SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 15: 2097-2104, 2018

Novel betulin derivative induces anti-proliferative activity by G_2/M phase cell cycle arrest and apoptosis in Huh7 cells

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Received January 26, 2016; Accepted July 11, 2017

DOI: 10.3892/ol.2017.7575

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 G_2 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-G_1 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.

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Key words: 3,28-di-(2-nitroxy-acetyl)-oxy-betulin, Huh7 cells, cell death, G_2/M phase, apoptosis

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_2/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'-tetrachloro-1,1',3,3'-tetramethyl benzimidazolyl-carbocyanine 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-bromo-acetyl)-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. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.83, 0.85, 0.86, 0.87, 0.89, 1.04 (s, 18H, 6xCH_3), 2.25 (m, 1H, H-19), 4.61 (d, 1H, *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, 2xCH_2ONO_2). ^{13}C NMR (100 MHz, $CDCl_3$) 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 $C_{34}H_{52}N_2O_{10}$, 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% CO_2 with RPMI-1640 medium, supplemented with 10% FBS.

Cell viability assay. Huh7 cells (5x10^3 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 (IC_{50}) value, using the DTX 880 Multimode Detector (Beckman Coulter, Inc., Brea, CA, USA).

Clonogenic assay. Cells (5x10^2 cells/well) in the exponentialgrowth 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 afluorescent probe, PI (Sigma-Aldrich, Merck KGaA), according to the manufacturer's protocol. Huh7 cells (3x10^4 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 wasused to evaluate the apoptosis rate (Nanjing Institute of Biological Engineering, Nanjing, China). Huh7 cells (3x10^4 cells/well) were inoculated 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 (3x10^4 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

