Anticancer Mechanism of Parthenolide Analogue PTLA-1 on Adriamycin Resistant Human Chronic Myeloid Leukemia Cell Line K562/ADR

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Abstract: *Objective: To investigate the anticancer effect and mechanism of parthenolide analogue (PTLA-1) on adriamycin resistant human chronic myeloid leukemia cell line K562/ADR cells. Methods K562/ADR cells were cultured in vitro. The inhibitory effects of PTLA-1 on cell proliferation were detected by MTT assay, and the effects of PTLA-1 on K562/ADR cell cycle, apoptosis, mitochondrial membrane potential (ΔΨm) and reactive oxygen species were detected by flow cytometry. The effects of PTLA-1 on K562/ADR cell cycle and apoptosis related proteins expressions were detected by Western blot. Results PTLA-1 had higher inhibitory activity on K562/ADR cells than parthenolide. It inhibited the expressions of Cyclin B1/D1 and CDK1/2/4/6, resulting in the arrest of K562/ADR cells in G0/G1 phase; PTLA-1 induced apoptosis of K562/ADR cells in mitochondrial pathway. PTLA-1 could induce mitochondrial membrane potential (ΔΨm) and down regulate the expressions of reactive oxygen species, Bcl-2 and p-Akt proteins, and up regulate the expressions of Bax, cyto c, caspase 9/3 proteins. The chromatin of K562/ADR cells contracted and apoptotic bodies appeared. Conclusion Parthenolide analogue PTLA-1 could inhibit cell proliferation by causing K562/ADR cells to block in G0/G1 phase. Secondly, it could induce K562/ADR cells to apoptosis through caspase dependent mitochondrial pathway, which was related to Akt signal pathway. It had certain anticancer therapeutic potential for chronic myeloid leukemia.*

Keywords: *Parthenolide analogue; Cell cycle; Apoptosis; Akt signaling pathway; Leukemia*

1. Introduction

Leukemia, or blood cancer, is a malignant tumor of the hematopoietic system. It seriously affects the normal hematopoietic function of the body, and also affects the balance of other non hematopoietic tissues and organs. Its incidence rate and mortality rate are among the top among young patients [1]. Nowadays, chemotherapy is one of the most important means to treat malignant tumors.

Cell cycle regulation is one of the main regulatory mechanisms of cell growth. Mitochondrial energy metabolism is strictly regulated in cell proliferation and tumor growth. In addition to the direct function of Cyclin B1/CDK1 complex in regulating the process of cell cycle, it can also coordinate the occurrence and development of cell cycle events with mitochondria [2]. The interaction between them may guide the biological function of mitochondria according to different stages of cell cycle progression and tumor growth state (such as tumor metastasis). Because different CDKs are required in the transformation of each cell cycle stage, under physiological and pathological conditions, the mechanism guiding the signal transmission between nucleus and mitochondria will help to understand the mitochondrial bioenergy of cancer cells under different stress conditions, so as to invent new metabolic targets to treat cancer.

Apoptosis is a form of programmed cell death used by the body to remove defective cells produced in the process of DNA damage or caused by other physical and chemical factors [3]. The disorder of apoptotic cell death mechanism is the sign of cancer. The change of apoptosis is not only related to the development and progress of tumor, but also related to the resistance of tumor to treatment. Among them, mitochondrial apoptosis pathway is an important apoptosis pathway [4]. After this pathway is activated, the expression protein of Pro apoptotic gene Bax competitively binds to the mitochondrial membrane, resulting in the increase of mitochondrial membrane permeability and the release of cytochrome c (cyto c). Cyto c forms a complex with apoptotic protease activator Apaf-1 in the cytoplasm, and cascade activates caspase 9 and caspase 3, so as to make the cell enter apoptosis [5, 6]. Akt pathway is also an important link in the process of apoptosis, and promotes cell proliferation. After Akt is phosphorylated,

it will lead to the inactivation of downstream pro apoptotic proteins such as Bax, bad and caspase 9, so as to reduce cancer cell apoptosis and promote cancer cell growth [7, 8]. Therefore, inhibiting Akt pathway to induce apoptosis has become another method for the treatment of cancer.

