Novel betulin derivative induces anti-proliferative activity by G2/M phase cell cycle arrest and apoptosis in Huh7 cells

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Abstract. Betulin (BT) has been identified to exhibit potential benefits for treating hepatocellular carcinoma (HCC). The results of the present study demonstrated that a new semisynthetic derivative of BT, 3,28-di-(2-nitroxy-acetyl)-oxy-BT, may effectively decrease the viability of Huh7 cells. Mechanistic studies revealed that 3,28-di-(2-nitroxy-acetyl)-oxy-BT inhibited the transition between G1 and M phase of the cell cycle by regulating cell cycle regulatory proteins. Additional study revealed that 3,28-di-(2-nitroxy-acetyl)-oxy-BT may trigger Huh7 cells to undergo caspase-dependent apoptosis as an increased proportion of cells were identified in the sub-G1 phase, which may be a result of poly(ADP-ribose) polymerase cleavage and caspase activation. Furthermore, 3,28-di-(2-nitroxy-acetyl)-oxy-BT-induced apoptosis was mitochondrion-mediated. The results of the present study demonstrated that Bcl-2-associated X protein translocated to the mitochondria from the cytosol following 3,28-di-(2-nitroxy-acetyl)-oxy-BT treatment. Notably, the phosphoinositide 3-kinase/protein kinase B signaling pathway was involved in 3,28-di-(2-nitroxy-acetyl)-oxy-BT-treated Huh7 cells. Therefore, the results of the present study demonstrated that 3,28-di-(2-nitroxy-acetyl)-oxy-BT may inhibit HCC, which may be a possible application to treat HCC.

Introduction

In recent years, people have an increased risk of hepatocellular carcinoma (HCC) due to living habits (heavy alcohol drinking and tobacco smoking) and living in a worsening environment (polluted air) (1). A previous study demonstrated that liver cancer or primary hepatic cancer is the fifth most common type of global malignancy and the third most common cause of cancer-associated mortality globally (2). An effective chemotherapy for HCC cancer has not yet been identified (3). Sorafenib is the first-line treatment; however, this only has a limited effect on increasing the survival time of patients with HCC, with the median OS extended by approximately 3 months (4). Therefore, it remains a challenge to identify a novel effective therapeutic agent with low toxicity for the treatment of HCC.

Betulin (BT), a member of pentacyclic lupane-type triterpenes primarily located in the white birch, has been demonstrated to exhibit a number of biological functions including anticancer, anti-human immunodeficiency virus and anti-inflammatory effects (5,6). BT is a traditional medicine and has been used for the treatment of actinic keratosis for a number of years in Germany (7). A previous study disproved the significance of BT in melanoma cells (8); however, subsequent studies have demonstrated the anticancer activity of BT in a number of types of human cancer including neuroblastoma (9), colon (10), breast (11), hepatocellular (12), lung (13), prostate (14), renal cell (15) and ovarian (16). In addition, it has been demonstrated that the apoptotic properties of BT are due to modulation of the B-cell lymphoma (Bcl-2) family and cell cycle regulatory proteins (12,13), and the activation of caspases and DNA fragmentation (15,17).

To identify an agent which exhibit increased inhibitory effects against distinct cancer cell lines and decreased toxicity compared with BT, a variety of BT derivatives have been synthesized (18-20). A previous study demonstrated that the C-3 or C-28 positions serve a function in the pharmacological activities of BT (21). On the basis of this principle, in the present study, a library of semisynthetic analogs of BT were synthesized, aiming at substituents with the C-3 or/and C-28 position. The results of the present study identified that...
3,28-di-(2-nitroxy-acetyl)-oxy-BT exhibited the most significant effect on Huh7 cells. To the best of our knowledge, the present study was the first to demonstrate that 3,28-di-(2-nitroxy-acetyl)-oxy-BT inhibited Huh7 cell growth, by inducing mitochondrion-mediated cell apoptosis and G_{1}/M cell cycle arrest. Furthermore, the results of the present study identified that 3,28-di-(2-nitroxy-acetyl)-oxy-BT inhibited the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway. These results suggested that 3,28-di-(2-nitroxy-acetyl)-oxy-BT may be used for the clinical treatment of HCC.

