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ORIGINAL RESEARCH

Oleanolic acid derivatives induce apoptosis in human leukemia K562 cell involved in inhibition of both Akt1 translocation and pAkt1 expression

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Abstract Oleanolic acid (OA) derivatives exhibit numerous pleiotropic effects in many cancers. The present study aimed to investigate the molecular mechanisms of 5′-amino-oleana-2,12-dieno[3,2-*d*]-pyrimidin-28-oic acid (compound 4) and oleana-2,12-dieno[2,3-*d*]isoxazol-28-oic acid (compound 5) inducing apoptosis in human leukemia K562 cell. We investigated the effects of the compounds on K562 cell growth, apoptosis and cell cycle. The compounds showed strong inhibitory effects on K562 cell viability in a dose-dependent manner determined by the 3-(4,5-dimethylthiazoyl)-2,5-diphenyltetrazolium bromide assay and significantly increased chromatin condensation and apoptotic bodies in K562 cells. Flow cytometry assay suggested that the compounds induced inhibition of K562 cell proliferation associated with G1 phase arrest. In addition, the compounds inhibited Akt1 recruiting to membrane in CHO cells which express Akt1-EGFP constitutively and down-regulated the expression of pAkt1 in K562 cell. These results suggested that the compounds can efficiently inhibit proliferation and induce apoptosis perhaps involved in inactivation of Akt1. The OA derivatives may be potential chemotherapeutic agents for the treatment of human cancer.

Keywords Oleanolic acid derivatives · Leukemia · Chronic myeloid · Apoptotic body · G1 phase · Akt1-EGFP

Abbreviations

OA	Oleanolic acid
DMSO	Dimethyl sulphoxide
FBS	Fetal bovine serum
PI	Propidium Iodide
MTT	3-(4,5-dimethylthiazoyl)-2,5-diphenyltetrazolium bromide
PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene fluoride

Introduction

Interest in the use of Chinese traditional medicines for cancer prevention and treatment is increasing (Yang et al. 2006). Pentacyclic triterpenes have contributed to the development of modern therapeutic drugs

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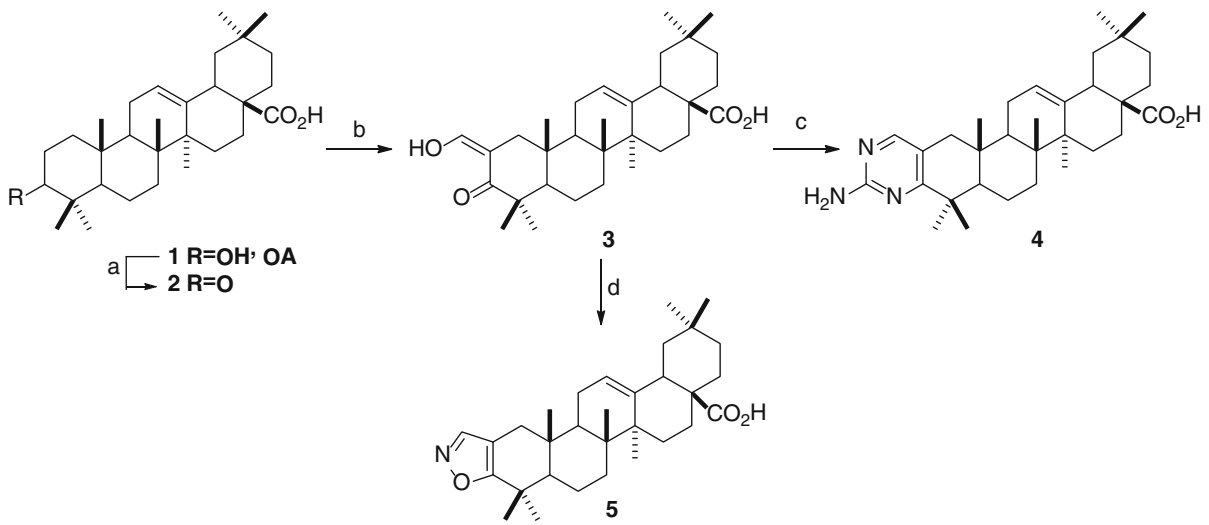
(Chadalapaka et al. 2008; Shyu et al. 2010). Oleanolic acid (OA) is a naturally occurring triterpenoid that has been used as antibacterial, antifungal, anticancer, and anti-inflammatory agent (Huang et al. 1994; Liu et al. 2012; Shyu et al. 2010). It is reported that OA can induce apoptosis in human leukemia cells through activation of caspases and cleavage of PARP (Zhang et al. 2007). Meanwhile, OA can induce apoptosis in HuH7 cells through a mitochondria-mediated pathway and downregulation of XIAP (Shyu et al. 2010). Recently, various OA derivatives have been synthesized focusing on the A-ring and C-28 to enhance the effect of anti-proliferative effects and reduce toxicity to normal cells (Chen et al. 2007; Chintharlapalli et al. 2007; Tanaka et al. 2001). Methyl-2-cyano-3, 12-dioxooleana-1,9 (11)-dien-28-oate (CDDO-Me), an OA derivative has strong growth inhibitory and apoptosis-inducing activity in ovarian cancer cells (Gao et al. 2011). Liang's (Liang et al. 2013) group found three oleanane-type triterpenes showing the potential in inhibiting the growth of HepG2 cells. All results indicate that OA and OA derivatives play a significant role in anti-tumor activity. In our previous work, OA derivatives with different azaheterocyclic groups at the 2,3-position of the A-ring were synthesized and had stronger inhibitory effects against human hepatocellular carcinoma BEL-7404 cells and human leukemia K562 cells compared to the parent OA (Gao et al. 2012a, b; Kang et al. 2012). Gao's group (Gao et al. 2011) found CDDO-Me, showing strong growth inhibitory and apoptosis-inducing activity for ovarian cancer cells through the inhibition of Akt/NF-κB/mTOR signaling pathway. The present study was undertaken to further investigate the mechanism of OA derivatives induced apoptosis of K562 cell and the relationship between Akt signal pathway and apoptosis. The CHO cells used in this research are a kind of AKT1-EGFP overexpressing cells model supplied by our collaborator. We used this cell model to investigate the translocations of AKT1 to the membrane.

