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Apoptosis induced by weisiensin B isolated from *Rabdosia weisiensis* C.Y. Wu in K562

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The *ent*-kaurane diterpenoid weisinensis B shows significant cytotoxicity to human chronic myeloid leukemia K562 cells. It inhibits cell growth at low concentration and kills cells at high concentration. The compound induced cell apoptosis and necrosis mainly associated with G2/M phase cell cycle arrest and the ROS generation is the early event in weisiensin B induced cell apoptosis.

1. Introduction

Diterpenoids are a group of naturally occurring or synthetic compounds. Because of their unique structural scaffold and interesting biological properties such as antioxidative (Liu et al. 2006), immunosuppressive (Chen et al. 2006) and antimutagenic activities (Di Sotto et al. 2009), they have attracted considerable attention. Many diterpenoids exhibit excellent anti-tumor effects (Abelson 1990). Oridonin, a widely studied and active diterpenoid was reported to have remarkable anti-proliferative activities and selectively induces apoptosis of leukemia cells through increasing intracellular reactive oxygen species (ROS) (Zhen et al. 2012). Not only inducing tumor cell apoptosis, adenanthin, a diterpenoid was recently reported to induce differentiation of acute promyelocytic leukemia NB4 cells in long time and low concentration treatment (Liu et al. 2012). *ent*-Kaurane diterpenoids are main secondary metabolites of the genus *Isodon* and more than 400 of these chemicals have been isolated from plants. Weisinensis B is one of the *ent*-kauranoids separated from *Isodon weisiensis* C.Y. Wu (Ding et al. 2008). Chronic myelogenous leukemia is categorized in the myelodysplastic-myeloproliferative spectrum of hematopoietic stem cell neoplasms. Although the pathological mechanisms involved in leukemogenesis have been extensively investigated and some chemotherapeutic agents have been developed, still most cases of leukemias are incurable. In the particular case of leukemias multi-drug resistance has become a major barrier to effective treatment (Jendiroba et al. 2002). So to develop new anti-cancer drugs to overcome tumor resistance is one of the promising strategies. Our early experiments demonstrated that weisinensis B has significant cytotoxicity and induces cell apoptosis to human hepatoma Bel-7402 (Yang et al. 2005; Ding et al. 2008). To explore the effect and the mechanism to other tumor cells, in this paper, the cytotoxicity and cell death induction of weisinensis B to human chronic myeloid leukemia K562 cell were tested and the relationship with ROS generation is investigated.

2. Investigations, results and discussion

2.1. Cytotoxic effect of weisiensin B on K562 cells

The addition of the substance to cultured cells dramatically inhibited the proliferation of K562 cells in a dose- and time-dependent manner. The cytotoxic effect of weisiensin B on K562 cells evaluated using SRB assays is shown in Fig. 2A, The IC_{50} (concentration inhibiting 50% cell growth) were 5.59 and 3.83 μM for 24 and 48 h, respectively.

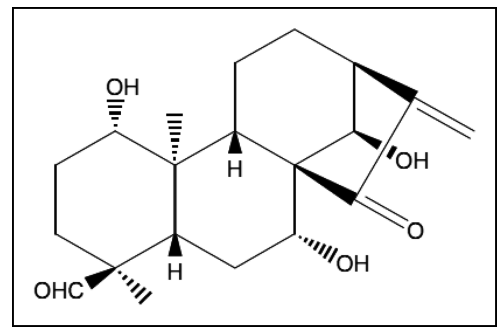

Fig. 1: Weisiensin B

The use of different methods confirms the cytotoxicity of the compound. Dose-response curves obtained using Trypan blue dye exclusion when the cells were treated over different time are shown in Fig. 2B. A decrease in cell viability was observed when the cultures were exposed to the substance from 24 h to 72 h at concentrations as high as 1.0 μM compared to control. But the cells were killed when the concentration arrived 2 μM. At higher concentrations (4 and 8 μM), the lethal effects were stronger. This indicates that weisiensin B inhibits cell growth at low concentration and kills cells at high concentration.

