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Alternol inhibits proliferation and induces apoptosis in mouse lymphocyte leukemia (L1210) cells

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Abstract Alternol is a novel compound purified from the fermenting products by microorganisms named as *Alternaria alternata var. monosporus* from the bark of Yew. In this study, we tested its effect on mouse lymphocyte leukemia L1210 cells. Alternol was found to inhibit the proliferation and induce apoptosis in L1210 cells. When the cells were treated with Alternol, chromatin condensation and phosphatidylserine externalization were observed with the down-regulation of the pro-survival gene Bcl-2 and the activation of caspase-3, caspase-9, but not caspase-8. Moreover, exposure of cells to Alternol resulted in a significant increase in reactive oxygen species (ROS) and mitochondrial transmembrane potential ($\Delta\Psi m$) depolarization. Taken together, these results demonstrate that Alternol is a potent agent in inducing L1210 cells to apoptosis, which involve caspase activation and ROS generation.

Keywords Alternol · L1210 · Apoptosis · Leukemia

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Introduction

Acute lymphocytic leukemia (ALL) is the most common type of leukemia in children under the age of 15 and it accounts for 25% of all cancers and 78% of leukemia. In the United States, morbidity of leukemia is a little higher than that in Asia countries and about 3,930 new cases of ALL are diagnosed each year (Surveillance, Epidemiology, and End Results Program, 2006). Chemotherapy is the standard treatment for ALL, but only about one-third of patients can achieve long-term survival after effective chemotherapy. Although some novel therapeutic strategies will cure the hematological malignancies without affecting normal cells [1–3], there are many problems such as drug resistance and inefficiency in transduction. It is considered that the unchecked proliferation and obstruction of differentiation/apoptosis in leukemia cells is the main pathologic foundation of leukemia. Therefore, selective apoptosis induction for the leukemia cells is a promising strategy of leukemia therapy. Apoptosis proceeds mainly through two routes, known as the extrinsic and intrinsic pathways. The extrinsic pathway of apoptosis is mediated by death receptors (FADD), which activate initiator caspase-8 or caspase-10 signaling that leads to activation of executioner caspases such as caspase-3, caspase-6, and caspase-7. Regulation of the intrinsic pathway-induced apoptosis involves Bcl-2-related family members such as Bcl-2, Bcl-xl, Bad, and Bax [4]. Bcl-2 and Bcl-xl exert their anti-apoptotic effect, at least in part by binding to Bax and related pro-apoptotic proteins.

Live or attenuated pathogenic microorganisms or their products were used in the therapy of cancer [5]. Recently, researchers have successfully extracted and purified a novel compound-Alternol (Fig. 1) from fermenting productions by microorganisms from the bark of Yew. It has

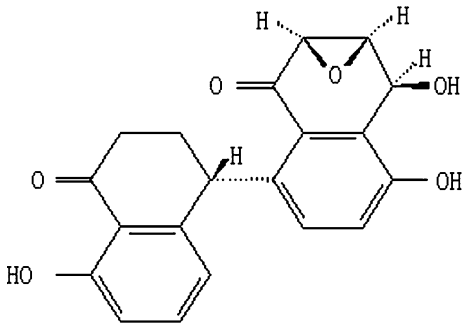

Fig. 1 The structure of Alternol ($C_{20}H_{16}O_6$, Mw352)

been found that Alternol could inhibit the proliferation of several malignant tumor cells such as human esophageal epithelial cancer cell line Eca-109, mouse melanoma cell line B16, human ovarian cancer cell line A2780 and human gastric cancer cell line MGC-803 [unpublished data]. In this study, we identified that Alternol could induce apoptosis in L1210 cells. We further demonstrated that Alternol potently induced caspase-3 and caspase-9 dependent apoptosis in which ROS might also play a role.