Sesquiterpene lactones [9] have been widely used in the treatment of high fever, headache, irregular menstruation and other inflammatory diseases. Parthenolide (PTL) is an effective chemical component of *chryanthemum parthenium* [10]. It has α-Methylene-γ-Butyrolactone and epoxy groups, whose nucleophilic properties enable them to interact with some enzymes and proteins and affect many important biological reactions in cells, such as information transmission, cell proliferation and apoptosis [11]. In recent years, in vitro studies have reported that PTL can significantly inhibit the growth and proliferation of a variety of tumor cells [12, 13], improve the sensitivity of tumor cells to anticancer drugs, and show a wide range of drug-resistant tumor activity [14]. However, due to its low water solubility, stability and bioavailability, PTL has limitations in clinical application. Our research group found a parthenolide analog (PTLA-1) could inhibit the proliferation of human leukemia adriamycin resistant cell K562/ADR and this study studied its anticancer mechanism.

2. Materials and methods

2.1. Drugs

The structural formulas of parthenolide (PTL) and its analogue (PTLA-1) are shown in Fig. 1A and Fig. 1B respectively. PTL was purchased from Energy Chemical (China), and PTLA-1 was provided by Dr. Hu Kun, Changzhou University, Jiangsu, China, Purity > 98% (HPLC).
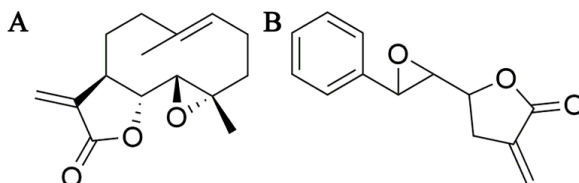

Figure 1: Chemical structure of parthenolide (PTL, A) and its analogue (PTLA-1, B).

2.2. Reagents

RPMI-1640 and fetal bovine serum were purchased from Gibco (USA); MTT and Hoechst 33258 were purchased from sigma Aldrich (USA); 4',6-diamidino-2-phenylindole (DAPI) was purchased from Beckman Coulter (USA); Annexin V-FITC apoptosis detection kit was purchased from KeyGEN Bio TECH (China); ROS detection kit and Rhodamine 123 Staining Kit were purchased from Beyotime (China); All antibodies were purchased from Abcam (UK).

3. Methods

3.1. Cell viability assay (MTT assay)

K562/ADR cells were treated with 5×10^4 cells/mL and inoculated into 96 well plates and cultured in cell incubator overnight. After the cells adhered to the wall, the experimental group was treated with different concentrations of PTLA-1 and PTL (1, 5, 10, 25, 50 μM) acted on K562/ADR cells for 24, 48 and 72 h. MTT assayW was used to detect the survival rate of cells. The absorbance OD value at 570-630nm was detected by multifunctional microplate detector. The ratio of the average OD value of the experimental hole to the average OD value of the blank control hole was the cell survival rate under the influence of PTLA-1 or PTL on K562/ADR cells.

3.2. Cell cycle analysis

K562/ADR cells were seeded into 6-well plates at a density of 1×10^5 cells/mL and cultured in cell incubator overnight. After the cells adhered to the wall, the experimental groups were respectively added PTLA-1 with final concentrations of 1, 2 and 4 μM for 48 h. Collect the cells, absorb and discard the supernatant after centrifugation, and add DAPI solution (so that the cell density was 2×10^4 cells /mL), which were kept away from light at room temperature for 15 min, and then detected by flow cytometry.