2.2. Weisiensin B induced morphological changes of K562cells

In order to further characterize the action on cells induced by weisiensin B, the morphological changes of cell nucleus were analyzed by Giemsa staining. As shown in Fig. 3, control K562 cells stained with Giemsa showed nuclei with dispersed chromatin and organized plasma membrane (Fig. 3A). The nuclei of cells exposed to weisiensin B (4.0 μM) for 24 h exhibited condensed and fragmented chromatin (Fig. 3B), which suggested apoptosis. Longer time (48 h) treated cells showing more serious chromatin condense and marginalization (Fig. 3C), these changes were clearly progressing in a time-dependent manner.

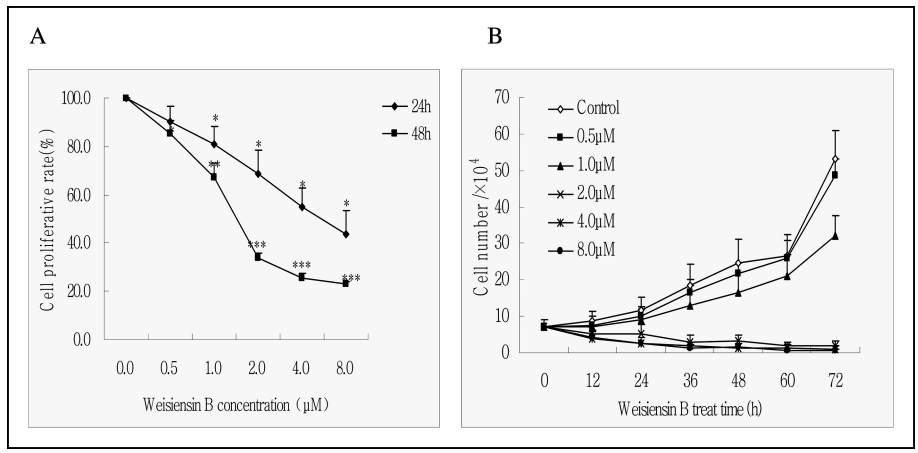

Fig. 2: The growth inhibition and lethal effects of Weisiensin B to K562 mearued by SRB assay (A) and Trypan blue exclusion (B) *$p \leq 0.05$,**$p \leq 0.01$,***$p \leq 0.001$ Vs Control.

2.3. Weisiensin B induced apoptosis and necrosis in K562cells

To determine whether the anti-proliferative effect of weisiensin B on K562 cells was related to apoptotic cell death or necrosis, we analyzed phosphatidylserine exposure as an early marker of apoptosis and PI stain positive as necrosis marker using AnnexinV–FITC/PI double staining. The percentages of apoptotic and necrosis cells were determined by flow cytometry.

As shown in Fig. 4, the percentages of cells with Annexin V/PI-positive staining increased gradually in a concentration-dependent manner after treatment, suggesting that weisiensin B could induce apoptotic and necrosis response in K562 cells. In the untreated control group, more than 97% cells were normal or viable (lower left, LL). While, after 24 h incubation with 2 μM and 4 μM weisiensin B, the early apoptotic ($Annexin^+/PI^-$,lower right, LR) cell population increased from 0.60% to 8.76% and 17.17%, respectively. The secondary necrotic or later apoptotic cells ($Annexin^+/PI^+$, upper right, UR) were 1.31% and 9.86%, respectively. When K562 cells were treated for 48 h, more double-positive cells appeared, the $Annexin^+/PI^+$ cells even up to 18.75% and 36.94%, respectively. These data obviously exhibited the time and concentration-dependent apoptosis and necrosis effect of weisiensin B to K562 cells.