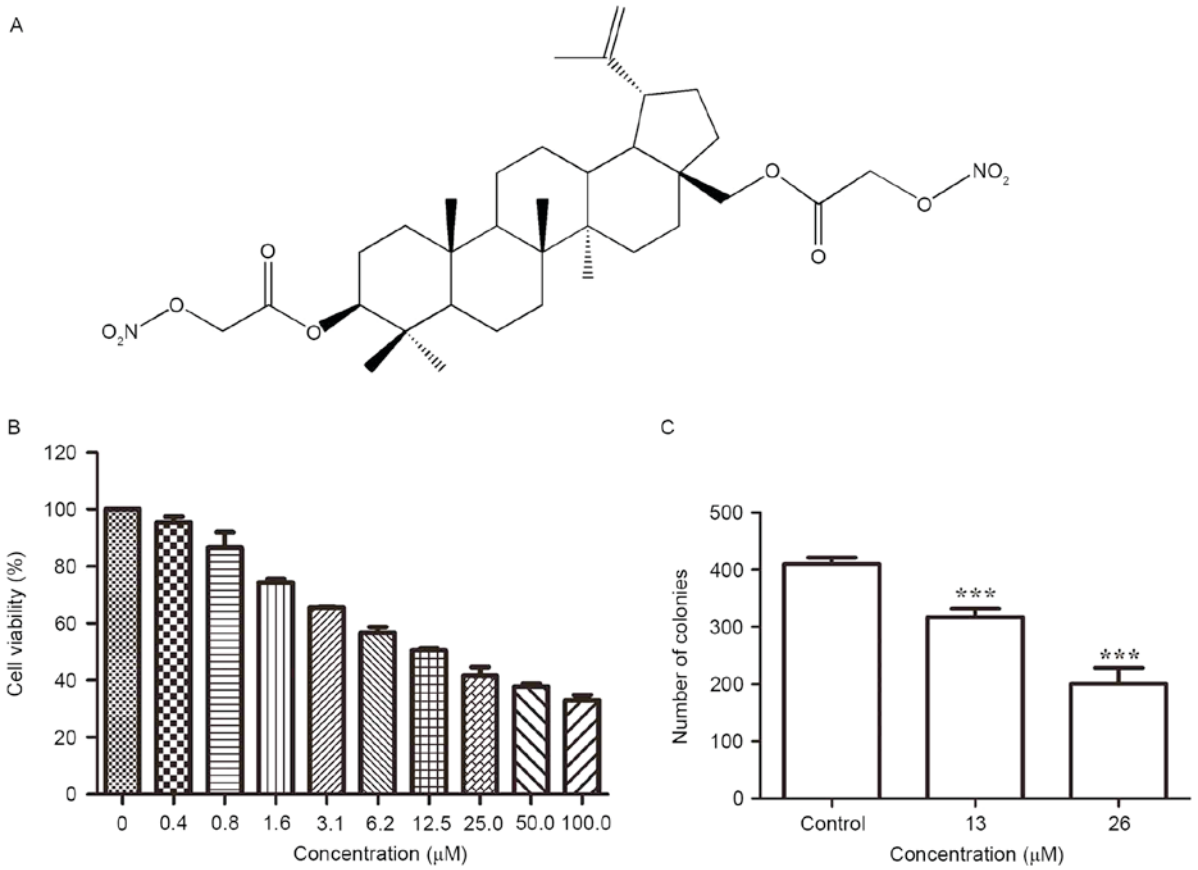

Figure 1. Inhibitory effect of 3,28-di-(2-nitroxy-acetyl)-oxy-betulin on the viability of Huh7 cells. (A) Chemical structure of 3,28-di-(2-nitroxy-acetyl)-oxy-betulin. (B) Huh7 cells seeded in 96-well plates were exposed to 3,28-di-(2-nitroxy-acetyl)-oxy-betulin (0, 0.4, 0.8, 1.6, 3.1, 6.2, 12.5, 25.0, 50.0 and 100.0 μM) for 48 h. Cell viability was determined using the MTT assay and demonstrated to decrease in a concentration-dependent manner. (C) The clonogenicity of Huh7 cells following treatment with 3,28-di-(2-nitroxy-acetyl)-oxy-betulin was determine using a colony-formation assay. The number of colonies decreased in a concentration-dependent manner. The data are expressed as the mean ± standard deviation of three independent experiments. ***P<0.001 vs. controlusing one-way analysis of variance followed by Tukey's test.

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 (6x10^5 cells/100 mm dish) in the exponential growth phase were incubated overnight with various concentrations of 3,28-di-(2-nitroxy-acetyl)-oxy-BT. 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 mMKCl] 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. 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.