Materials and methods

Cell culture and reagents

L1210 cells and Alternol were provided by Strand Biotech Company Limited. The cells were cultured in RPMI-1640 medium (Gibco Invitrogen Grand Island, NY, USA), supplemented with 10% fetal bovine serum(FBS) (Saiyue Bioscience Shanghai, China), 2 mM glutamine, 50 μg/ml gentamicin at 37°C in a 5% CO_2 humidified atmosphere. Paclitaxel (6 mg/ml) was purchased from Sihuan Pharmaceutical Co. (Beijing, China). Rabbit anti-Bcl-2 polyclonal antibody (sc-783), rabbit anti-Bcl-x_L polyclonal antibody (sc-7195), rabbit anti-Bax polyclonal antibody (sc-526), rabbit anti-Bak polyclonal antibody (sc-7873) and rabbit anti-caspase-3 polyclonal antibody (sc-7148) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradishperoxidase-conjugated goat anti-rabbit/mouse IgG and mouse anti-α-tubulin monoclonal antibody were purchased from Sigma (St. Louis, MO, USA). All other chemicals were purchased from Sigma unless otherwise specified.

Cell proliferation assay

For cell proliferation and viability assays, 2×10^5 L1210 cells were seeded into 96-well culture plates. After 24 h of cultivation in FBS-free medium, the cells were treated with 100 μl of FBS-free medium supplemented with Alternol/Paclitaxel and further cultivated for the indicated period. The Alternol, dissolved in ethanol, was added to the culture medium at a final concentration of 2, 4, 6, and 8 μM. Paclitaxel was added to the culture medium at a final concentration of 200 nM. The final concentration of ethanol in the culture medium was 0.1% (v/v). FBS-free medium with vehicle was used as a control. Cell proliferation was evaluated by using a Cell Counting Kit-8 (Dojin Laboratories, Kumamoto, Japan).

Morphological study of apoptotic cells

After exposed to 8 μM Alternol for 72 h, 2×10^5 L1210 cells were collected and washed with PBS and fixed with 4% paraformaldehyde for 15 min. Fixed cells were washed with 0.5 ml PBS again and dropped on clean slides, then the cells were stained with 10 μg/ml Hoechst 33342 (Sigma, St. Louis, MO, USA) in PBS for 15 min at room temperature in the dark. Cells were washed three times with PBS and analyzed using a fluorescence microscopy (IX71, Olympus, Japan). At least 200 cells were counted.

Quantitative analysis of apoptosis

Annexin V-FITC and PI double staining were used for quantitative analysis of apoptosis. Briefly, L1210 cells were treated as described above for cell proliferation assay for 24 and 48 h. A total of 1×10^6 cells were harvested and washed with cold PBS, resuspended in 200 μl binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH7.4), and incubated with 5 μl AnnexinV-FITC for 15 min in the dark. Then the cells were resuspended and counterstained with 5 μg/ml PI for 5 min. The percentage of apoptotic cells was estimated using a BD FACScan flow cytometer and the data were obtained and analyzed with Cell Quest software.

Determination of intracellular ROS production

Dichlorodihydrofluorescein diacetate (DCFH-DA), a fluorescent probe whose fluorescence intensity is proportional to the amount of peroxides produced by the cells, was used to measure the intracellular ROS production [6]. Briefly, L1210 cells were treated as described above for cell proliferation assay for 2, 6, 12, and 48 h. A total of 1×10^6 cells were harvested and suspended in PBS. DCFH-DA was then added to a final concentration of 10 μM and incubated for 30 min at 37°C. The fluorescence emission from DCF was determined at an excitation wavelength of 503 nm with flow cytometer. Data were obtained and analyzed with Cell Quest software.

Flow cytometric analysis of changes in mitochondrial transmembrane potential ($\Delta\Psi m$)

The change in $\Delta\Psi m$ of L1210 cells in response to Alternol was examined using Rodamin123, a fluorescent probe. L1210 cells were treated as described above for cell proliferation assay for 48 and 72 h. A total of 1×10^6 cells were harvested and washed twice in PBS, resuspended with 50 μl (10 μg/ml) Rodamin123 and incubated at 37°C for 30 min. Analysis of $\Delta\Psi m$ was preformed by flow cytometer with excitation at 510 nm. Data were obtained and analyzed with Cell Quest software.