Materials and methods

Chemicals and antibodies

Oleanolic acid (**1**) was oxidized with PCC to produce 3-oxo-olean-12-en-28-oic acid (**2**), which reacted with ethyl formate in presence of TEA to produce 2-(hydroxymethylene)-3-oxo-olean-12-en-28-oic acid (**3**). Compound 3 reacted with guanidine hydrochloride in the presence of NaOEt and produced 5′-amino-oleana-2,12-dieno[3,2-*d*]pyrimidin-28-oicacid (**4**). Treatment of (**3**) with hydroxylamine hydrochloride in aqueous EtOH at reflux temperature produced oleana-2,12-dieno[2,3-*d*]isoxazol-28-oic acid (**5**). The synthesis procedure is described in Scheme 1. Primary antibodies against pAkt1, GAPDH were purchased from cell signaling technology and anti-rabbit secondary antibody was purchased from LI-COR



Scheme.1 Reagents and conditions: a) Jones' reagent, acetone, rt.; b) HCO_2Et, NaOMe, toluene, rt.; c) guanidinium chloride, NaH, anhydrous ethanol, reflux; (d) $NH_2OH{\cdot}HCl$, ethanol, reflux

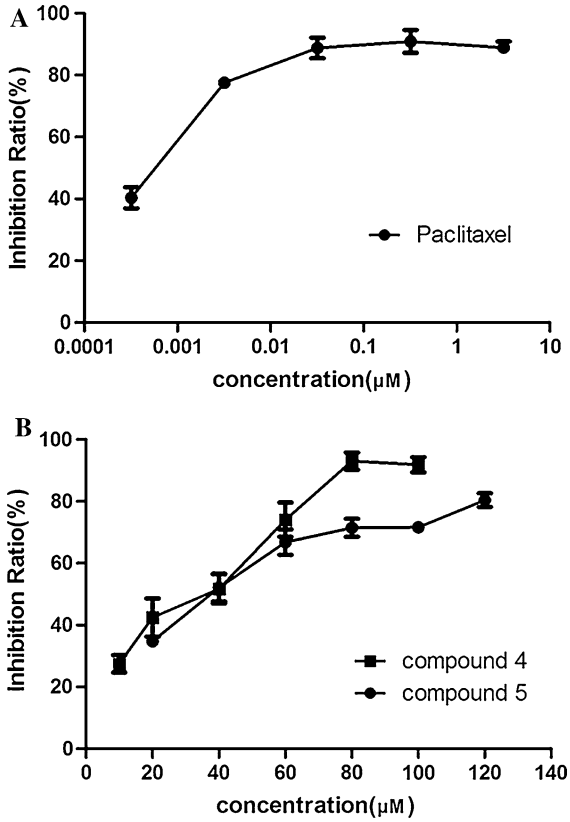


Fig. 1 Effects of compound 4 and compound 5 on the growth of K562 cells in a dose-dependent manner. **a** Treatment with paclitaxel at various doses for 72 h; **b** treatment with compound 4 and compound 5 at various doses for 72 h. The data shown are representative of three independent experiments with similar results and each point represents the mean ± SD (n = 3)