3.3. Annexin V-FITC/PI double staining and detect apoptosis

The same as 3.2 cell culture and administration method. Collect the cells, absorb and discard the supernatant after centrifugation, and add 200 μL Bing buffer and 5 μL annexin V-FITC/PI, and then detected by flow cytometry after being kept away from light at room temperature for 15 min.

3.4. Mitochondrial membrane potential (ΔΨm) analysis

The same as 3.2 cell culture and administration method. Collect the cells, absorb and discard the supernatant after centrifugation, and add 1 mL rhodamine 123 (5 μg/mL), and then detected by flow cytometry after being kept away from light at 37 ℃ for 30 min.

3.5. Reactive oxygen species (ROS) detection

The same as 3.2 cell culture and administration method. Collect the cells, absorb and discard the supernatant after centrifugation, and add 1 ml of DCFH-DA (10 μM), avoid light at 37 ℃ for 30 min, gently beat it with serum-free medium and then detect it by flow cytometry.

3.6. Western blot

The same as 3.2 cell culture and administration method. Add 80 μL/well RIPA lysate on ice for 30 min, and centrifuged (4 ℃, 15000 rpm, 15 min). The supernatant (test proteins) was quantified by BCA quantitative kit. Proteins (40 μg/lane) of different molecular weights were separated by a 6%-15% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidenefluoride (PVDF) membrane. The blotted PVDF membrane was incubated with a horseradish peroxidase (HRP) conjugated secondary antibody after reaction with the primary antibody (CDK1/2/4/6, Cyclin D1, Cyclin B1, caspase 3, caspase 9, PARP1, Bcl-2, Bax, cyto c, Akt and p-Akt) overnight at 4 ℃. Band intensity was measured using chemiluminescence western blot detection system.

3.7. Hoechst 33258 staining and nuclear morphology

K562/ADR cells were seeded into 24-well plates with pre placed cell climbing tablets at a density of 5×10^4 cells/mL and cultured in cell incubator overnight. After the cells adhered to the wall, the experimental groups were respectively added PTLA-1 with final concentrations of 1, 2 and 4 μM for 48 h. After fixing, add 500 μL Hoechst 33258 solution (0.5 μg/mL) dark staining for 30 min. The slices were made with glycerol and then observed by fluorescence inverted microscope.

3.8. Statistical analysis

The experimental results were analyzed by prism 5 software, and the data of each group were expressed as means $\pm$ SD (n = 3). Statistical significance was assessed by using one-way ANOVA. $^*P < 0.05$ was considered statistically significant.

4. Results

4.1. Effect of PTLA-1 on the viability of K562/ADR cells

MTT assay was used to evaluate the cytotoxicity of PTLA-1 on K562/ADR cells. As shown from Fig. 2, compared with the blank control group, PTLA-1 and PTL could significantly inhibit the cell viability of K562/ADR cells, and the higher the drug concentration, the longer the treatment time, and the stronger the inhibition of cell proliferation. When the cells were treated for 72 h, IC_{50} of PTLA-1 and PTL on K562/ADR cells were 9.59 μM and 10.77 μM, respectively. It could be seen that the inhibitory effects of PTLA-1 and PTL on the proliferation of K562/ADR cells were concentration and time-dependent, and the inhibitory effect of PTLA-1 on the proliferation of K562/ADR cells was stronger than that of PTL.
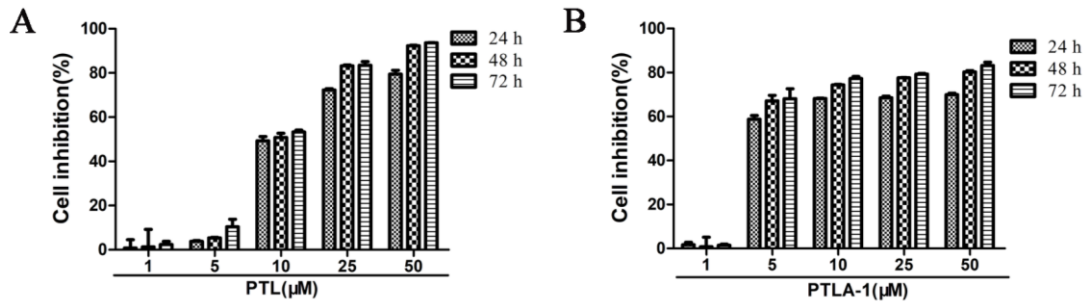
Figure 2: Effects of PTLA-1 and PTL on the proliferation of K562/ADR cells.