**Materials and methods**

**Reagents.** RPMI-1640 culture medium, fetal bovine serum (FBS), trypsin, penicillin-streptomycin were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). MTT, dimethyl sulfoxide (DMSO), propidium iodide (PI) and RNase A were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). An Annexin V-fluorescein isothiocyanate (FITC)/PI double staining kit was purchased from Nanjing Institute of Biological Engineering (Nanjing, China) and 5,5′,6,6′-tetraochloro-1,1′,3,3′-tetramethyl benzimidazolyl-carboxyiane iodide (JC-1) was obtained from Invitrogen; Thermo Fisher Scientific, Inc. All antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

**Synthesis of 3,28-di-(2-nitroxy-acetyl)-oxy-BT.** BT (purity >95%) was obtained from XiaoGan ShenYuan Chemical Co., Ltd. (XiaoGan, China). The reaction of BT with bromoacetyl bromide (Thermo Fisher Scientific, Inc.) yielded 3,28-di-(2-bromoacetyl)-oxy-BT. This compound reacted with silver nitrate to form 3,28-di-(2-nitroxy-acetyl)-oxy-BT (Fig. 1A). The structure of 3,28-di-(2-nitroxy-acetyl)-oxy-BT was identified by infrared spectroscopy (IR), 1D nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS). IR (KBr/cm⁻¹): 2918, 2850, 1742, 1655, 1467, 1384, 1292. ¹H NMR (400 MHz, CDCl₃): δ: 0.83, 0.85, 0.86, 0.87, 0.89, 1.04 (s, 18H, 6xCH₃), 2.25 (m, 1H, H-19), 4.61 (d, 1H, J 7.5 Hz, H-29a), 3.83 (m, 2H, J 7.5 Hz, H-29b), 4.64 (d, 1H, J 7.5 Hz, H-29a), 3.83 (m, 2H), 4.89, 4.88 (s, 2xCH₂,ONO), 13C NMR (100 MHz, CDCl₃): δ: 38.5 (C-1), 23.6 (C-2), 83.6 (C-3), 40.8 (C-4), 55.5 (C-5), 18.1 (C-6), 34.6 (C-7), 43.3 (C-8), 51.0 (C-9), 37.1 (C-10), 21.5 (C-11), 22.6 (C-12), 37.6 (C-13), 51.0 (C-14), 28.2 (C-15), 31.9 (C-16), 37.9 (C-17), 52.1 (C-18), 48.8 (C-19), 144.1 (C-20), 29.3 (C-21), 34.9 (C-22), 27.9 (C-23), 16.7 (C-24), 16.5 (C-25), 15.5 (C-26), 14.1 (C-27), 67.0 (C-28), 109.6 (C-29), 21.0 (C-30), 165.6 (C-31, C-31′), 67.4 (C-32, C-32′). HRMS (m/z) calculated for C₄₈H₃₂N₂O₆: 648.3622; identified 648.3621. 3,28-di-(2-nitroxy-acetyl)-oxy-BT was dissolved in DMSO until use.

**Cell culture.** The human hepatocellular carcinoma cell line Huh7 was purchased from the Chinese Academy of Sciences (Shanghai, China). Cells (passages <20) were cultured in 37°C in an atmosphere containing 5% CO₂ with RPMI-1640 medium, supplemented with 10% FBS.

**Cell viability assay.** Huh7 cells (5x10⁵ cells/well) in the exponential growth phase (24 h following passage) were seeded in 96-well plates, cultured overnight in 37°C and added with various concentrations of 3,28-di-(2-nitroxy-acetyl)-oxy-BT (0, 0.4, 0.8, 1.6, 3.1, 6.2, 12.5, 25.0, 50.0, 100.0 µM) at 37°C for 24 h. Subsequently, MTT (5 mg/ml) was added to the cells and incubated in the dark at 37°C for 4 h. The resulting formazan crystals were dissolved using DMSO and the optical density was measured at 595 nm to determine the half-maximal inhibitory concentration (IC₅₀) value, using the DTX 880 Multimode Detector (Beckman Coulter, Inc., Brea, CA, USA).

**Clonogenic assay.** Cells (5x10² cells/well) in the exponential growth phase were seeded in 6-well plates and cultured overnight at 37°C. The cells were treated with indicated concentrations of 3,28-di-(2-nitroxy-acetyl)-oxy-BT (0, 13, 26 µM) and cultured overnight at 37°C. Subsequently, the cells were maintained for 14 days with fresh RPMI-1640 medium. Plates were washed with PBS, and subsequently fixed with 75% methanol at 37°C for 30 min and stained with 1% crystal violet at 37°C for 30 min. The mixture was removed and the plates were washed with PBS and allowed to dry at room temperature. The number of colonies >0.5 mm in diameter with 5 fields of view were counted under an inverted phase-contrast IX51 microscope using x10 magnification (Olympus Corporation, Tokyo, Japan).