Biosciences. Paclitaxel was purchased from East China Pharmaceutical Group Limited Co, Ltd. (China), and the concentration were 3.2×10^{-4} μM, 3.2×10^{-3} μM, 3.2×10^{-2} μM, 3.2×10^{-1} μM, 3.2 μM and 32.0 μM. The CHO cells were provided by State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China. All other chemicals were of analytical grade.

Cell culture and cell viability assays

K562 cells were grown in RPMI-1640 medium (Thermo, Logan, UT, USA) supplemented with 10 % (v/v) FBS (GIBCO-Invitrogen, Carlsbad, CA, USA), 2 mM-glutamine, and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) (Beyotime Institute of Biotechnology, Jinagsu, China), at density of 3,000 cells/well in 96-well plates at the condition of 37 °C, 95 % air, 5 % carbon dioxide atmosphere. Cells were treated with various concentrations of compound 4 and compound 5. The DMSO concentrations in the cell culture medium did not exceed 0.2 % (v/v), and the controls were treated with the same amount of DMSO as that used in the corresponding experiments. The parallel cell cultures were incubated 72 h. At the end of the treatment, 3-(4,5-dimethylthiazoyl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well, and the cells were incubated for another 4 h. The precipitated MTT-formazan was dissolved in 150 μL DMSO and then the amount of formazan was measured at 490 nm using microplate reader. Inhibition rate (%) = $[(OD_{Control} - OD_{Treated})/OD_{Control}] \times 100$ %. Cell viability was expressed as a percentage relative to the control culture. CHO cells overexpressing AKT1, were provided by the State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China). They were grown as adherently as monolayer in culture, using the complete medium including Ham's F12, FBS (10 %), pen/strep (1 %) and G418 (0.5 mg/mL).

Nuclear staining with Hoechst 33342

K562 cells (1×10^6 cells/dish) were plated in 6 cm dish and then treated with or without compound 4 and compound 5. After 24 h incubation, cells were stained with 0.5–10 μg/mL Hoechst 33342 (Beyotime Institute of Biotechnology, China) for 30 min at 37 °C. The cells were washed with PBS, and their nuclear morphology was observed by fluorescent microscopy (Nikon, Tokyo, Japan).

Flow cytometry assay of apoptotic cell

Cell cycle assay was performed using a cell cycle phase determination kit. K562 cells were treated with compound 4 and compound 5 (40 μM) for 24 h. Then cells were centrifuged and washed with PBS, and fixed with 70 % ethanol at −20 °C overnight. Then cells were washed twice with PBS and incubated with 0.25 mg/mL RNase A at 37 °C for 30 min. Then the cells were incubated with PI (Beyotime Institute of Biotechnology, China) (50 μg/mL) for 30 min in the dark. Cells were filtered and analyzed in the FL2 channel of flow cytometer (Becton Dickinson, San Jose, CA, USA).