2.4. Weisiensin B induced K562 cell cycle arrest in G2/M phase

To explore the relationship of weisiensin B-induced apoptosis and necrosis with cell cycle progression, PI staining and flow cytometry was used to quantify the cell cycle. The changes of the cycle distribution are shown in Fig. 5 and the apoptotic or necrosis cells were counted based on sub-G1 DNA contents. When treated with 2 μM weisiensin B (Fig. 4 A), G2/M phase was increased significantly, from 8.1% to 35.6%. The percentage of S phase almost sustained at the same level and cells of sub-G1 displayed a slight increase. A significant and time-dependent increase, from 7.3% to 61.7% (Fig. 5 B), of G2/M phase was observed following the treatment with 4 μM substance. In the meantime of this increase, the sub-G1 cells were found to be increased significantly, too, though not as high as the percent tested by AnnexinV–FITC/PI double staining.

These data suggested that weisiensin B reduced K562 cell proliferation and induced cell death mainly associated with G2/M phase cell cycle arrest.

2.5. The ROS generation in K562 cells treated by Weisiensin B

ROS play an important physiological role as secondary messengers and interfere with the expression of a number of genes and signal transduction pathways. To investigate whether generation of intracellular ROS is a part of the mechanism by which weisiensin B inhibits cell proliferation and induces apoptosis in K562 cell, the generation of ROS was assessed by using fluorescent probes DCFH-DA. Figure 6 shows the flow cytometric analysis of the ROS produced by K562 cells treated with 8 μM weisiensin B within 60 min. After 10 min treatment, fluorescence intensity increased and resulted 1.4-fold to control. From 40 min to 60 min it increased significantly and attained to 4.55-fold at last. The generation and accumulation of ROS dramatically increased along with the incubation time. ROS have not been detected to increase compared to control cells after 6 h, 12 h, 24 h treatment (data not shown). This implicated that ROS have generated within 6 h and ROS formation may be an early event in weisiensin B induced apoptosis in K562 cells.

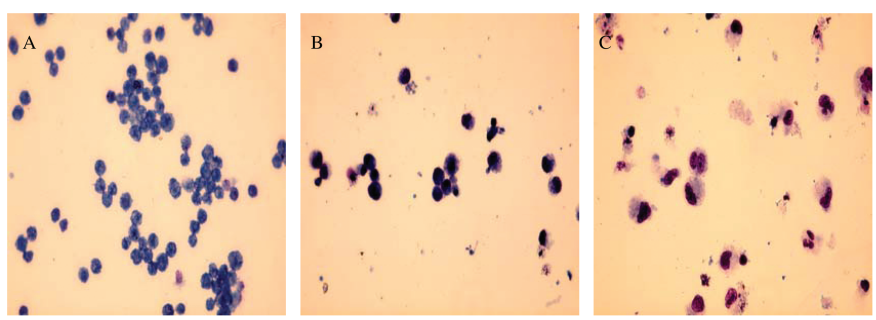

Fig. 3: Chromosome condensation of K562 induces by Weisiensin B (× 400) A.Control, B. Weisiensin B treated 24 h, C. Weisiensin B treated 48 h.