MTT assay was used to detect the cell viability after treatment with different concentrations of PTL or PTLA-1 for 24, 48 and 72 h, respectively. Data shown are the mean from three parallel experiments.

4.2. Effect of PTLA-1 on K562/ADR cell cycle

In order to explore whether the proliferation inhibition of PTLA-1 on K562/ADR cells was related to cell cycle arrest, this study could be further analyzed from the results of flow cytometry (Fig. 3A and 3B). When the concentration of PTLA-1 was 1 and 2 μM, K562/ADR cells were blocked in G0/G1 phase; when the concentration of PTLA-1 was 4 μM, the number of subG1 cells increased, indicating a large number of apoptosis or cell necrosis. Western blot results (Fig. 3C and 3D) further showed that after 48 h of treatment with different concentrations (1, 2, 4 μM) of PTLA-1, the expressions of CDK1/2/4/6, Cyclin B1 and Cyclin D1 in K562/ADR cells decreased in a dose-dependent manner. It showed that PTLA-1 blocked the cell cycle by inhibiting Cyclin/CDK proteins, which was consistent with the results of flow cytometry.
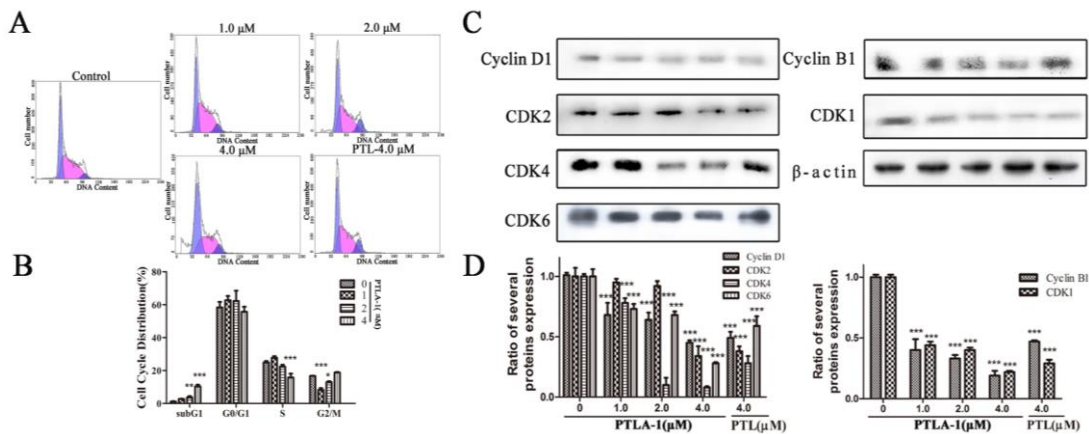

Figure 3: Effect of PTLA-1 on K562/ADR cell cycle

(A) Cycle changes of K562/ADR cells treated with PTLA-1; (B) The proportion (%) of the number of cells in each period of K562/ADR cell cycle (subG1 phase, G0/G1 phase, S phase and G2/M phase) in the total number of detected cells; (C) After K562/ADR cells were treated with PTLA-1 for 48 h, the expressions of CDK1, CDK2, CDK4, CDK6, Cyclin B1 and Cyclin D1 proteins were detected by Western blot, β-actin as internal parameter; (D) The proportion of change in the expressions of K562/ADR cell cycle associated proteins by PTLA-1 (%). Compared with the blank control group, $^{***}p<0.001$.