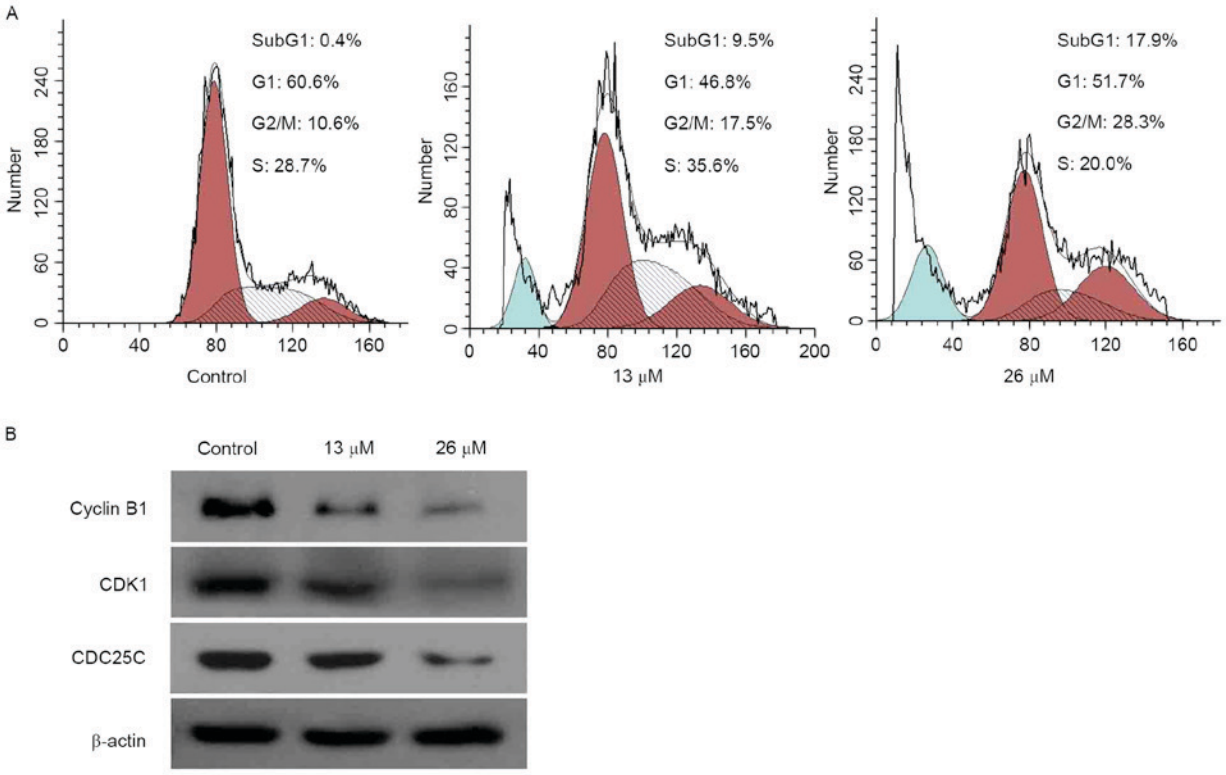

Figure 2. G_2/M cell cycle arrest is induced by 3,28-di-(2-nitroxy-acetyl)-oxy-betulin in Huh7 cells. (A) Cell cycle profiles, determined using flow cytometry, following treatment of the cells with 0, 13 and 26 μM 3,28-di-(2-nitroxy-acetyl)-oxy-betulin. The proportion of the cell population accumulated in G_2/M and the proportion of sub-G_1 cells following treatmentincreased, indicating apoptosis. (B) Huh7 cells were treated with 0, 13 and 26 μM 3,28-di-(2-nitroxy-acetyl)-oxy-betulin. Western blot analysis was performed to determine cyclin B1, CDK1 and CDC25C. β-actin was used as a loading control. Treatment inhibited cyclin B1 expression, and decreased the expression levels of CDK1 and CDC25C. Represented images are presented from two independent experiments. CDK1, cyclin-dependent kinase; CDC25C, cell division cycle 25C.

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-BTon Huh7 cells was as a result of cell cycle arrest, induced by 3,28-di-(2-nitroxy-acetyl)-oxy-BT, the cell cycle phase ratio was determined using flow cytometry and PI staining. The results of the present study demonstrated that, following exposure to 26 μM 3,28-di-(2-nitroxy-acetyl)-oxy-BT exposure (Fig. 2A), compared with the control, the proportion of cell population accumulation in G_2/M phase increased (10.6 vs. 28.3%, respectively). Furthermore, compared with the control, the proportion of sub-G_1 cells, following treatment with 26 μM 3,28-di-(2-nitroxy-acetyl)-oxy-BT, increased (0.4 vs. 17.95%, respectively), indicating apoptosis. To investigate the underlying molecular mechanism by which 3,28-di-(2-nitroxy-acetyl)-oxy-BT induced G_2/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 of cyclin-dependent kinase (CDK)1, compared with the control. In addition, the expression level of cell division cycle 25C (CDC25C), which acts 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