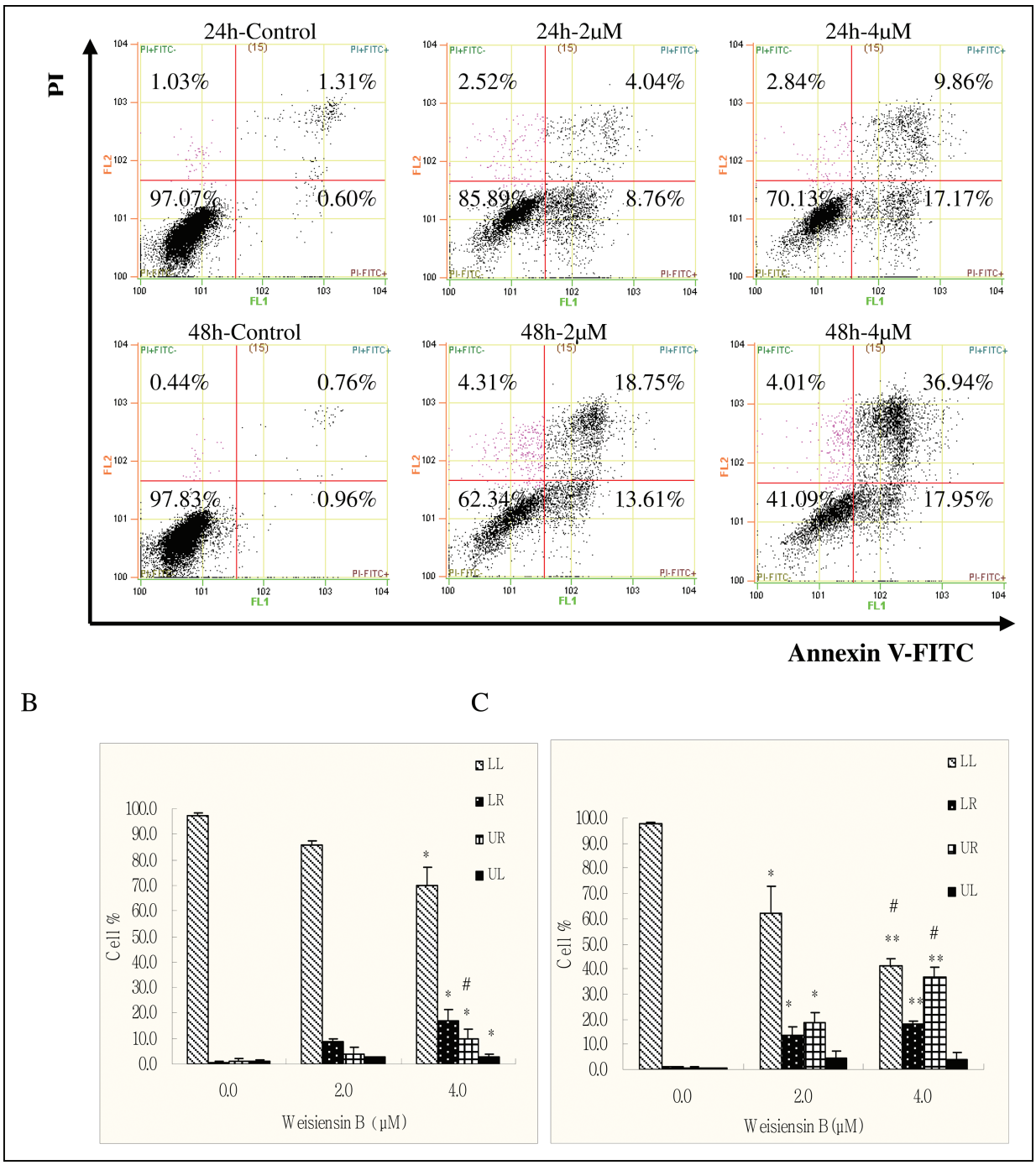

Fig. 4: Weisiensin B induced apoptosis and necrosis in K562 by Annexin-V-PI dual staining method A: Quadrant analysis of four panels showed their fluorescence characteristics: Live cells are AnnexinV(-)/PI(-), early apoptotic cells are AnnexinV(+)/PI(-), and necrotic or advanced apoptotic cells are both AnnexinV(+)/PI(+). B and C: Quantitative analysis of the histograms expressed as the percentage of apoptotic or necrotic cells in total K562 cells treated by Weisiensin B for 24 h and 48 h. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ Vs. Control, $^{\#}p \leq 0.05$ *vs.* 2 μM Weisiensin B treatment.

This was similar to chlorogenic acid induced apoptosis in CML cells (Rakshi et al. 2010).