4.3. Effect of PTLA-1 on apoptosis of K562/ADR cells

4.3.1. Effects of PTLA-1 on apoptosis and nuclear morphology of K562/ADR cells

In order to detect the effect of PTLA-1 on K562/ADR cell apoptosis, after Annexin V-FITC/PI double staining, the results of flow cytometry (Fig. 4A and 4B) showed that PTLA-1 increased the proportion of apoptotic cells in a dose-dependent manner. When the concentrations of PTLA-1 and PTL were both 4 μM, the proportion of apoptotic cells in PTLA-1 group was 61.89%, while the proportion of apoptotic cells in PTL group was only 9.52%. Therefore, compared with PTL, PTLA-1 had a stronger ability to induce apoptosis of K562/ADR cells. When apoptosis occurs, the chromatin in the nucleus shrinks. After Hoechst 33258 stained the nucleus, the fluorescence inverted microscope image (Fig. 4C) could be

observed that the K562/ADR nuclei of the blank control group were evenly stained and the edge contours were clear. However, after 48 h of PTLA-1 treatment, the nuclear edge contours of K562/ADR became rough, the chromatin gradually shrunk, and apoptotic bodies appeared. These results suggested that PTLA-1 could induce apoptosis of K562/ADR cells.
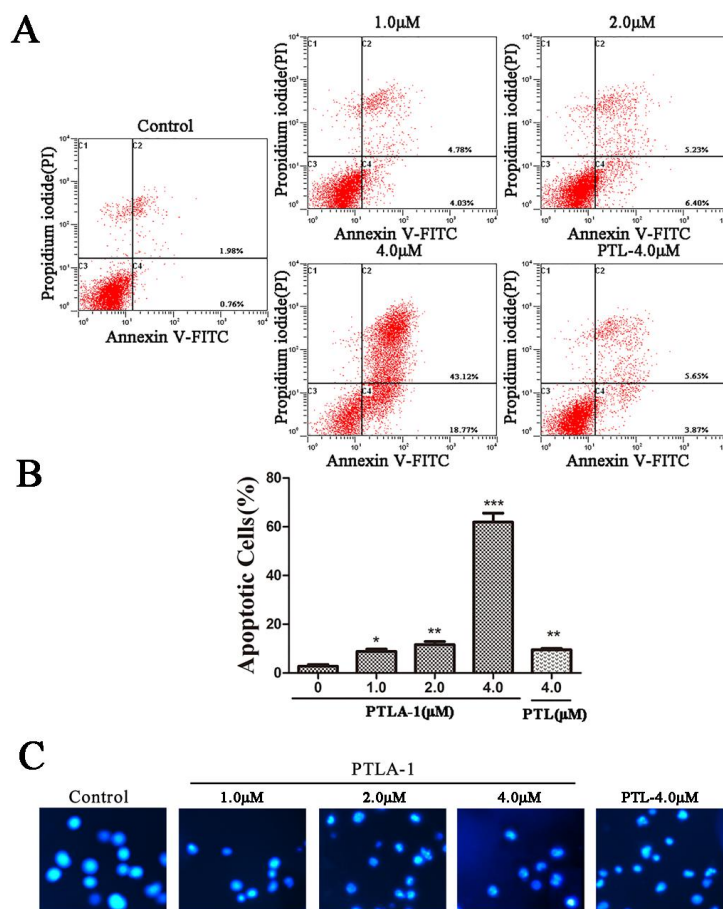

Figure 4: Effect of PTLA-1 on apoptosis and nuclear morphology of K562/ADR cells detected (Hoechst 33258 fluorescence, × 200)

(A) Distribution of apoptosis in K562/ADR cells treated with PTLA-1 for 48 h; (B) Apoptosis rate of K562/ADR cells treated with PTLA-1 for 48 h; (C) After PTLA-1 treated K562/ADR cells for 48 h, the changes of cell nuclear morphology were observed under fluorescence inverted microscope. Compared with the blank control group, *p<0.05, **p<0.01, ***p<0.001.