**DNA content analysis.** DNA content analysis was performed using an autofocus probe, PI (Sigma-Aldrich, Merck KGaA), according to the manufacturer's protocol. Huh7 cells (3x10⁴ cells/well) in the exponential growth phase were seeded in 6-well plates, cultured at 37°C for 24 h and added various concentrations of 3,28-di-(2-nitroxy-acetyl)-oxy-BT (0, 13, 26 µM) at 37°C for 24 h. The cells were selected, washed three times with PBS and fixed with 75% ethanol at 4°C overnight. Each sample was incubated with 0.05 mg/ml PI and 0.1 mg/ml RNase A for 30 min at 37°C. The DNA content was determined using an Epics XL flow cytometer (excitation, 488 nm; emission, 620 nm). The data were analyzed with ModFit LT software (version 3.2; BD Biosciences, Franklin Lakes, NJ, USA).

**Apoptosis detection using Annexin V-FITC/PI.** The Annexin V-FITC/PI staining assay kit was used to evaluate the apoptosis rate (Nanjing Institute of Biological Engineering, Nanjing, China). Huh7 cells (3x10⁶ cells/well) were incubated in 6-well plates and incubated with various concentrations of 3,28-di-(2-nitroxy-acetyl)-oxy-BT (0, 13, 26 µM) at 37°C for 24 h. Subsequently, the cells were selected, washed three times with PBS and incubated with Annexin V-FITC/PI at 37°C in the dark for 15 min. The apoptosis ratio was determined using the Epics XL flow cytometer (Annexin V-FITC: Excitation, 488 nm; emission, 525 nm; PI: Excitation, 488 nm; emission, 620 nm).

**Detection of mitochondrial membrane potential.** The JC-1 kit (Thermo Fisher Scientific, Inc.) was used to detect mitochondrial membrane potential. Huh7 cells (3x10⁴ cells/well) in the exponential growth phase were seeded in 6-well plates and incubated with various concentrations of 3,28-di-(2-nitroxy-acetyl)-oxy-BT (0, 13, 26 µM) at 37°C for 24 h. The cells were selected, washed three times with PBS and incubated with 10 µg/ml JC-1 in 37°C for 30 min in the dark. Subsequently, the
Mitochondrial membrane potential alterations were determined using the Epics XL flow cytometer (J-aggregates: Excitation, 488 nm; emission, 575 nm. JC-1 monomers: Excitation, 488 nm; emission, 525 nm).

Western blot analysis. Huh7 cells (6x10⁵ cells/100 mm dish) in the exponential growth phase were incubated overnight with various concentrations of 3,28-di-(2-nitroxy-acetyl)-oxy-betulin. Following treatment, the cells were selected, incubated with radioimmunoprecipitation assay buffer (0.1 M phenylmethylsulfonyl fluoride protease and phosphatase inhibitor cocktail; Sigma-Aldrich; Merck KGaA) for 30 min on ice, centrifuged at 10,000 x g at 4°C for 15 min, and the supernatant was stored at -80°C. To isolate the cytosolic fraction, the cells were selected, lysed in cytosolic lysis buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 300 mM sucrose, 0.5% NP-40, 10 mM KCl] with protease and phosphatase inhibitor cocktail (Sigma-Aldrich; Merck KGaA) for 30 min on ice and centrifuged at 10,000 x g at 4°C for 15 min. The supernatant fluid was selected as part of the cytoplasm. To isolate the mitochondrial fraction, cell pellets were lysed in mitochondrial lysis buffer (50 mMTris-HCl, 150 mMNaCl and 1% NP-40) with protease and phosphatase inhibitor (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) on ice for 30 min, centrifuged at 10,000 x g at 4°C for 15 min and the supernatant was selected.

The cell lysates (50 µg) were separated using 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane, blocked at 37°C for 1 h with 5% bovine serum albumin (Beyotime Institute of Biotechnology, Haimen, China) and with the primary antibody [cyclin B1, cat. no. 12231; CDK1, cat. no. ab133327 (Abcam, Cambridge, MA, USA); CDC25C, cat. no. 4688; PARP, cat. no. 9542; C-PARP, cat. no. 5625; caspase 3, cat. no. 9662; caspase 9, cat. no. 9502; Bcl-2, cat. no. 7382; Bax, cat. no. 5023; cytochrome c, cat. no. 11940; VDAC, cat. no. 4866; PI3K p110α, cat. no. 4249; Akt, cat. no. 4691; p-AKT (Thr308), cat. no. 2965; p-AKT (Ser473), cat. no. 4060; β-actin, cat. no. 4967; Cell Signaling Technology, Inc.] at 4°C overnight (dilution, 1:1,000) and the secondary antibody (anti-rabbit IgG, HRP-linked antibody, cat. no. 7074; dilution, 1:2,000; Cell Signaling Technology, Inc.). Immunoreactive bands were visualized using an enhanced chemiluminescence substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a X-ray film processor (Kodak, Rochester, NY, USA).