2.6. NAC reverses the growth rate decrease of K562 cell induced by weisiensin B

To confirm the finding that weisiensin B treatment induced ROS generation, we investigated whether the free radical scavenger NAC (N-acetylcysteine), a thiol-protecting antioxidant precursor of glutathione (GSH) (Oh et al. 2006) could neutralize intracellular ROS production by weisiensin B and therefore increase cell viability. As shown in Fig. 7A, K562 cells growth rates significantly decreased to 64.97% and 32.13% after treatment with 4 μM and 8 μM weisiensin B, respectively. By co-incubation with NAC, the cell viabilities were increased to 75.05% and 73.98%, respectively. NAC exhibited more effective protection in 8 μM weisiensin B treated cells. This also means that more ROS were produced in this concentration weisiensin B and NAC reversed cell growth decrease more effectively. These was in agreement with cell morphological changes observed by phase contrast microscopy (Fig. 7 B). Except cell density decrease, morphological changes such as cell rounding, cell shrinkage and blebs formation could be observed in 8 μM weisiensin B treated cells

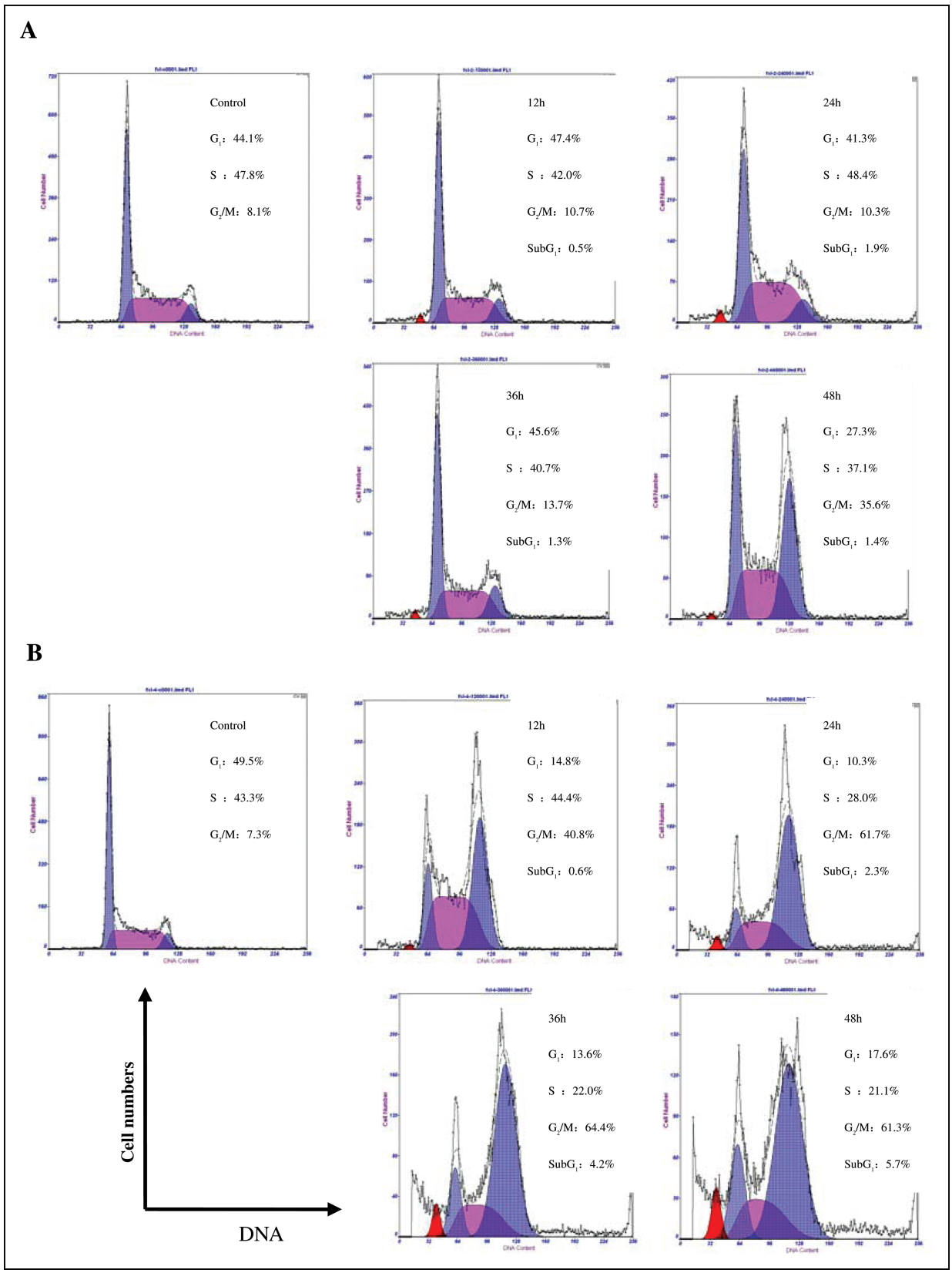

Fig. 5: Cell cycle assay of K562 cells with flow cytometry when cells treated by 2 μM (A) and 4 μM Weisiensin B (B).

(Fig. 7 B c), exhibiting morphological features of apoptotic cells. Co-incubation with NAC led to a significant reversion (Fig. 7 B d).

These results confirmed that weisiensin B inhibited cell growth by producing ROS and NAC reverted the effect by scavenging ROS.

3. Experimental

3.1. Materials and reagents

Weisiensin B was isolated from dry leaves and stem of *Rabdosia weisiensis* C. Y. Wu produced in the Gansu province of China. The structure was elucidated by 2D-NMR techniques and single-crystal X-ray-diffraction analysis (Ding et al. 2005) and shown in Fig. 1. Before use, the compounds were