Western blot analysis

L1210 cells were treated as described above for cell proliferation assay for 72 h, then the cells were collected and incubated with Lysate Buffer (20 mM Hepes pH7.5, 200 mM NaCl, 0.2 mM EDTA-Na_2, 1%Triton X-100, 0.05% SDS, 0.5 mM DTT, 1 mM Na_3VO4, 20 mM β-glycero-phosphate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotintin, 20 μg/ml leupeptin) on ice for 30 min. The lysate was centrifuged at 12,000 rpm for 10 min to obtain the supernatant. The protein concentration was determined with a BCA protein assay kit (Applygen Technologies Beijing, China). Proteins from total cell lysates (50 μg) were resolved on 10% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-C Amersham Vienna, Australia) by electroblotting. The nitrocellulose membrane was incubated in milk block buffer (TBS-T) containing 10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween-20 involving 5% nonfat milk at room temperature for 2 h, and was probed with each of the primary antibodies overnight at 4°C. The antibodies were as follows: Bcl-2 (1:500), Bcl-xl (1:500), Bax (1:500), Bak (1:500), caspase-3 (1:1,000) and α-tubulin (1:5,000). The membranes were washed three times for 10 min in TBS-T, and were incubated with horseradish-peroxidase-conjugated goat anti-rabbit/mouse secondary antibodies in TBS-T. After the membranes were washed three times for 10 min in TBS-T, fluorescent bands were developed with 150 μl Super Signal Luminol Enhancer solution and 150 μl Super Signal Peroxidase solution (Pierce Rockford IL, USA). The X-ray films were exposed to the membranes and then developed.

Caspase activity assay

The caspase-3, caspase-8, and caspase-9 activity was determined using Caspase Activity Assay Kits (Biolife, Beijing, China). A total of 3×10^6 treated cells were washed twice with ice cold PBS and then were lysed in 50 μl cold buffer for 20 min on ice. After the lysate was centrifuged at 12,000 rpm for 15 min, the supernatant was collected and the protein concentration was determined with BCA protein assay kit. Aliquots (50μg protein) of the supernatant in triplicate were transferred to the 96-well plate and incubated in the dark with 100 μl of buffer containing caspase substrates at 37°C for 4 h. Absorbance at 405 nm was measured using a microplate reader (Titertek Multiskan Plus, Labsystems, Finland).

Statistical analysis

Values are expressed as means ± SD. The significance of the difference from the respective controls for each experimental test condition was assayed performed using Student's t test analysis, with P values <0.05 or 0.01 considered significant.

Results

Alternol inhibits the proliferation of L1210 cells

To test the effect of Alternol on the proliferation of L1210 cells, the cells were treated with different concentrations of Alternol or paclitaxel in indicated time. The growth inhibition of L1210 cells was determined by using CCK-8 assay. The amount of the yellow colored formazan dye

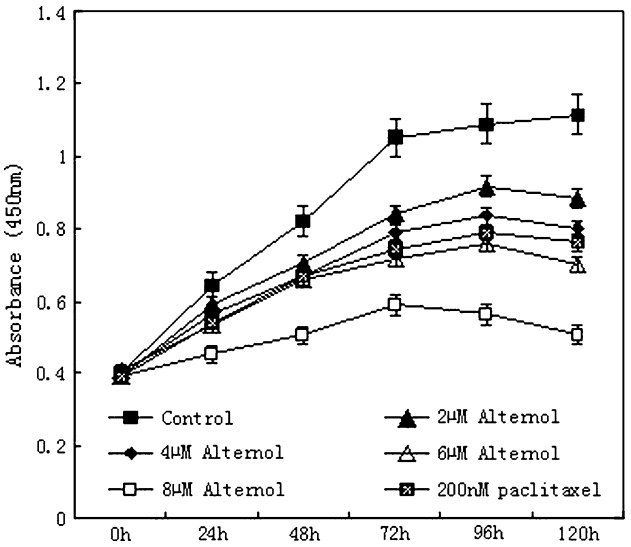

Fig. 2 Alternol inhibits the proliferation of L1210 cells. L1210 cells in 96-well plates were treated with Alternol at the concentrations indicated. Cell proliferation was measured using CCK-8 assay at the indicated times. The number of viable cells is proportional to the absorbance. Data are means ± SD for three independent experiments

generated by dehydrogenases in cells is directly proportional to the number of viable cells in a culture medium. The results showed that the cell proliferation of L1210 was inhibited in a dose- and time-dependent manner compared to control group (Fig. 2).