Statistical analysis. The data are presented as the mean ± standard deviation. GraphPad Prism (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. A one-way analysis of variance followed by Tukey’s test was performed. P<0.05 was considered to indicate a statistically significant difference.
Results

3,28-Di-(2-nitroxy-acetyl)-oxy-BT inhibits the growth of Huh7 cells in vitro. Determined using an MTT assay, 3,28-di-(2-nitroxy-acetyl)-oxy-BT led to an anti-proliferative effect on Huh7 cells and markedly decreased the viability of Huh7 cells in a dose-dependent manner (Fig. 1B). The IC_{50} value was identified to be 13.1±1.37 µM, following 24 h of treatment. To evaluate the long-term effect on cell survival, a colony-formation assay was performed (Fig. 1C). The inhibitory effect of 3,28-di-(2-nitroxy-acetyl)-oxy-BT on colony formation was demonstrated to be concentration-dependent, which validated the cytotoxic effect of 3,28-di-(2-nitroxy-acetyl)-oxy-BT against Huh7 cells.

3,28-Di-(2-nitroxy-acetyl)-oxy-BT induces cell cycle arrest and is associated with cell cycle regulatory proteins. In order to determine whether the anti-proliferative effect of 3,28-di-(2-nitroxy-acetyl)-oxy-BT led to an anti-proliferative effect on Huh7 cells and markedly decreased the viability of Huh7 cells in a dose-dependent manner (Fig. 1B). The IC_{50} value was identified to be 13.1±1.37 µM, following 24 h of treatment. To evaluate the long-term effect on cell survival, a colony-formation assay was performed (Fig. 1C). The inhibitory effect of 3,28-di-(2-nitroxy-acetyl)-oxy-BT on colony formation was demonstrated to be concentration-dependent, which validated the cytotoxic effect of 3,28-di-(2-nitroxy-acetyl)-oxy-BT against Huh7 cells.

3,28-Di-(2-nitroxy-acetyl)-oxy-BT induces apoptosis. To investigate the underlying mechanism by which 3,28-di-(2-nitroxy-acetyl)-oxy-BT induced G2/M phase arrest, the expression of proteins involved in cell cycle regulation were identified using western blot analysis (Fig. 2B). The results revealed that 3,28-di-(2-nitroxy-acetyl)-oxy-BT treatment inhibited cyclin B1 expression and decreased the expression levels of CDK1 and CDC25C. In addition, the expression level of cell division cycle 25C (CDC25C), which acts as an upstream regulator of the CDK-cyclin complex, was inhibited by 3,28-di-(2-nitroxy-acetyl)-oxy-BT.

3,28-Di-(2-nitroxy-acetyl)-oxy-BT induces caspase-dependent apoptosis. To validate the occurrence of apoptosis, an Annexin V-FITC/PI double-staining assay was performed. As presented in Fig. 3A, the proportion of apoptotic cells (including early and late apoptotic cells) increased as the concentration of 3,28-di-(2-nitroxy-acetyl)-oxy-BT increased (0 µM, 1.6%; 26 µM, 27.0%). Validated using western blotting, cleaved poly(ADP-ribose) polymerase (PARP) was markedly increased in 3,28-di-(2-nitroxy-acetyl)-oxy-BT-treated Huh7 cells, compared with the control. In addition, it was identified that cleaved caspase 3 and cleaved caspase 9 were markedly increased in 3,28-di-(2-nitroxy-acetyl)-oxy-BT-treated Huh7 cells (Fig. 3B).