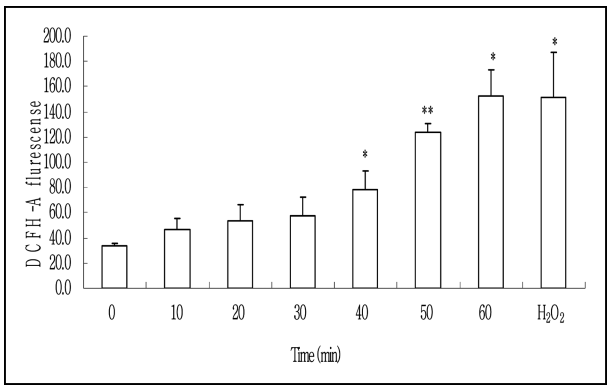

Fig. 6: The ROS generation in K562 cells treated by Weisiensin B or H_2O_2 (200 μM) $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ Vs Control.

dissolved in dimethylsulfoxide, stored at 4°C and then subjected to experimental assays.

RPMI-1640 and DMEM from Gibco Invitrogen Corporation (Carlsbad, CA, USA); trypsin, propidium iodide, DAPI (2-(4-amidinophenyl)- 6-indolecarbamidine dihydrochloride and Giemsa were purchased from Sigma Chemical Co. (St Clara, CA,USA); Annexin-V-FITC from eBioscience (America); Sulforhodamine B from Tokyo KaSei Industry Co. Ltd; N-acetylcysteine and ROS assay kit bought from Beyotime Biotech (China).

3.2. *Methods*

3.2.1. *Cell culture and viability assay*

K562, human chronic myelogenous leukemia cells, were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C in humidified air with 5% CO_2. Sulforhodamine B (SRB) assay was performed according to Papazisis et al. (1999) with minor modifications. Cells were seeded into 96-well culture plates at a density of 0.5×10^4 cells/mL and grown for one day prior to treatment with various concentrations of weisiensin B alone or with 10 mM NAC. After further 24 h and 48 h of culture, 50 μL 50% cold TCA were added and the plate were left for 1 h at 4 °C for cell fixation. Then the plates were washed five times with deionized water and left to dry at room temperature. SRB in 1% acetic acid solution was added to each well and plates were placed on a plate shaker for at least 10 min. Absorbance was read in a 96-well plate reader at 515 nm, dose-response curves were plotted (values expressed as percentage of control optical density) and IC_{50} values were estimated by regression analysis.