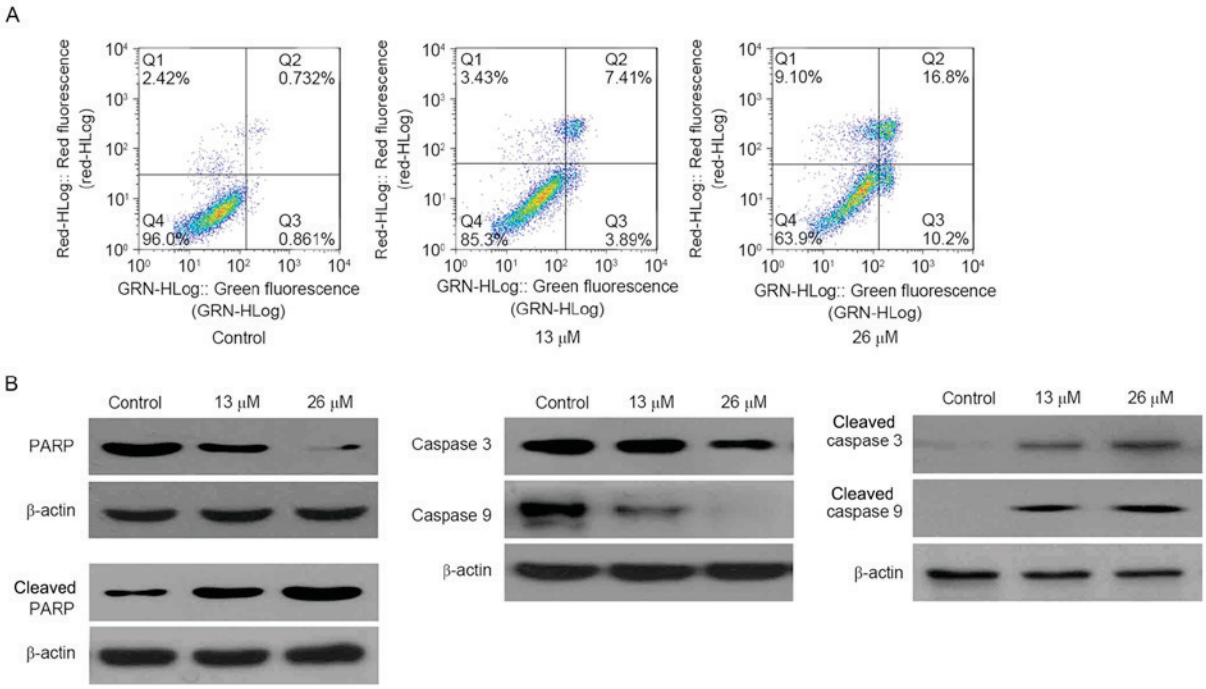

Figure 3. Caspase-dependent apoptosis is induced by 3,28-di-(2-nitroxy-acetyl)-oxy-betulin in Huh7 cells. (A) Flow cytometric analysis of 3,28-di-(2-nitroxy-acetyl)-oxy-betulin in Huh7 cells using Annexin V-fluorescein isothiocyanate/propidium iodide staining. Cells in the lower right quadrant represent early apoptotic cells and those in the upper right quadrant represent late apoptotic cells. The proportion of Huh7 cells in early and late apoptosis increased in a concentration-dependent manner. (B) Huh7 cells were treated with 0, 13 and 26 μM 3,28-di-(2-nitroxy-acetyl)-oxy-betulin. Western blot analysis was performed to determine the expression level of PARP, caspase 3 and caspase 9. β-actin was used as a loading control. The expression levels of PARP, caspase 3 and caspase 9 markedly increased following treatment. Represented images are presented from two independent experiments. PARP, poly(ADP-ribose) polymerase.

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 G_2/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 G_2/M transition by forming the CDK1-cyclin B1 complex (28). In the present