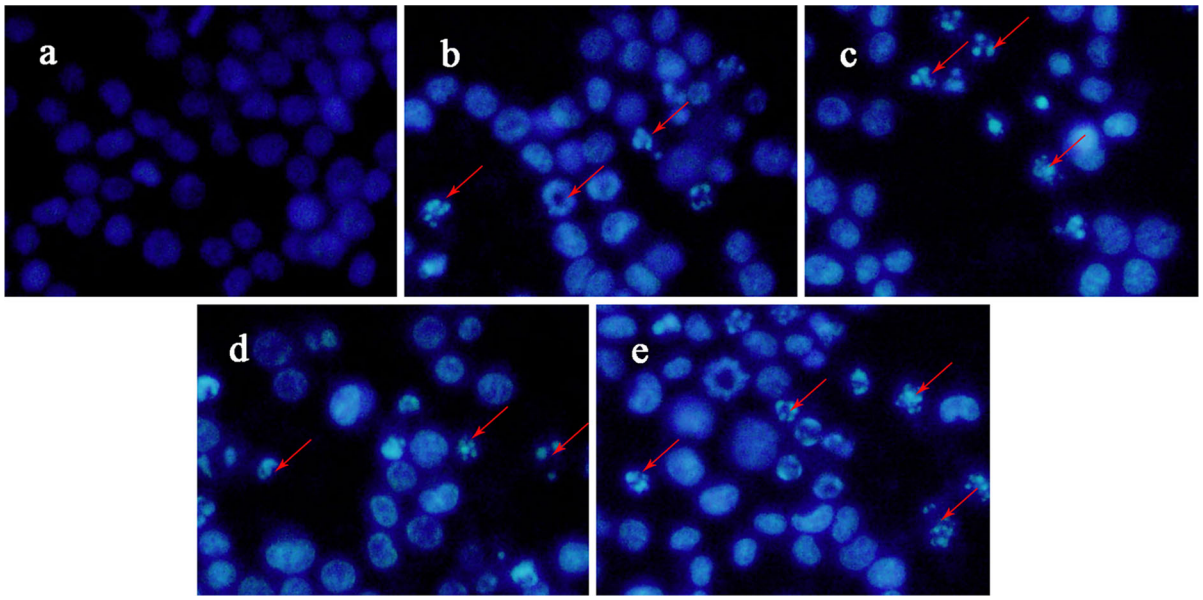


Fig. 2 Morphological changes of K562 cells following treatment with compound 4 and compound 5 for 24 h. Observation of K562 cells after staining by Hoechst 33342 under an inverted fluorescent microscope (×200). Apoptotic bodies are indicated by *red arrows*. **a** Control cells; **b** cells treated with compound 4 (20 μM); **c** cells treated with compound 4 (40 μM); **d** cells treated with compound 5 (20 μM); **e** cells treated with compound 5 (40 μM). Both compound 4 and compound 5 induced apoptosis in K562 cell. (Color figure online)

Western blot analysis

Cells were collected after treatment with compound 4 and compound 5 at various concentrations and then lyzed in ice-cold lysis buffer. Protein content was determined by the Bradford test kit (Beyotime Institute of Biotechnology, China). The cell lysates (60 mg) were fractionated by SDS-PAGE on a 12 % polyacrylamide gel and transferred to a PVDF membrane. The membrane was blocked with a solution containing 5 % fat-free dry milk TBST buffer (50 mM Tris–HCl, 150 mM NaCl and 0.1 % Tween 20, pH 7.4) for 1 h and washed with TBST buffer. The membrane was incubated with anti-pAkt1 and GAPDH antibodies overnight at 4 °C. Then the membranes were incubated with secondary antibodies for 2 h at room temperature. Membranes scanned by infrared fluorescence scanning imaging (LI-COR, Lincoln, NE, USA).

In Cell Analyzer 1000 assay

To monitor the cytoplasm-to-membrane translocation, the images of Akt1-EGFP redistribution assays were analyzed using the IN Cell Analyzer 1000 Membrane Spot analysis module. The membrane Spot analysis module algorithm can identify spots at the edge of the cell and the EGFP fluorescence intensity accumulation at the perimeter of the cells could be identified by using the analysis parameters (Lundholt et al. 2005). CHO cells which expressed Akt1-EGFP constitutively were plated in 96-well plates. After 24 h incubation, cells were incubated with the compounds for 1 h. Thereafter, 16.7 nM insulin was added to the cells for 10 min before analyzing by the In Cell Analyzer 1000 (GE Healthcare, Piscataway, NJ, USA). The average average area of identified spots per cell was calculated for each well and taken as a measurement of the cytoplasm-to-membrane translocation of EGFP. The analysis parameters were optimized for each of the assays.

Statistical analysis

Data were analyzed and presented as mean ± SD for three different grounds. Statistical significance testing was performed using the SPSS software 17.0. A p value <0.05 is considered as statistically significant.