For trypan blue exclusion assay, cells were seeded in 24-well plates at a concentration of 5×10^4 cells/mL and incubated for 24 h. After varying concentrations of weisiensin B added for different times, 0.4% trypan blue solution was added to 0.4 mL cell suspension. After mixture and dye 3 min, the viable ones that excluded trypan blue dye were counted for estimation the cytotoxicity.

3.2.2. *Assessment of cell morphology by Giemsa staining*

To examine the change in cell morphology, the control and Weisiensin B treated cells were centrifuged and washed twice with PBS. The cells were fixed in methanol- acetic acid (3:1) for 30 min and were drawn onto glass

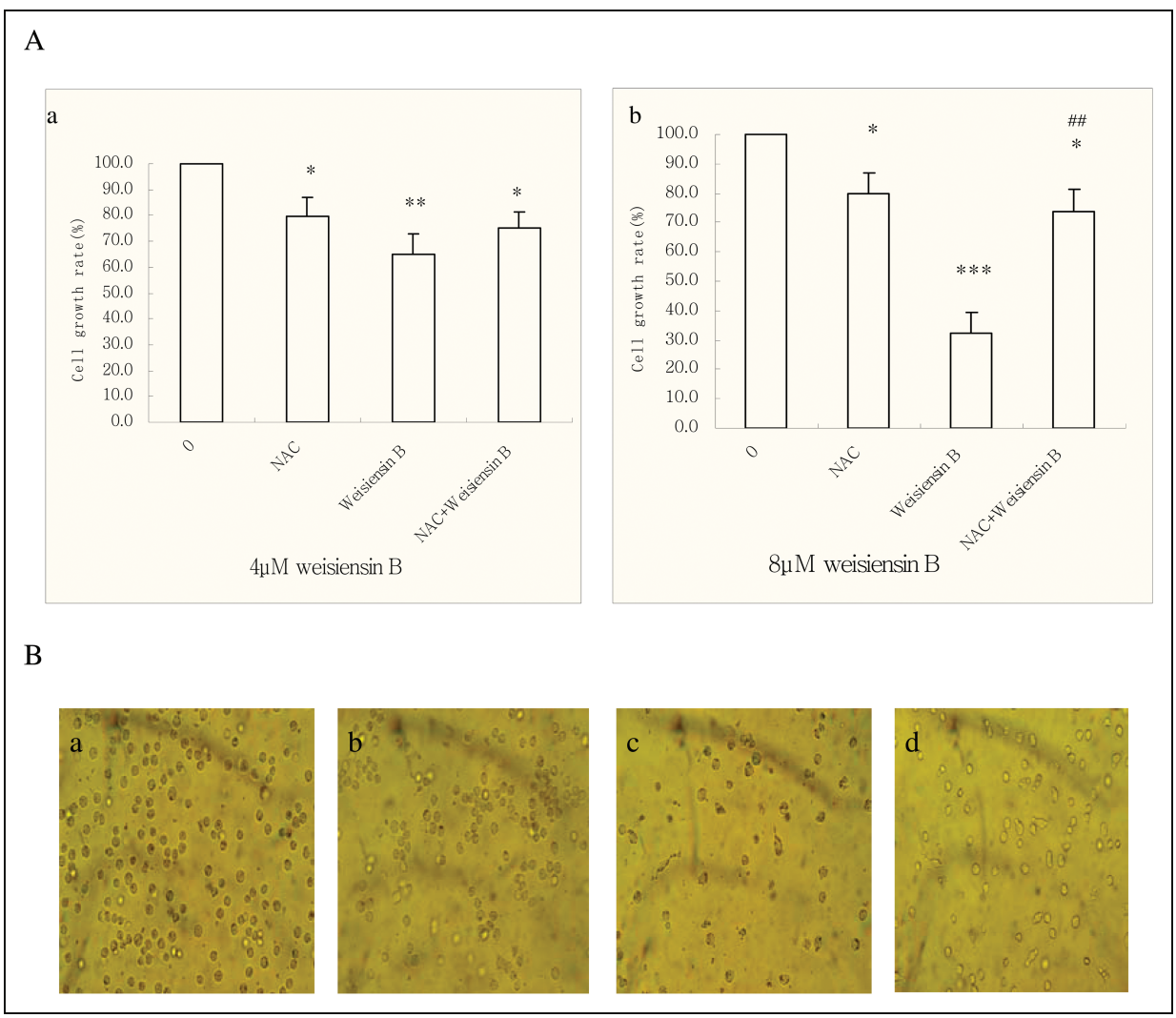

Fig. 7: NAC reversed cell growth rate decrease and morphological changes in K562 cell induced by Weisiensin B A The growth rate of K562 cells treated by 4 μM (a) or 8 μM(b) Weisiensin B and 10 mM NAC *Vs Control; $^{\#}$Vs Weisiensin B; $^{*}p \leq 0.05$,$^{**}p \leq 0.01$,$^{***}p \leq 0.001$; $^{\#}p \leq 0.05$,$^{\#\#}p \leq 0.01$ B Protective effect of NAC in K562 cells morphological changes induced by Weisiensin B(× 200) a.Control; b. 10 mM NAC; c.8 μM Weisiensin B; d 8 μM Weisiensin B combined 10 mM NAC.

slides. After air dried and stained 20 min with Giemsa stain, the slides were washed with distilled water, observed and photographed under microscope.

3.2.3. Apoptotic and necrosis cells measured with an Annexin V/propidium iodide (PI) flow cytometric analysis

Chemically induced cell death can be mediated by either necrosis or the apoptotic pathway. For cell death pathway assay, 1×10^6/mL K562 cells were exposed to weisiensin B (2 and 4 μM) for 24 h and 48 h, respectively. After incubation, the cells were harvested, washed with PBS, and re-suspended in 190 μL binding buffer. Annexin V-FITC staining solution (5 μL) was added in the dark for 10 min and PI for another 10 min. The Annexin V-FITC and PI fluorescence of cultured cells were immediately determined by flow cytometry (Liang et al. 2008).