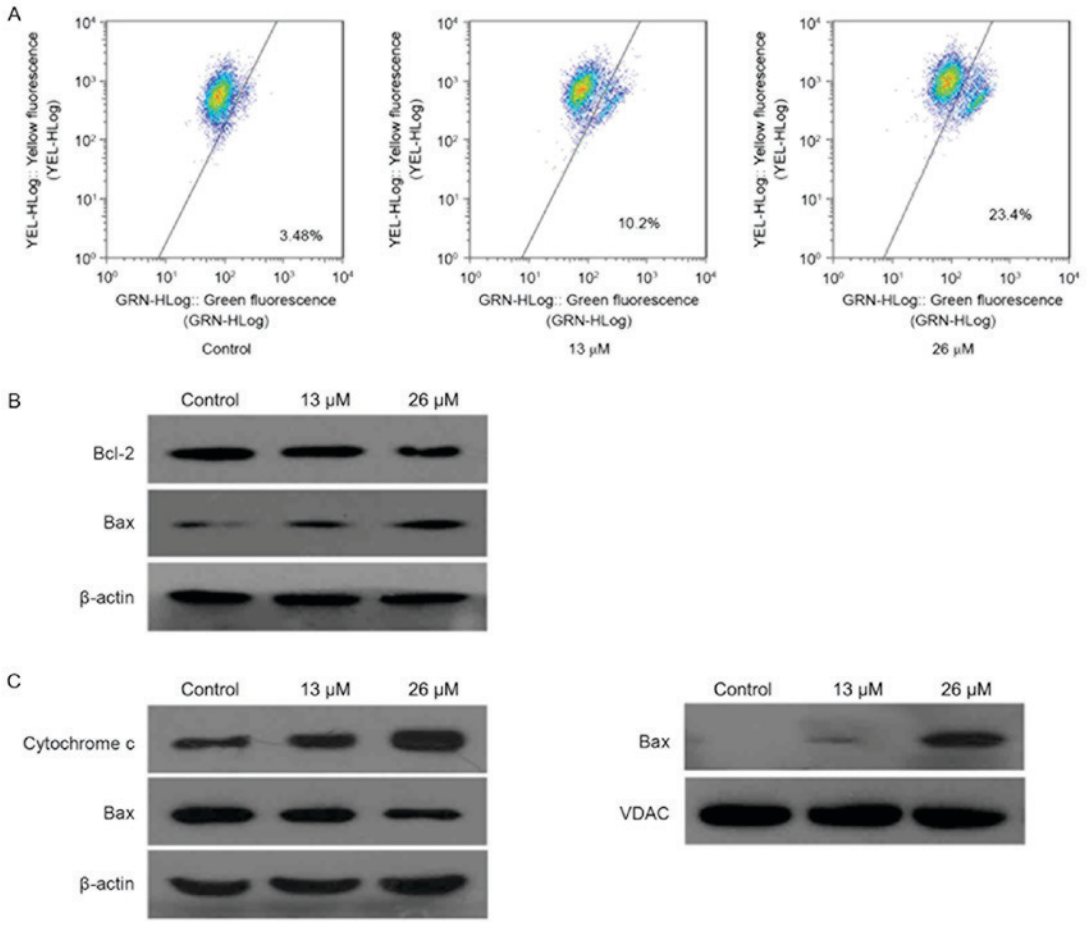

Figure 4. Apoptosis is induced by 3,28-di-(2-nitroxy-acetyl)-oxy-betulin via the mitochondrial pathways in Huh7 cells. (A) Decrease in mitochondrial potential induced by 3,28-di-(2-nitroxy-acetyl)-oxy-betulin. Following treatment with 3,28-di-(2-nitroxy-acetyl)-oxy-betulin, the cells were stained with JC-1 and analyzed using flow cytometry. Changes in mitochondrial potential were determined by the ratio of red fluorescence with green fluorescence. (B) The total expression of Bcl-2 and Bax in Huh7 cells treated with or without 3,28-di-(2-nitroxy-acetyl)-oxy-betulin were analyzed using western blotting. Bcl-2 and Bax were identified to be decreased and increased, respectively, following treatment. β-actin was used as a loading control. (C) The cytosolic and mitochondrial levels of the pro-apoptotic proteins, cytochrome *c* and Bax, in Huh7 cells with or without 3,28-di-(2-nitroxy-acetyl)-oxy-betulin, determined using western blot analysis. β-actin was used as the loading control. Following treatment, the cytosolic cytochrome *c* level was markedly increased and the translocation of Bax from the cytosol to the mitochondria was increased. Represented images are presented from two independent experiments. JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetramethyl benzimidazolyl-carbocyanine iodide; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; VDAC, voltage-dependent anion channel.

study, the downregulation of CDK1 and cyclin B1 suggested that 3,28-di-(2-nitroxy-acetyl)-oxy-BT induced G_2/M arrest viathe modulation of CDK1 and cyclin B1. CDC25C is required for the full activation of CDK1-cyclin B1 during the G_2/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 G_2/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-G_1 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

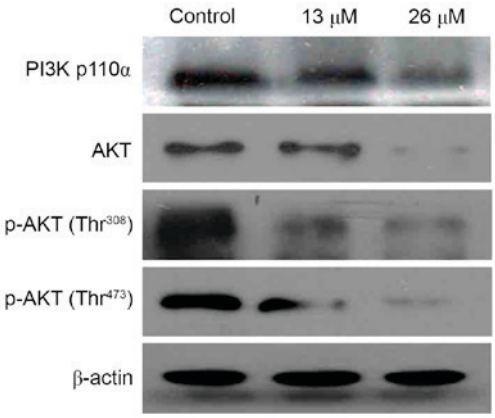

Figure 5. Apoptosis is induced by 3,28-di-(2-nitroxy-acetyl)-oxy-betulin via Akt inactivation in Huh7 cells. Total cell lysates were prepared and subjected to SDS-PAGE followed by western blot analysis to determine the protein levels of PI3K p110α, Akt and p-Akt. β-actin was used as the protein loading control. Following treatment, the levels of PI3K p110α, Akt and p-Akt were decreased in a concentration-dependent manner. Represented images are presented from two independent experiments. Akt, protein kinase B; p-, phosphorylated; PI3K,phosphoinositide 3-kinase; p100α, catalytic subunit of PI3K.

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 (p110α) 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 (p110α) 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 (p110α) 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.

Acknowledgements

The present study was supported by the Guangdong Provincial Science and Technology Plan (grant no. 2012A030100015) and Guangzhou University of Chinese Medicine (grant no. XH20150103).

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