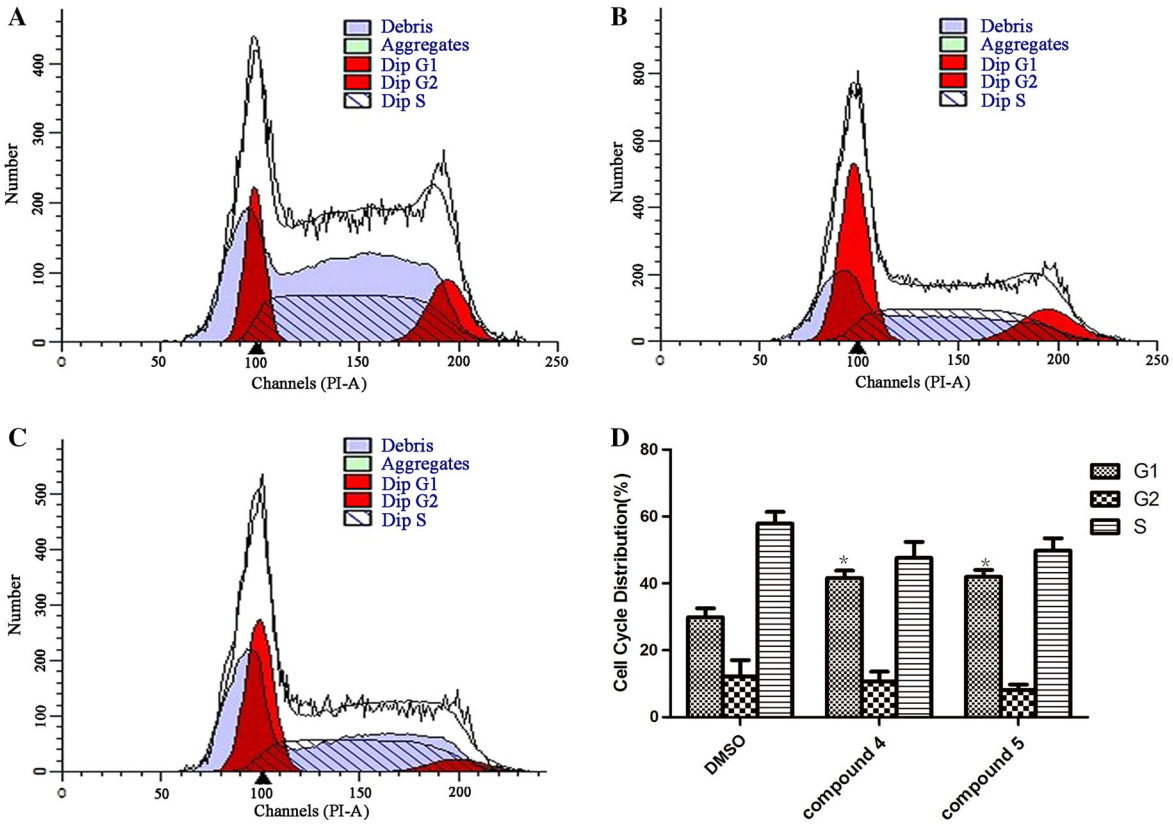


Fig. 3 K562 cells were treated with compound 4 and compound 5 for 24 h. The relative number of cells within each cell cycle was determined by flow cytometry. **a** Control cells; **b** cells treated with compound 4 (40 μM); **c** cells treated with compound 5 (40 μM). Each point represents the mean ± SD (n = 3). *$p < 0.05$, compared with untreated groups; **d** The cell cycle distribution (%) of K562 cell after treating with DMSO, compound 4 and 5 analyzed by using Flow cytometry

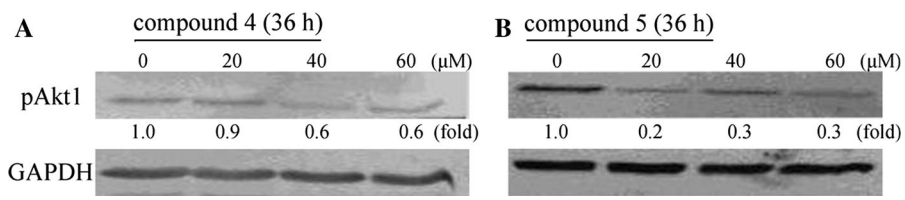


Fig. 4 Effects of compound 4 and compound 5 on expression of pAkt1 protein. **a** K562 cells treated with compound 4 at concentrations of 0, 20, 40, 60 μM, respectively, for 36 h; **b** K562 cells treated with compound 5 at concentrations of 0, 20, 40, 60 μM, respectively