Alternol induces apoptosis in L1210 cells

To examine whether Alternol inhibits the proliferation of L1210 cells by inducing apoptosis, we used Hoechst 33342 staining to observe the morphological changes of L1210 cells induced by Alternol. Control cells exhibited round nuclei with well-distributed chromatin, whereas typical apoptotic morphology characterized by condensed chromatin, nuclear fragmentation, and appearance of apoptotic bodies, was demonstrated after 72 h exposure to 8 μM Alternol (Fig. 3). To further characterize the death process, we measured phosphatidylserine exposure, a defining morphological characteristic of apoptotic cells. Alternol induced the increase in the size of the Annexin V positive population in a time- and dose-dependent manner as compared to an untreated control (Fig. 4). The effect was maximal for cells exposed to 8 μM Alternol for 48 h.

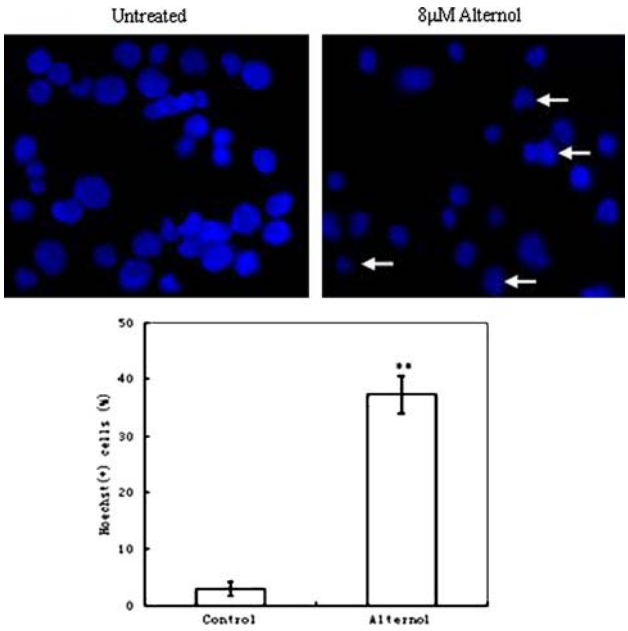

Fig. 3 Apoptosis in L1210 cells induced by Alternol. L1210 cells were treated with 8 μM Alternol for 72 h, followed by fixation, stained with Hoechst33342, and visualized using a fluorescent microscopy. Arrows indicate the condensed, fragmented, brightly stained nuclei. Bar diagram showing the ratio of Hoechst positive cells. Data are the means ± SD of three independent experiments with each count of no less than 200 cells. $^{**}P < 0.01$ significantly different from the control as analyzed by Student's *t*-test

Alternol promotes ROS generation in L1210 cells

ROS levels are increased in cells exposed to various stress agents, including anticancer drugs [7] and an emerging view is that it is a key effector of apoptosis (ROS, stress-activated kinases, and stress signaling in cancer). We examined whether the Alternol induced apoptosis was associated with intracellular ROS generation. As shown in Fig. 5, all treatment of different concentrations of Alternol for 6, 12, and 48 h caused significant increase in intracellular ROS level. Even after treatment of 2 h, Alternol at 4, 6, and 8 μM also significant by increased the intracellular ROS. These results suggested that intracellular ROS generation might be one of the early events in Alternol induced apoptosis in L1210 cells.

Alternol induces the loss of mitochondrial transmembrane potential (ΔΨm) in L1210 cells

The dissipation of the inner mitochondrial transmembrane potential (ΔΨm) was considered as a mark during the apoptotic program. Also, the mitochondrial depolarization was associated with outer mitochondrial membrane permeability which is induced by many physiological effectors including ROS and the blockade of the respiratory chain [8]. In an attempt to discover whether the ΔΨm depolarization exist in Alternol-inducing apoptosis, the ΔΨm of L1210 cells were measured by a fluorescence-activated cell sorter. Rodamin123 was applied in control cells and treated cells for 48 and 72 h and then level of fluorescence was analyzed with flow cytometry. The results showed that the ΔΨm of Alternol groups (2, 4, 6, and 8 μM) decreased significantly compared with the control group, so did the paclitaxel group (Fig. 6).

Alternol up-regulates csapase-3 and down-regulates Bcl-2 proteins in L1210 cells

The Bcl2 family of intracellular proteins is the central regulator of caspase activation, and its opposing factions of anti- and pro-apoptotic members arbitrate the life-or-death decision [9]. We examined the expression of the Bcl-2 family intracellular proteins in L1210 cells after Alternol treatment of different concentrations for 72 h by western blot. As shown in Fig. 7, the expression of Bcl-2 decreased significantly while there was no detectable change in Bcl-X_L. With the increase in the concentration of Alternol, there was no significant change in the expression level of Bax and Bak. In addition, the expression level of caspase-3 was increased after Alternol treatment, especially in 8 μM.

