaging drugs are highly effective anticancer drugs that are in current clinical use; however, side-effects, including vomiting, have been reported in several studies (34). RCCs are the most common malignant tumours found in the adult kidney, and unlike other malignancies, they are generally resistant to conventional therapies. Therapeutic resistance in RCCs occurs as a result of genetic changes that confer tumorigenic potential and survival advantages during chemotherapy (35,36). Most of the established resistance-modifying agents (RMA) are excessively toxic at the required doses. The search for P-gp inhibitors among libraries of natural products may be more promising, as many natural products and phytotherapeutics are appreciated for their low incidence and severity of side-effects and good tolerability (5). Previous studies have revealed that the first intron of the human MDR1 gene binds to NF-κB complexes (37). In the present study, betulin alone or in combination with etoposide, significantly increased the protein level of cleaved PARP, while etoposide alone did not affect cleavage of PARP. Furthermore, the combination of betulin and etoposide significantly suppressed MDR1 expression, whereas either drug alone did not. Betulin inhibited the translocation of NF-κB, yet failed to affect the expression of MDR1. Thus, we suggest that betulin and anticancer drugs synergistically enhance toxicity in multidrug-resistant human renal carcinoma cells. Further research is necessary to identify the sensitising molecular mechanism and in vivo therapeutic benefits of combination therapy involving betulin and anticancer drugs.

In conclusion, we demonstrated that betulin induced apoptosis in RCC4 cells via activation of death-inducing receptors, including TRAIL receptors and TNFR1, and regulation of anti-apoptotic or pro-apoptotic proteins. Furthermore, the combination of betulin and an anticancer agent synergistically inhibited PARP expression and upregulated MDR1 expression in RCC4 cells. Therefore, we suggest that betulin is a promising candidate chemopreventive and chemotherapeutic agent for multidrug-resistant human renal cancer.

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References