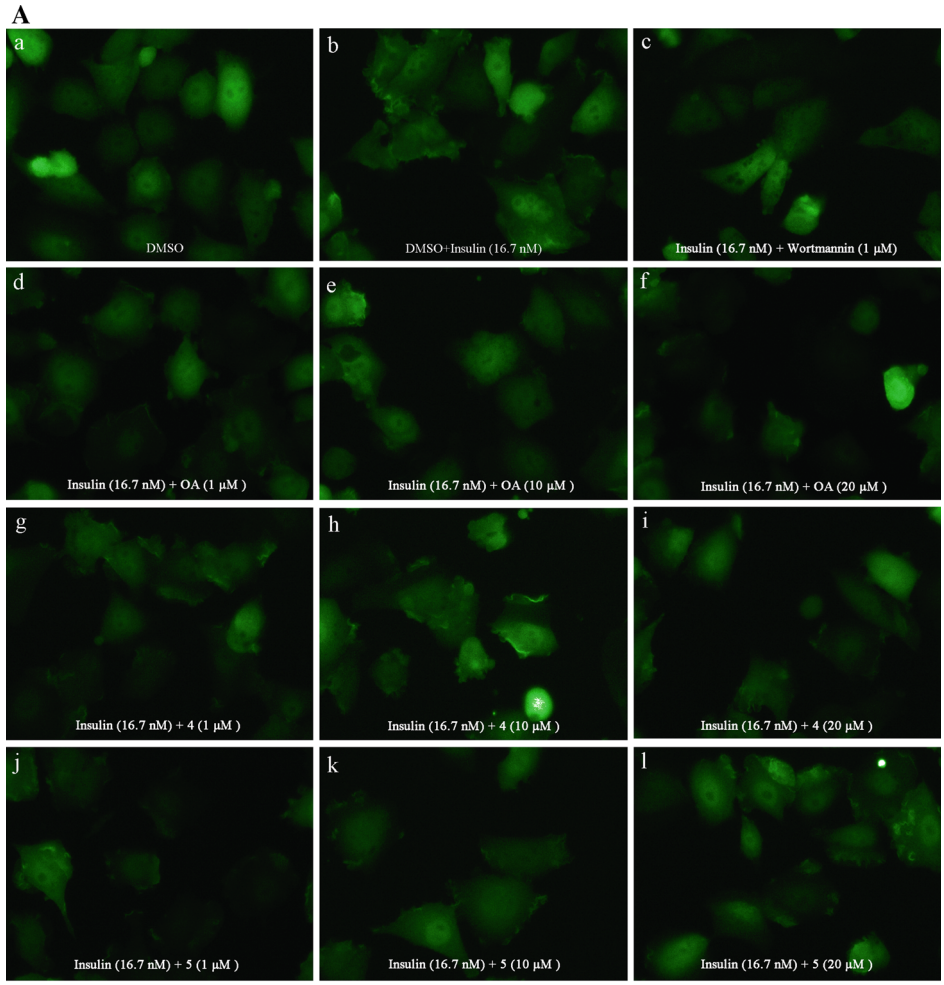
Results

Effects of compound 4 and compound 5 on cell proliferation in K562 cell line

To investigate the effects of compound 4 and compound 5 on cell proliferation, K562 cells were treated with various doses of compound 4, compound 5 and paclitaxel (positive control), and then were examined by MTT assay (Fig. 1). Compound 4 and compound 5 possessed strong inhibitory effects and inhibited K562 cell proliferation in a dose-dependent manner. After K562 cells were exposed to compound 4 and compound 5 for 72 h, the IC_{50} were valued 32.686 and 39.252 μM, respectively.

Effects of compound 4 and compound 5 on apoptosis induction

Apoptosis involves typical morphological events including chromatin condensation, DNA fragmentation, cell