3.2.4. Cell cycle analysis by DAPI staining

To study the effect of weisiensin B on the cell cycle, 10×10^4/mL K562 cells were cultured for 24 h. The compound was added every 12 h in concentrations of 2 and 4 μM. After treatment, the cells were harvested and washed twice with PBS, then fixed in 70% ice-cold ethanol overnight. Cells were then washed again and finally stained with 10 μg/mL DAPI in the dark for 30 min. The DAPI-elicited fluorescence of individual cell was measured by flow cytometry.

3.2.5. Intracellular ROS determination by DCFH-DA stain

2',7'-Dichlorofluorescin diacetate (DCFH-DA) is a stable compound that readily diffuses into cells where it is broken down into cell impermeable, non-fluorescent reduced dichlorofluorescin (DCFH) and diacetate by cellular esterases. DCFH is oxidized by ROS such as H_2O_2, hydroxyl and peroxyl radicals to produce fluorescent DCF. Thus, the fluorescence intensity is proportional to the amount of ROS produced by the cells and ROS was monitored by flow cytometry (Patil et al. 2012).
For the short time (≤ 2 h) treatment, the fluorescence probe were loaded first and then weisiensin B was added. Briefly, cells incubated with 10 μM DCFH-DA for 30 min in the dark at 37 °C and washed twice. Then the probe loaded cells were treated with weisiensin B or combined with NAC for a period time. After washing, the cells were re-suspended and tested. For long time treatment (≥ 6 h), cells were loaded with 10 μM DCFH-DA and incubated for 30 min in the dark at 37 °C. Cells were then harvested, washed and re-suspended in PBS and analyzed immediately via flow cytometry (Liang et al. 2008). The values were calculated as the relative intensity of DCF fluorescence compared with control.

3.2.6. Statistics

All the data are expressed as mean ± standard deviation (S.D.) from three independent experiments. Significant differences were calculated using Student's t-test. The p-value of < 0.05 was adopted as statistically significant.

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