3,28-Di-(2-nitroxy-acetyl)-oxy-BT induces apoptosis through mitochondrial signaling pathways. To investigate the
The molecular mechanism underlying 3,28-di-(2-nitroxy-acetyl)-oxy-BT-induced apoptosis in Huh7 cells, the loss of mitochondrial transmembrane potential was determined using JC-1. As presented in Fig. 4A, the green fluorescence of the JC-1 monomers increased, compared with the control, following treatment with 3,28-di-(2-nitroxy-acetyl)-oxy-BT (control, 3.48%; 26 µM, 23.4%), suggesting that 3,28-di-(2-nitroxy-acetyl)-oxy-BT induced a loss of mitochondrial membrane potential in Huh7 cells in a concentration-dependent manner. Subsequently, the expression level of the Bcl-2 family of apoptosis regulator proteins were determined using western blot analysis. As presented in Fig. 4B, Bcl-2 and Bcl-2-associated X protein (Bax) were identified to be decreased and increased, respectively, following treatment with 3,28-di-(2-nitroxy-acetyl)-oxy-BT, compared with the control. In addition, the cytosolic cytochrome c level was determined, which demonstrated that treatment with 3,28-di-(2-nitroxy-acetyl)-oxy-BT resulted in a marked increase, compared with untreated cells (Fig. 4C). Furthermore, 3,28-di-(2-nitroxy-acetyl)-oxy-BT treatment increased the translocation of Bax from the cytosol to the mitochondria.

3,28-Di-(2-nitroxy-acetyl)-oxy-BT inhibits the PI3K/Akt signaling pathway in Huh7 cells. As the PI3K/Akt signaling pathway is a critical regulator of cellular survival and apoptosis, the effect of 3,28-di-(2-nitroxy-acetyl)-oxy-BT on this pathway, and whether PI3K/Akt served a function in 3,28-di-(2-nitroxy-acetyl)-oxy-BT-induced apoptosis, was investigated. As presented in Fig. 5, 3,28-di-(2-nitroxy-acetyl)-oxy-BT treatment decreased the level of the catalytic (p110) subunit of PI3K, and the level of Akt and phosphorylated (p-)Akt in a concentration-dependent manner, compared with the controls.

Discussion

Despite advancements in the therapeutic strategies for the majority of types of cancer, the systemic treatment for HCC remains ineffective (22). Therefore, identification of novel agents that may prevent the progression of HCC is required. In the present study, a novel semi-synthetic derivative of BT was developed to target tumor cells, to improve patient outcome, was synthesized. To the best of our knowledge, the present study was the first to investigate and demonstrate the antitumor effect and cytotoxic mechanisms of 3,28-di-(2-nitroxy-acetyl)-oxy-BT against Huh7 cells.

Deregulation of cell cycle progression is a typical hallmark of cancer (23). Therefore, targeting the regulatory components of the cell cycle machinery has been identified as an important strategy for the treatment of cancer (24). Diverse natural compounds inhibit cancer cell growth by arresting the cell cycle (25,26). To determine whether 3,28-di-(2-nitroxy-acetyl)-oxy-BT induced cell cycle arrest of Huh7 cells, a DNA content assay was performed, which demonstrated that Huh7 cells were arrested in G2/M phase following treatment with 3,28-di-(2-nitroxy-acetyl)-oxy-BT. The cell cycle is regulated by proteins which are divided into two classes of molecule: CDKs, a family of serine/threonine kinases, and the cyclin-binding partners (27). CDK1 and cyclin B1 serve regulatory functions in the G2/M transition by forming the CDK1-cyclin B1 complex (28). In the present...
The downregulation of CDK1 and cyclin B1 suggested that 3,28-di-(2-nitroxy-acetyl)-oxy-BT induced G2/M arrest via the modulation of CDK1 and cyclin B1. CDC25C is required for the full activation of CDK1-cyclin B1 during the G2/M transition (29). The decreased expression of CDC25C, identified in the present study, indicated that 3,28-di-(2-nitroxy-acetyl)-oxy-BT may decrease CDC25C and thus suppress the activation of CDK1-cyclin B1, resulting in Huh7 cell G2/M arrest.

Apoptosis is a process of programmed cell death, which serves a function in maintaining cellular homeostasis between cell division and cell death (30). Previous studies have demonstrated that apoptosis is an important mechanism by which chemotherapeutic agents kill susceptible cells (31,32). As the results of the present study demonstrated that 3,28-di-(2-nitroxy-acetyl)-oxy-BT caused a marked sub-G1 apoptotic peak in Huh7 cells, the molecular mechanisms underlying the anti-hepatic effect of 3,28-di-(2-nitroxy-acetyl)-oxy-BT on Huh7 cells were investigated. Annexin V-FITC/PI double staining assay indicated that 27.0% of Huh7 cells underwent apoptosis following treatment.
treatment with 26 μM 3,28-di-(2-nitroxy-acetyl)-oxy-BT for 24 h. Additionally, activated caspases 9 and 3, the initiator and executioner caspases in the mitochondrial apoptotic signaling pathway (33), were determined, using western blot analysis, in Huh7 cells following 3,28-di-(2-nitroxy-acetyl)-oxy-BT treatment. A previous study demonstrated that once the specificity substrate, including cleaved PARP, has been cleaved by caspases, apoptosis will be induced (34). In the present study, western blot analysis revealed that PARP was cleaved from a 116 kDa fragment to an 85 kDa fragment during 3,28-di-(2-nitroxy-acetyl)-oxy-BT-induced apoptosis. Notably, a decreased proportion of apoptotic cells (11.3%) was observed at the IC_{50} concentration, which may be because cell death processes, induced by 3,28-di-(2-nitroxy-acetyl)-oxy-BT, are complex and include necrosis and autophagy. Additional study is required to investigate the involvement of other types of cell death in 3,28-di-(2-nitroxy-acetyl)-oxy-BT-treated cells, including necrosis and autophagy.