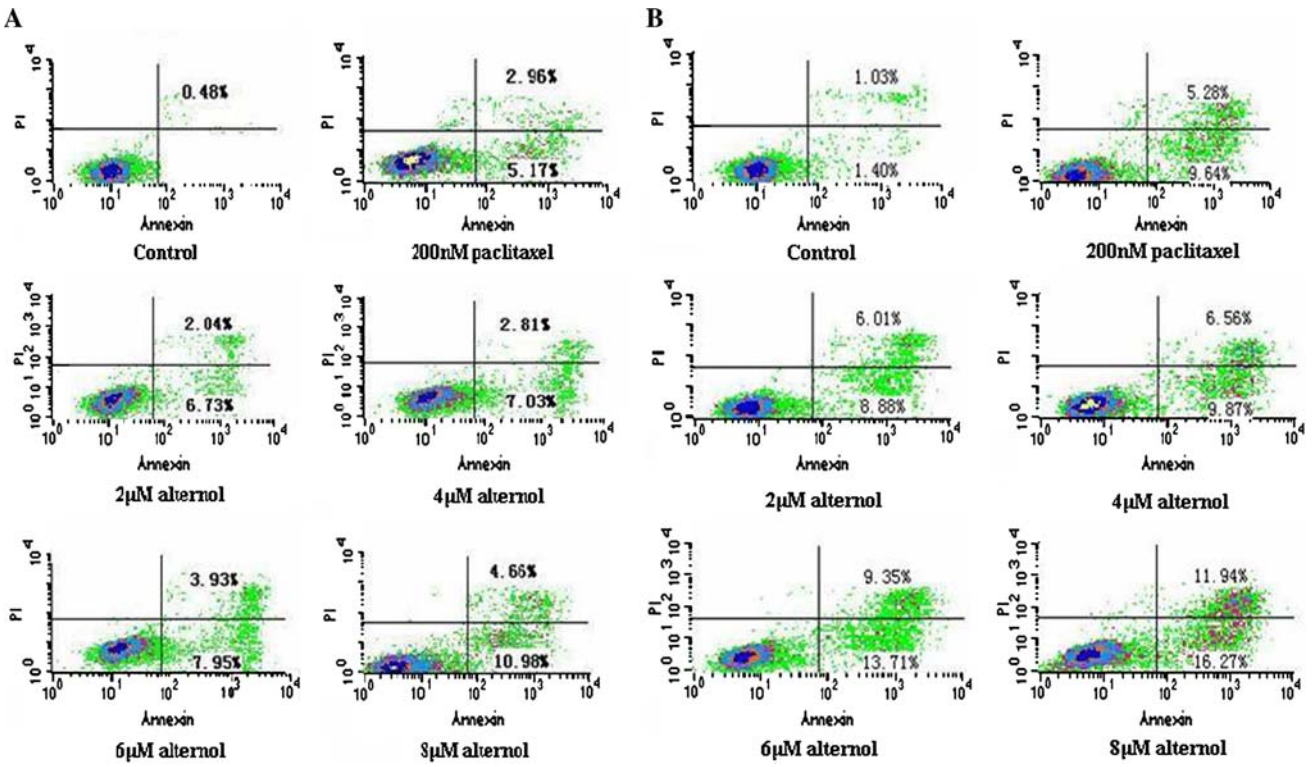

Fig. 4 Quantitative analysis of apoptosis. Flow cytometric analysis of apoptosis in L1210 cells which were treated with indicated concentrations of Alternol for 24 h (A) and 48 h (B). The percentage of apoptotic cells was determined by Annexin V-FITC/PI staining. This combination allows the differentiation among early apoptotic cells (annexin V positive, PI negative), necrotic cells (annexin V positive, PI positive), and viable cells (annexin V negative, PI negative). The figure represents results of three independent experiments