4.3.2. Effect of PTLA-1 on mitochondrial apoptosis in K562/ADR cells

In order to study whether PTLA-1 induced apoptosis of K562/ADR cells through mitochondrial pathway, mitochondrial membrane potential ($\Delta\Psi m$) was detected by flow cytometry after Rhodamine 123 staining. The results are shown in Fig. 5A and 5B, K562/ADR cells treated with different concentrations of PTLA-1 (1, 2 and 4 μM) for 48 h, mitochondrial membrane potential ($\Delta\Psi m$) decreased significantly. In order to further verify the conjecture, Western blot was used to detect the expression of mitochondrial apoptosis pathway related proteins in K562/ADR cells after 48 h of drug action. Western blot results (Fig. 5C and 5D) showed that after treated with different concentrations of PTLA-1 for 48 h, the expression gap between pro apoptotic protein Bax and anti apoptotic protein Bcl-2 in K562/ADR cells gradually widened with the increase of administration concentration. Meanwhile, as shown in Fig. 5E, the expressions of mitochondrial pathway related proteins cyto c, caspase 9, caspase 3 and their cleavage product PARP1 developed towards apoptosis. These results suggested that PTLA-1 might induce apoptosis of K562/ADR cells through mitochondrial pathway.
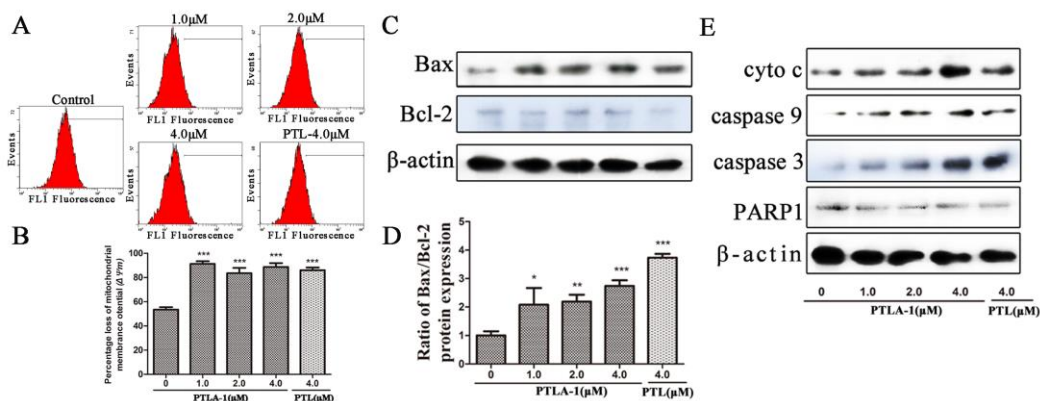
Figure 5: Effect of PTLA-1 on mitochondrial apoptosis in K562/ADR cells

(A) Changes in mitochondrial membrane potential (ΔΨm) of K562/ADR cells treated with PTLA-1 for 48 h by flow cytometry; (B) PTLA-1 induced the decrease of mitochondrial membrane potential (ΔΨm) in K562/ADR cells (%); (C) Expressions of Bax and Bcl-2 protein in K562/ADR cells treated with PTLA-1 for 48 h by Western blot, β-actin as internal parameter; (D) The ratio of optical density of Bax and Bcl-2 protein bands after PTLA-1 acted on cells for 48 h; (E) After treatment with PTLA-1 for 48 h, the expressions of cyto c, caspase 9, caspase 3 and PARP1 proteins in K562/ADR cells by Western blot, β-actin as internal parameter. Compared with the blank control group, *p<0.05, **p<0.01, ***p<0.001.