A
a
DMSO
b
DMSO+Insulin (16.7 nM)
c
Insulin (16.7 nM) + Wortmannin (1 μM)
d
Insulin (16.7 nM) + OA (1 μM)
e
Insulin (16.7 nM) + OA (10 μM)
f
Insulin (16.7 nM) + OA (20 μM)
g
Insulin (16.7 nM) + 4 (1 μM)
h
Insulin (16.7 nM) + 4 (10 μM)
i
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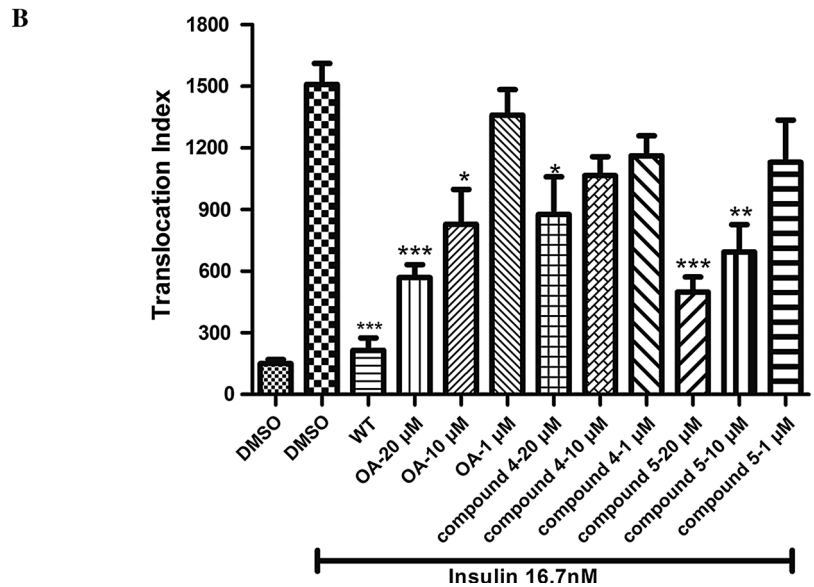
B
Translocation Index
1800
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DMSO
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OA-1 μM
compound 4-20 μM
compound 4-10 μM
compound 4-1 μM
compound 5-20 μM
compound 5-10 μM
compound 5-1 μM
Insulin 16.7nM

◀ **Fig. 5** **a** Effects of insulin, Wortmannin (WT), OA, compound 4 and compound 5 on Akt1-GFP translocation. **b** Quantification of the effects of the compounds on the Akt1 translocation using the In Cell Analyzer 1000. *First bar*: cells treated with DMSO; *Second bar*: cells treated with DMSO plus Insulin (16.7 nM); *Third bar*: cells treated with WT (1 μM), *Fourth to twelfth bars*: cells treated with OA, compound 4, compound 5 at various doses for 1 h before stimulation with insulin for 10 min, followed by fixing and scoring for Akt1 membrane translocation by fluorescence microscopy: the results implied that OA, compound 4 and compound 5 can inhibit translocation of Akt1 in a dose-dependent manner. Each point represents the mean ± SD (n = 3). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, compared with untreated group

shrinkage, the plasma membrane blebbing, cytosol vacuoles, and apoptotic bodies. To determine whether apoptosis contributing to compounds-induced reduction in the viability of K562 cells, after the cells treated with compound 4 and compound 5 for 24 h, the nuclear morphology of the cells was observed using Hoechst 33342 staining. As shown in Fig. 2, the control cells exhibited normal nuclear morphology, whereas the cells treated with the compounds displayed chromatin condensation. We also analyzed the hypodiploid DNA content (sub-G1 phase) using flow cytometry after the cellular DNA had been stained with PI. Compound 4 and compound 5 increased the percentage of cells in the sub-G1 phase, which raised from 29.89 % (control) to 41.65 % and 42.15 % respectively. The compounds induced G1 phase arrest (Fig. 3). Overall, these results clearly indicate that compound 4 and compound 5 exerted its anti-proliferative effect via the induction of apoptotic cell death.

Effects of compound 4 and compound 5 on expression of pAkt1

Akt1 is activated by phosphorylation at Thr308 and Ser473. Activated Akt1 promotes cell growth, survival pathways and inactivates various components of the apoptotic cascade (Zhou et al. 2012). As shown in Fig. 4, the compounds can effectively suppress expression of pAkt1in K562 cells.

Effects of compound 4 and compound 5 on the cytoplasm-to-membrane translocation of Akt1

Before Akt1 phosphorylated by PDK1/PDK2, it needs to migrate from the cytoplasm to the membrane surface and activated Akt1 produced the relevant effects. We used In Cell Analyzer 1000 to analyze the distribution of Akt1 within the CHO cells which expressed Akt1-EGFP steadily. Insulin is a positive Akt agonist, can significantly stimulate phosphorylation of Akt; Wortmannin (WT) is positive Akt inhibitors and can inhibit phosphorylation of Akt. As shown in Fig. 5, OA, compound 4 and compound 5 do indeed reverse the insulin-stimulated translocation of Akt1 to the plasma membrane.