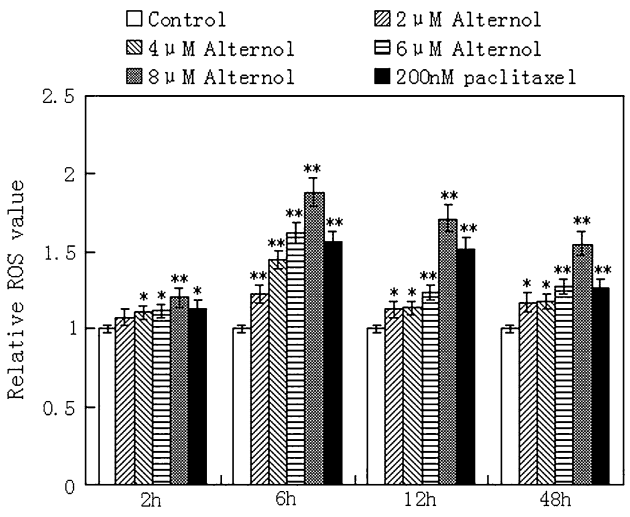

Fig. 5 Alternol promotes ROS generation in L1210 cells. L1210 cells were, respectively, treated with indicated concentrations of Alternol for 2, 6, 12, and 48 h, followed by DCFH-DA addition for 30 min. The fluorescence intensity of the cells was detected by flow cytometric analysis. Results are expressed as fold of controls and data are means ± SD of three independent experiments. $*P < 0.05$ and $**P < 0.01$ significantly different from the control as analyzed by Student's *t*-test

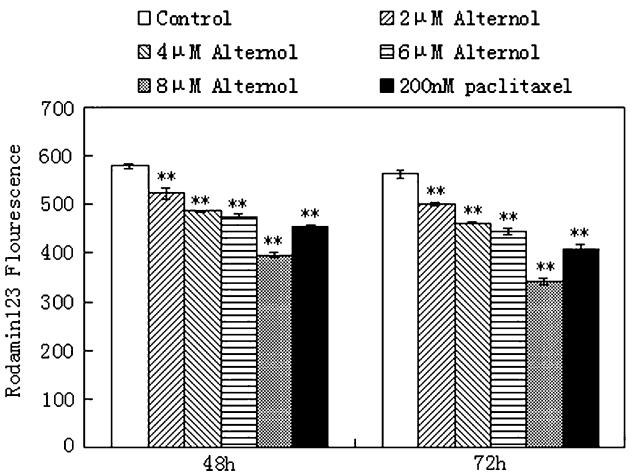

Fig. 6 Alternol causes the loss of ΔΨm in L1210 cells. ΔΨm was assessed by measuring the fluorescence intensity of the membrane potential dependent dye Rhodamin123 using a flow cytometric analysis. L1210 cells were treated with indicated concentrations of Alternol for 48 and 72 h. Data are the means ± SD of three independent experiments. $**P < 0.01$ significantly different from the control as analyzed by Student's *t*-test

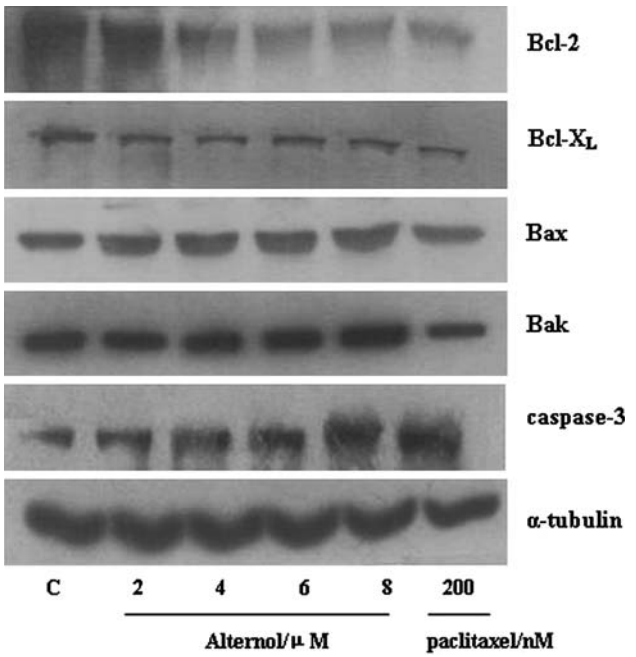

Fig. 7 Effect of Alternol on the expression level of Bcl-2 family proteins and caspase-3 protein. L1210 cells were treated with indicated concentrations of Alternol for 72 h. The whole-cell lysate was used for Western blot analysis with antibodies for Bcl-2, Bcl-X_L, Bax, Bak, caspase-3, and α-tubulin. The figure represents results of three independent experiments