4.3.3. Effects of PTLA-1 on reactive oxygen species (ROS) and Akt pathway in K562/ADR cells

ROS plays an important role in the induction of DNA damage and its interference in the development of tumor cells. According to the results of flow cytometry (Fig. 6A), the fluorescence intensity of DCFH-DA decreased after 48 h of treatment with different concentrations of drugs, indicating that ROS in K562/ADR cells decreased, indicating that PTLA-1 and PTL had antioxidant ability. Both of them might affect the intracellular redox balance to affect the apoptosis of cells. Akt signaling pathway regulates a variety of biological processes in cells and plays an important role in regulating apoptosis. In order to explore whether the upstream signal of the anticancer mechanism of PTLA-1 came from Akt, the effect of PTLA-1 on the phosphorylation level of Akt in K562/ADR cells was detected by Western blot. The results of Western blot (Fig. 6B and 6C) showed that the proportion of p-Akt/Akt decreased in K562/ADR cells treated with PTLA-1 or PTL, and showed a certain concentration dependence. These results suggested that PTLA-1 might act on Akt signaling pathway, regulate and induce apoptosis of K562/ADR cells together with mitochondrial apoptosis pathway.
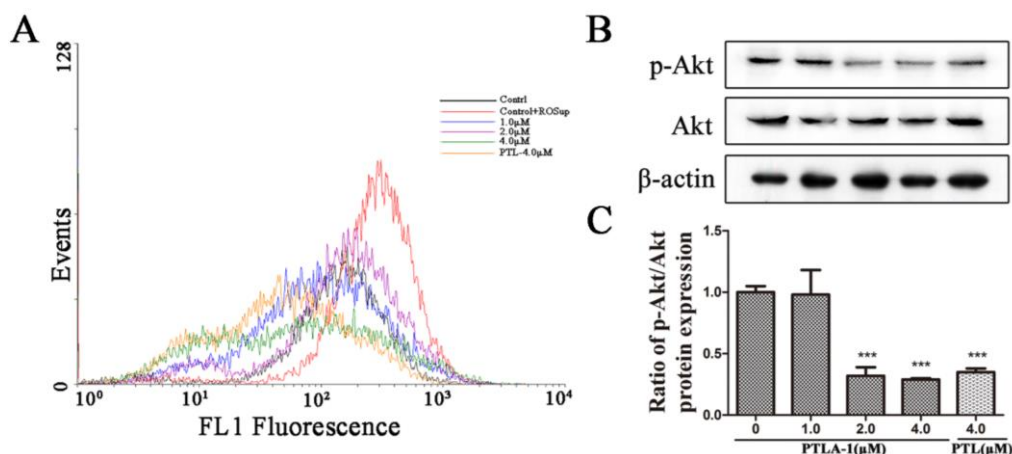

Figure 6: Effect of PTLA-1 on reactive oxygen species and Akt signaling pathway related protein expressions of K562/ADR cells

(A) After K562/ADR cells were treated with PTLA-1 for 48 h, the level of intracellular reactive oxygen species was detected by flow cytometry with fluorescent probe DCFH-DA; (B) The expressions of p-Akt and Akt protein in K562/ADR cells treated with PTLA-1 for 48 h, β-actin as internal parameter; (C) The ratio of p-Akt and Akt protein bands optical density after PTLA-1 acted on cells for 48 h. Compared with the blank control group, ***p<0.001.

5. Discussion

Leukemia is caused by the carcinogenic transformation of leukocytes in the process of hematopoiesis. Hematological malignancies quickly produce abnormal leukocytes. By occupying space, they eventually damage the bone marrow, which will reduce the production of functional cells such as erythrocytes, platelets and normal leukocytes. Leukemia has different types, affected populations, prognosis and treatment options, but a common theme of leukemia is the metabolic imbalance of leukemia cells and leukemia stem cells relative to non cancer cells [15]. Therefore, an accurate and thorough understanding of its unique metabolic state can provide a more selective, robust and persistent target for anti leukemia treatment.