Discussion

Recently, various triterpenes have been described to induce apoptosis in different type cells (Cheng et al. 2013; Gao et al. 2011; Han et al. 2009). Among these compounds, pentacyclic triterpenes play a part in inhibiting tumor cell proliferation and tumor growth, and inducing differentiation and apoptosis (Deeb et al. 2012; Yan et al. 2013). The signaling pathway involving phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt/PKB) plays a major role in cancer cell proliferation (Campbell et al. 2001; Marone et al. 2008). The aim of this work was to determine the effects of compound 4 and compound 5 on cell apoptosis, and the relation of Akt/PKB signal pathway associated with apoptosis on human leukemia K562 cell.

Our study demonstrated that compound 4 and compound 5 inhibited cell proliferation and induced apoptosis in a human leukemia K562 cell. In Fig. 1, compound 4 and compound 5 inhibited the growth of the K562 cells in a dose-dependent manner and compound 4 with a [2,3-*d*]pyrimid in ring A possessed a stronger effect than compound 5 with a [2,3-*d*]isoxazolon inhibiting cell proliferation and induced apoptosis in a human leukemia K562 cell. In Fig. 2, cell morphology changed after K562 cells treated with compound 4 and compound 5 at 20 μM and 40 μM for 24 h. The typical morphological characteristic of apoptosis such as karyopyknosis, conglomeration and apoptosis body were observed. Thus, based on Figs. 1 and 2, this implied that compound 4 and compound 5 possessed antitumor activity through inducing cell apoptosis.

Inhibition of cancer cell proliferation was usually accompanied by cell cycle arrest (Arul and Subramanian 2013; Chuang et al. 2005; Zeng et al. 2013). Cell

cycle arrest was determined by flow cytometry. The results showed that compound 4 and compound 5 arrested cell cycle of K562 cells at G1 phase resulting in the cell cycle prevented from entering S phase.

It has been documented that the progress of apoptosis was regulated by a variety of regulatory genes in the cell (Alvarez et al. 2006). Many studies have found that pentacyclic triterpene compounds induced apoptosis through regulating apoptosis-related protein (Liu et al. 2007; Zhang et al. 2007; Zheng et al. 2006). Akt/PKB, a serine threonine kinase which is a critical signaling molecule promoting cell growth and survival pathways, is frequently dysregulated in many cancers and diabetes (Carnero et al. 2008; Gershtein et al. 2007; Mahajan and Mahajan 2012; Renner et al. 2008). To date, three members of the Akt family denominated Akt1, Akt2, and Akt3 have been identified. Three events appear to be required for full activation of its enzymatic activity. Firstly, Akt rapidly translocates to membrane in response to specific stimuli, subsequently is phosphorylated at Thr308 and Ser473 and thus transforms into a potent kinase (Dong and Liu 2005; Sarbassov et al. 2005). Fully activated Akt then translocates to the cytosol to phosphorylate BAD, MDM2, GSK3β or to the nucleus to phosphorylate pro-apoptotic FOXOs protein. These proteins regulate cell cycle progression, survival, apoptosis and translation (Ghayad and Cohen 2010; Huang and Tindall 2007; Rao et al. 2011). Antrocin from Antrodia camphorata can inhibit Akt/mTOR signaling pathway and cause cleavage of caspase-3 and poly (ADP-ribose) polymerase in MDA-MB-231 cells. It also decreased the expression of Bcl-2, Bcl-xL and survivin, and increased the expression Bax and released cytosolic cytochrome C to induce the apoptosis (Rao et al. 2011). In Fig. 5a, fluorescence microscopy was used to confirm that these compounds do indeed inhibit Akt1 translocation. In order to quantify the effects of compounds 4 and 5 on Akt1 translocation, we use high-throughput imaging assay of In Cell Analyzer 1000 to validate assay, as shown in Fig. 5b. In our research, compound 4 and compound 5 inhibited insulin-stimulated Akt1 translocation to the membrane in CHO cells significantly and the expression of pAkt1 in K562 cell lines. It reveals that these two compounds may down-regulated the expression of pAkt1 protein through inhibiting Akt1 translocation to the membrane. It is possible that down - regulation of pAkt1 protein may influence the proteins associated with apoptosis such as Bcl-2, Bcl-xL. Our previous work has illustrated that compound 4 and compound 5 could inhibit proliferation and induce apoptosis via the mitochondrial pathway in human hepatocellular carcinoma BEL-7404 cells. In our present work, we found that compound 4 and compound 5 inducing apoptosis in K562 cells also were associated with the inhibition of the expression of pAkt1 in PI3K/Akt pathway.

In summary, the results of the present study revealed that compound 4 and compound 5 might be a promising lead compound suitable for developing new drug as leukemia therapy.

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