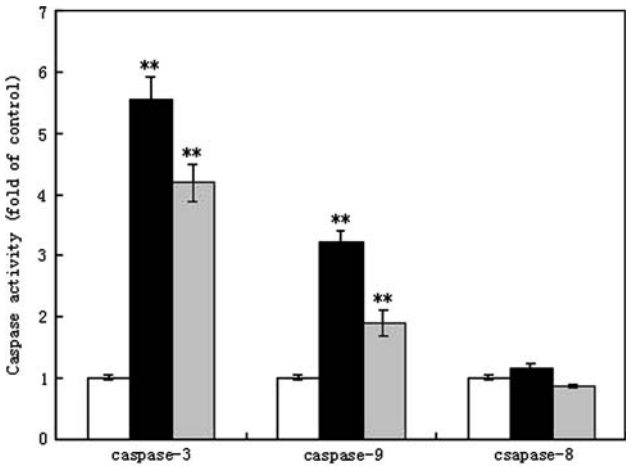

Fig. 8 Spectrophotometric detection of csapase-3, csapase-9, and csapase-8 activities in L1210 cells treated without (control) or with 8 μM Alternol for 48 and 72 h. Caspase activities were determined using the fluorescent substrates Ac-DEVD-MCA (caspase-3), Ac-LEHD-MCA (caspase-9), and Ac-IETD-MCA (caspase-8). The results are given as fold of controls. The open, black and gray bars show treatment with Alternol for 0, 48, and 72 h. Data are the means ± SD of three independent experiments. $^{**}P < 0.01$ significantly different from the control as analyzed by Student's *t*-test

Alternol induces the activation of caspase-3 and csapase-9

Activation of caspase cascades has been shown to occur in apoptosis [10]. We examined the activity of csapase-3, caspase-9, and caspase-8 in L1210 cells treated with 8 μM at 48 and 72 h. As shown in Fig. 8, after treatment for 48 h, caspase-3 activity increased to the peak of about 5.56 times the activity of the control treatment and 4.19 times in 72 h. The peak activation of caspase-9 was also found in 48 h. However, there was no detectable change in the activity of caspase-8.

Discussion

Alternol is a novel compound obtained by bioengineering technology. Microorganisms named as *Alternaria alternata var. monosporus* (ST-026-R CGMCC No.0899) are acquired from the bark of Yew, and organic extracts are isolated from cultured microorganisms or supernatant. Compound then are purified from the fermenting production. In this study, we find that Alternol inhibits the proliferation of L1210 cells and induces them to apoptosis, as characterized by nuclear fragmentation, appearance of apoptotic bodies and Annexin V-FITC-positive staining. Moreover, the results suggest that ROS are possibly involved in Alternol-induced apoptosis of L1210 cells and act priority to the loss of $\Delta\Psi m$.

The induction of apoptosis is now considered to be an attractive strategy for cancer therapy [11, 12]. Various genes are involved in the regulation of apoptosis, among which P53 gene is the most important one. High-level expression of wild-type P53 will induce cell cycle arrest or apoptosis [13, 14]. However, in most human cancer cells, loss of functional P53 impairs the response to apoptotic stimuli [15, 16] and leads to poor prognosis in patients. Therefore, development of agents that are capable of inducing apoptosis in P53-independent manner is highly desirable [9, 17]. In our research, we found that Alternol induced apoptosis of L1210 cells with mutated and dysfunctional P53 and the significant founding drove us to detect the possibly associated mechanism.

Mitochondria play a critical role in the regulation of apoptosis induced by some death stimuli [18, 19]. Early cell changes of apoptosis are associated with mitochondrial changes mediated by the Bcl-2 families including anti-apoptotic (e.g., Bcl-2 and Bcl-xl) and pro-apoptotic (e.g., Bak and Bax) proteins [20, 21]. Bcl-2 or related gene products facilitate recovery from DNA damage in apoptosis [22] and is also involved in preventing oxidant-induced cell death [14, 23]. In addition, over-expression of Bcl-2 or Bcl-xl can increase the stability of $\Delta\Psi m$ and can protect against caspase activation and DNA fragmentation [19, 24]. Our results showed the expression of Bcl-2 protein was down-regulated pronouncedly after Alternol treatment. Though

the change of expression of Bax protein was not obvious, the ratio of Bcl-2/Bax protein was decreased, which was considered to determine how a cell responds to apoptotic or survival signals [25]. These findings suggested that Bcl-2 family proteins would be involved in the apoptosis induced by Alternol in the L1210 cells.