Mitochondria are important organelles which regulate cell apoptosis (35). In order to clarify the underlying molecular mechanism by which 3,28-di-(2-nitroxy-acetyl)-oxy-BT induces apoptotic cell death, the mitochondria-mediated apoptotic signaling pathway was evaluated. The results of the present study indicated that the mitochondrial transmembrane potential was decreased following 3,28-di-(2-nitroxy-acetyl)-oxy-BT treatment, compared with the control. Previous studies have identified that the mitochondrial membrane permeability is regulated by Bcl-2 family members, which are the central regulators of caspase activation (36,37). Bax, a pro-apoptotic member of the Bcl-2 family, serves as a gateway for the release of apoptotic proteins, including cytochrome c (38). The results of the present study demonstrated that Bax was translocated to the mitochondria in marked amounts following 3,28-di-(2-nitroxy-acetyl)-oxy-BT treatment. In addition, the Bax/Bcl-2 ratio was markedly increased in treated cells which validated that the intrinsic mitochondrial pathway triggered 3,28-di-(2-nitroxy-acetyl)-oxy-BT-induced Huh7 cell apoptosis. Furthermore, downregulated Bcl-2, an inhibitor of mitochondrial cytochrome c release (39), observed following 3,28-di-(2-nitroxy-acetyl)-oxy-BT treatment, may participate in the apoptosis of Huh7 cells. Therefore, it may be hypothesized that 3,28-di-(2-nitroxy-acetyl)-oxy-BT treatment resulted in the decrease in the mitochondrial transmembrane potential and subsequent apoptosis of Huh7 cells.

Activation of the PI3K/Akt signaling pathway is a typical feature in a number of types of human cancer (40). Phosphorylation of the serine/threonine kinase Akt is known to trigger the inactivation of proapoptotic factors, which in turn confers a survival advantage on tumor cells (41). The significance of the PI3K/Akt signaling pathway and its potential as a therapeutic target for cancer treatment have been investigated in preclinical studies of several types of human cancer, including renal, lung, breast, glioblastoma, neuroblastoma and HCC (42). The results of these studies suggested that PI3K/Akt and its downstream signaling pathways are promising targets for therapeutic intervention (43,44). The PI3K/Akt pathway is known to serve a function in cell cycle progression, apoptosis and tumorigenesis; therefore, it is hypothesized that the PI3K/Akt signaling pathway may serve functions in 3,28-di-(2-nitroxy-acetyl)-oxy-BT-induced apoptosis. The results of the present study demonstrated that 3,28-di-(2-nitroxy-acetyl)-oxy-BT decreased the expression level of the catalytic (p110\alpha) subunit of PI3K, and the expression levels of Akt and p-Akt, in a concentration-dependent manner. Furthermore, treatment of Huh7 cells with 3,28-di-(2-nitroxy-acetyl)-oxy-BT resulted in decreased expression of PI3K (p110\alpha) and decreased phosphorylation of Akt at Ser^{473} and Thr^{308}.

The results of the present study indicated that 3,28-di-(2-nitroxy-acetyl)-oxy-BT may trigger Huh7 cells to undergo apoptosis, with the decreased expression level of the catalytic (p110\alpha) subunit of PI3K, Akt and p-Akt, in a concentration-dependent manner. Additionally, 3,28-di-(2-nitroxy-acetyl)-oxy-BT treatment downregulated the protein expression of Bcl-2 and resulted in a loss of mitochondrial membrane potential, and consequent release of cytochrome c. Therefore, the present study demonstrated the potential usefulness of 3,28-di-(2-nitroxy-acetyl)-oxy-BT as an anti-liver cancer therapeutic candidate.