Sesquiterpene lactones are common in natural plants and have multiple biological activities [16, 17]. Parthenolide (PTL), as a Sesquiterpene lactones, is a kind of bioactive compound derived from *chryanthemum parthenium* and has α-Methylene-γ-butyrolactone structure. It has been used in western countries to treat diseases such as fever, rheumatoid arthritis and skin infection [18, 19]. In 1973, Wiedhopf research group first reported that parthenolide had anticancer and anti-inflammatory activities [19]. In 1997, Bork research group reported that parthenolide could inhibit the activity of NF-κB [20], and was patented as an anticancer drug in 2005 [21]. In recent years, parthenolide showed a variety of pro apoptotic and anticancer characteristics. Many studies in vitro reported that parthenolide could inhibit the growth and proliferation of various tumor cells (such as breast cancer, bladder cancer, prostate cancer, melanoma and neuroma) [22-25] through different mechanisms of action, which proved that parthenolide had a wide range of anticancer activities. Our research group found a parthenolide analogue PTLA-1, which could significantly inhibit the growth and proliferation of human leukemia adriamycin resistant K562/ADR cells with the better effect than parthenolide. Therefore, this study intended to further explore the anticancer effect and mechanism of PTLA-1 from two aspects of cell cycle and apoptosis through Akt signaling pathway.

Many compounds with anticancer activity inhibit the growth and proliferation of cells by cell cycle arrest or apoptosis induction [26]. Firstly, through DAPI staining and flow cytometry, we observed that PTLA-1 effectively induced K562/ADR cells to block the G0/G1 cell cycle, and even caused a large number of apoptosis or necrosis. At the same time, Western blot detected that the expressions of cycle associated proteins CDK1/2/4/6, Cyclin B1 and Cyclin D1 decreased in K562/ADR cells treated with PTLA-1. In view of its superior therapeutic effect and structural advantages over parthenolide, it suggested that PTLA-1 might become a potential anticancer drug.

Reactive oxygen species (ROS) are mainly produced in mitochondria and are closely related to the development, growth, proliferation and differentiation of multicellular organisms. At present, it is believed that the excessive increase of ROS level will lead to the destruction of DNA genome, and this imbalance will eventually lead to genetic instability, which is also a great possibility to promote the development of tumor [27]. Experiments have shown that parthenolide can regulate the expression level of ROS in prostate cancer cells by affecting NADPH oxidase, cause the cascade reaction of Akt pathway and FOXO3a, increase mitochondrial membrane permeability, release smac/DIABLO and cyto c, and cascade activate two apoptosis families of Bcl-2 and caspase, and finally make the cells apoptosis [28, 29]. In this study, it was found that PTLA-1 could significantly inhibit the production of ROS, which was helpful to control the occurrence and development of tumors. With the down regulation of Akt phosphorylation level, the mitochondrial membrane potential ($\Delta\Psi m$) in K562/ADR cells decreased, the expressions of Bax/Bcl-2 proteins developed towards promoting apoptosis, and the apoptosis family related proteins cyto c and caspase 9/3 were involved. Especially after Hoechst 33258 staining, the shrinkage of chromatin and the appearance of apoptotic bodies in K562/ADR cells caused by PTLA-1 treatment could be observed. These confirmed that PTLA-1 could induce apoptosis of K562/ADR cells through Akt signaling pathway and caspase dependent mitochondrial apoptosis pathway, with the better effect than parthenolide.

In conclusion, this study preliminarily discussed the anticancer activity and mechanism of parthenolide analogue PTLA-1 on adriamycin resistant human chronic myeloid leukemia cell line K562/ADR cells. The results showed that PTLA-1 might play a certain anticancer effect by controlling the cell cycle development, down regulating Akt signal pathway and up regulating caspase dependent mitochondrial apoptosis pathway, and showed higher activity than parthenolide. It provided a preliminary experimental basis for the possibility of developing parthenolide analogues into new anticancer drugs.

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