The caspases play a central role in both the extrinsic and intrinsic pathways of apoptosis [26]. Caspase-8, a caspase activated by the Fas ligand in the extrinsic pathway, acts as the major initiator and activates downstream effecter caspases (e.g., caspase-3) [23]. While in the intrinsic pathway, the release of some apoptogenic factors from mitochondria results in the activation of caspase-9 [27, 28] and further activates downstream effecter caspases (e.g., caspase-3). The activated caspase-3 is capable of cleaving many cellular substrates, including ICAD (inhibitor of caspase-activated DNase), PARP (a DNA repair enzyme) and lamin. Once the ICAD is cleaved, CAD enters the nucleus and degrades chromatin into DNA fragments, disassembly of the cell structure that eventually leads to cell death [29, 30]. Results of the present study show that activation of caspase-3 and -9 but not caspase-8, was identified in Alternol-induced apoptosis. The activation of caspase-3 may also explain the morphological change of L1210 cells after Alternol treatment. The data reinforce the idea that Alternol-induced apoptosis occurs through mitochondria-dependent caspase activation.

Reports show that the cytotoxicity of some chemical agents is related to the production of ROS [31]. ROS can affect mitochondrial function through the opening of mitochondrial permeability transition pore (MTP), which has been considered as an early event in apoptosis [32]. It is also reported that oxidative stress by ROS exposure lead to a loss of $\Delta\Psi m$ and the positive feedback can drive the decrease of $\Delta\Psi m$ into an irreversible process [33]. In our study, ROS production of L1210 cells increased in 2 h Alternol treatment and a peak production were noted at 6 h. Afterwards, the production of ROS decreased gradually but maintained in a high level. In contrast, no obvious change of $\Delta\Psi m$ was monitored after Alternol treatment for 6 h [32]; the reduction of $\Delta\Psi m$ was obvious after Alternol treatment for 48 h. Though we did not confirm the role of ROS in cell viability, the increased ROS production by Alternol may lead to the reduction of $\Delta\Psi m$. This in turn increases mitochondrial membrane permeability and subsequently results in the release of mitochondrial apoptogenic factors into cytosol.

Paclitaxel is now considered a standard of care in the treatment of breast and ovarian cancer. Researchers continue to explore the potential benefits of paclitaxel in treating a variety of different tumors and studies indicate that paclitaxel can inhibit the growth and induce apoptosis in human and murine leukemia cells [34–36]. In this research, 200 nM Paclitaxel was chosen as positive control and results show that it can induce apoptosis in L1210 cells, which was consistent with the relevant reports. At the cellular level, paclitaxel induces tubulin polymerization and microtubule formation [37], blocks the cell cycle in G_2/M phase [38]. While in our study, different finding of G_1 arrest was detected after L1210 cells exposed to Alternol [data not shown]. In addition, although scientists have invented a semi-synthetic form of paclitaxel to increase the availability for patient care and reduce the environmental impact, obtaining the bulk substances for drug production is still difficulty. Alternol is obtained from the bark of Yew by microorganisms fermenting technology, which has an advantage over paclitaxel in availability and production in bulk without destroying the source of yew. Our study demands further investigations to establish the detailed mechanism of paclitaxel on L1210 cells and to explore how Alternol differs from paclitaxel.

In conclusion, we demonstrate for the first time that Alternol can inhibit the proliferation of L1210 cells and induce apoptosis. The apoptosis is thought to be mediated by mitochondria-dependent pathway through reducing the $\Delta\Psi m$, inhibiting the Bcl-2/Bax protein ratio, and activating the caspase-3 and -9, but not caspase-8. Moreover, the accumulation of ROS may contribute to the Alternol-induced cell death. Although the concrete receptor of Alternol is unclear and further research on apoptosis-promotion mechanism need to be done, these findings suggest that Alternol might be a promising candidate agent for the research and development in leukemia chemotherapy.

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