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**References**

1. Bosetti C, Turati F and La Vecchia C: Hepatocellular carcinoma epidemiology. Best Practice & Res Clin Gastroenterol 28: 753-770, 2014.
2. Forner A, Llovet JM and Bruix J: Hepatocellular carcinoma. Lancet 379: 1245-1255, 2012.
3. Fares N and Peron JM: Epidemiology, natural history and risk factors of hepatocellular carcinoma. Kev Prat 63: 216-217, 2013 (In French).
4. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, et al: SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 359: 378-390, 2008.
5. Alakurtti S, Makela T, Koskimies S and Yli-Kauhaluoma J: Pharmacological properties of the ubiquitous natural product betulin. European journal of pharmaceutical sciences: Official journal of the European Federation for Pharmaceutical Sciences 29: 1-13, 2006.
6. Tolstikov GA, Flekhter OB, Shultz EE, Baltina LA and Tolstikov AG: Betulin and its derivatives. Chemistry and biological activity. Chemistry for Sustainable Development. 13: 1-29, 2005.
7. Huyke C, Reuter J, Rodig M, Kersten A, Laszczyn M, Scheffler A, Nashan D and Schemp C: Treatment of actinic keratoses with a novel betulin-based oleogel. A prospective, randomized, comparative pilot study. J Dtsch Dermatol Ges 7: 128-133, 2009 (In English, German).
8. Soica C, Dehelean C, Danciu C, Wang HM, Wenz G, Ambrus R, Bojin F and Anghel M: Betulin complex in gamma-cyclodextrin derivatives: properties and antineoplastic activities in in vitro and in vivo tumor models. Int J Mol Sci 13: 14902-15011, 2012.
9. Rzeski W, Stepulak A, Szymanski M, Juszczak M, Grabarska A, Siffringer M, Kaczor J and Kandeler-Szerszen M: Betulin elicits anti-cancer effects in tumour primary cultures and cell lines in vitro. Basic Clin Pharmacol Toxicol 105: 425-432, 2009.
10. Hwang RJ, Chen HP, Wang G, Anghel M, Kardono H, Pezzuto JM and Kinghorn AD: Cytotoxic triterpenes from the twigs of Celtis philippinensis. Phytochemistry 62: 197-201, 2003.
11. Hsu RJ, Hsu YC, Chen SP, Fu CL, Yu JC, Chang FW, Chen YH, Liu JM, Ho JY and Yu CP: The triterpenoids of H Hibiscus syriacus induce apoptosis and inhibit cell migration in breast cancer cells. BMC Complement Altern Med 15: 65, 2015.
BETULIN DERIVATIVE INDUCES CELL CYCLE ARREST AND APOPTOSIS IN Huh7 CELLS

12. Li Y, He K, Huang Y, Zheng D, Gao C, Cui L and Jin YH: Betulin induces mitochondrial cytochrome c release associated apoptosis in human cancer cells. Mol Carcinogene 49: 630-640, 2010.

13. Li XD, Zhang YJ and Han JC: Betulin inhibits lung cancer cell proliferation through activation of AMPK signalling. Tumour Biol 35: 11153-11158, 2014.

14. Gauthier C, Legault J, Lavoie S, Rondeau S, Tremblay S and Pichette A: Synthesis and cytotoxicity of bidesmosidic betulin and betulinic acid sapons. J Natl Prod 72: 72-81, 2009.

15. Yim NH, Jung YP, Kim A, Kim T and Ma JY: Induction of apoptotic cell death by betulin in multidrug-resistant human renal carcinoma cells. Oncol Rep 34: 1058-1064, 2015.

16. Dehelean CA, Soica C, Ledeti I, Alus A, Zipko I, G Luscan A, Cinta-Pinsar S and Munteanu M: Study of the betulin enriched birch bark extracts effects on human carcinoma cells and ear inflammation. Chem Central J 6: 137, 2012.

17. Saudagar P and Dubey VK: Molecular mechanisms of in vitro betulin-induced apoptosis of Leishmania donovani. TAM J Trop Med Hyg 90: 354-360, 2014.

18. Santos RC, Salvador JA, Marin S and Cascante M: Novel semi-synthetic derivatives of betulin and betulinic acid with cytotoxic activity. Bio Organic Med Chem Letters 20: 3409-3412, 2010.

19. Yang SJ, Liu MC, Xiang HM, Zhao Q, Xue W and Yang S: Synthesis and in vitro antitumor evaluation of betulin acid ester derivatives as novel apoptosis inducers. Eur J Med Chem 102: 4814-4817, 2009.

20. Santos RC, Salvador JA, Marin S and Cascante M: Novel semi-synthetic derivatives of betulin and betulinic acid with cytotoxic activity. Bio Organic Med Chem 17: 6241-6250, 2009.

21. Santhosh K and Hernandez-Gea V: Hepatocellular carcinoma: reasons for phase III failure and novel perspectives on trial design. Clin Cancer Res 20: 2072-2079, 2014.

22. Hanahan D and Weinberg RA: The hallmarks of cancer. Cell 100: 57-70, 2000.

23. Weber AM and Ryan AJ: ATM and ATR as therapeutic targets in cancer. Pharmacol Ther 149: 124-138, 2015.

24. Lee YS, Choi KM, Kim W, Jeon YS, Lee YM, Hong JT, Yun YP and Yoo HS: Hinokitiol induces cell growth through induction of S-phase arrest and apoptosis in human colon cancer cells and suppresses tumor growth in a mouse xenograft experiment. J Nat Prod 76: 2195-2207, 2013.

25. Shin EM, Kim S, Merfort I and Kim YS: Glycerol induces apoptosis in human Jurkat T cell lymphocytes via the Fas-Fasl caspase-8 pathway. Plantamedica 7: 242-247, 2011.

26. Lim S and Kaldis P: Cdks, cyclins and CKIs: Roles beyond cell cycle regulation. Development 140: 3079-3093, 2013.

27. Tamura D, Arao T, Tanaka K, Kanedo H, Matsumoto K, Kudo K, Aomatsu K, Fujita Y, Watanabe T, Saijo N, et al: Bortezomib potentially inhibits cellular growth of vascular endothelial cells through suppression of G2/M transition. Cancer Sci 101: 1403-1408, 2010.

28. Boutros R, Lobjois V and Ducumon B: CDC25 phosphatases in cancer cells: key players? Good targets? Nature reviews. Cancer 7: 495-507, 2007.

29. Thornberry NA and Lazebnik Y: Caspases: enemies within. Science 281: 1312-1316, 1998.

30. Brown JM and Attardi LD: The role of apoptosis in cancer development and treatment response. Nature reviews. Cancer 5: 231-237, 2005.

31. Fulda S and Debatin KM: Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 25: 4798-4811, 2006.

32. Li P, Nijhawan D, Budihardjo I, Srinivasa SU, SM, Ahmad M, Alnemri ES and Wang X: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91: 479-489, 1997.

33. Ghavami S, Hashemi M, Ande SR, Yeganeh B, Xiao W, Esraghi M, Bus CJ, Kadkhoda K, Wiechez E, Halayko AJ, et al: Apoptosis and cancer: mutations within caspase genes. J Med Gen 46: 497-510, 2009.

34. Sola S, Morgado AL and Rodrigues CM: Death receptors and mitochondria: Two prime triggers of neural apoptosis and differentiation. Biochem Biophys Acta 1830: 2160-2166, 2013.

35. Sasi N, Hwang M, Jaboin J, Csiki I and Lu B: Regulated cell death pathways: new twists in modulation of BCL2 family function. Mol Cancer Ther 8: 1421-1429, 2009.

36. Salvesen GS and Dixit VM: Caspase activation: The induced-proximity model. Proc Natl Acad Sci USA 96: 10964-10967, 1999.

37. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsokopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB and Korsmeyer SJ: Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. Science 292: 727-730, 2001.

38. Shi Y: Mechanisms of caspase activation and inhibition during apoptosis. Molecular Cell 9: 459-470, 2002.

39. Yuan TL and Cantley LC: PI3K pathway alterations in cancer: Variations on a theme. Oncogene 27: 5497-5510, 2008.

40. Manning BD and Cantley LC: AKT/PKB signaling: Navigating downstream. Cell 129: 1261-1274, 2007.

41. Mayer IA and Arteaga CL: The PI3K/AKT pathway as a target for cancer treatment. Ann Rev Med 67: 11-28, 2016.

42. Neri LM, Cani A, Martelli AM, Simionmi C, Junghanss C, Tabellini G, Ricci F, Tazzari PL, Pagliaro P, McCubrey AJ, et al: Targeting the PI3K/Akt/mTOR signaling pathway in B-precursor acute lymphoblastic leukemia and its therapeutic potential. Leukemia 28: 739-748, 2014.

43. Slomovitz BM and Coleman RL: The PI3K/AKT/mTOR pathway as a therapeutic target in endometrial cancer. Clin Cancer Res 18: 5856-5864, 2012.

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