

Novel anti-leukemia activities of pipoxolan operate via the mitochondria-related pathway in human leukemia U937 cells and attenuate U937 cell growth in an animal model

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Abstract. Pipoxolan HCl (5,5-diphenyl-2-(2'-piperidino-ethyl)-1,3-dioxolane-4-one hydrochloride) is a compound containing a dioxolan moiety that was reported to induce apoptosis in cancer cells. In this study, we investigated the anti-leukemia effects of pipoxolan on U937 leukemia cells both *in vivo* and *in vitro*. Cell viability, reactive oxygen species (ROS) production, mitochondrial membrane potential, apoptosis and caspases-9 and -3 activity were examined following treatment of U937 leukemia cells with 10 μ M pipoxolan by flow cytometry and caspase-activity assay. The apoptosis-associated Bcl-2 family proteins, Bax, Bcl-2 and Bcl-xL, were examined by Western blotting. We found that pipoxolan inhibited U937 cell proliferation in a dose- and time-dependent manner. Morphological assessment and cell cycle analysis indicated that pipoxolan induced the apoptosis of the U937 cells. Pipoxolan (10 μ M) increased ROS production and decreased mitochondrial membrane potential 1 h after pipoxolan treatment. Pre-treatment of pipoxolan-treated cells with N-acetyl-L-cysteine (a ROS chelator) inhibited the increase in ROS production. After treatment with 10 μ M pipoxolan for 24 h, there was an increase in pro-apoptotic Bax and a decrease in anti-apoptotic Bcl-2 and Bcl-xL proteins. *In vivo*, we found that pipoxolan significantly suppressed tumor growth in BALB/c^{nu-/nu-} mice inoculated with U937 cells. Taken together, the data from our studies indicate that pipoxolan possesses potent anti-leukemia activity and is a potential novel alternative cancer therapeutic agent for human leukemia.

Introduction

Leukemia is a heterogeneous group of diseases characterized by uncontrolled proliferation of abnormal blood cells of the hematopoietic system (1). As conventional chemotherapy does not seem to be very effective in the treatment of leukemia, there has been an effort to find new therapeutic strategies that possess apoptotic potential (2). Apoptosis is a suicide program of cell death that is essential for the development and maintenance of tissue homeostasis (3). Inhibition of apoptosis has been observed in some forms of cancer (4), and the induction of apoptosis in tumor cells is the most common anti-cancer mechanism in cancer therapy (3).

Apoptosis is characterized by a series of morphological changes involving cell shrinkage and chromatin condensation (5). Two apoptotic pathways are involved in cell death (6,7). The intrinsic pathway involves disrupting the mitochondrial membrane and releasing cytochrome c, apaf-1 and pro-caspase-9 into the cytosol (8). Bcl-2 family proteins regulate mitochondrial membrane permeability through either pro-apoptotic proteins, such as Bax, or anti-apoptotic proteins, such as Bcl-2 and Bcl-xL (9). Regulation of the relative levels of Bcl-2 and Bax may play an important role in modulating the susceptibility of cells to apoptosis (10,11). High Bcl-2/Bax ratios favor cell survival, while low ratios promote apoptosis (12). The extrinsic pathway of apoptosis requires death receptors, such as Fas/FasL, to activate downstream caspase-8 (13). In the final stage of apoptosis, both the intrinsic and extrinsic pathways induce the activation of caspase-3, the executioner of the caspase cascade, which results in cell death (6,7).

Pipoxolan HCl (5,5-diphenyl-2-(2'-piperidino-ethyl)-1,3-dioxolane-4-one hydrochloride), a 1,3-dioxolan derivate also known as rowapraxin, was synthesized by Pailer *et al* in 1968 (14) (Fig. 1). Clinically, pipoxolan is used as an anti-spasmodic drug for relief of smooth muscle spasm in the digestive tract, bronchial tree, urinary tract and gynecological system (15,16). However, there are very few reports to date describing anti-cancer actions of pipoxolan or the mechanisms of those actions. In this study, we examined the growth-inhibitory effects and apoptotic mechanisms of pipoxolan in human U937 leukemia cells both *in vitro* and *in vivo*, in order to investigate

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its anti-leukemia activities and determine its potential as a novel cancer therapeutic agent.

Materials and methods

Chemicals and reagents. Propidium iodide (PI), RNase A, DCFH-DA, DiOC6, proteinase K and N-Acetyl-L-Cysteine (Sigma, MO, USA) were dissolved in double-distilled water. Pipoxolan was a gift from The Wide Pharmaceutical Co., Ltd. (Taichung, Taiwan) Pipoxolan was dissolved in dimethylsulfoxide (DMSO) (Sigma) and diluted in tissue culture medium before use.

Cell culture. Human U937 leukemia cells were obtained from the Culture Collection and Research Center (CCRC, Taiwan, R.O.C.), originally from the American Type Culture Collection (ATCC, USA). Cells were cultured in RPMI-1640 cell culture medium (Gibco BRL, Life Technologies, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, UT, USA), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml) (Gibco BRL, Life Technologies) and incubated at 37°C in a humidified 5% CO₂ atmosphere.

Propidium iodide exclusion assay and flow cytometry for cell viability and cell cycle analysis. For cell viability and cell cycle analysis, 2.5x10⁵ cells/ml were seeded in a 24-well culture plate (Falcon, CA, USA). Different concentration (5, 10 and 20 μM) of pipoxolan was added to each well and the plates were incubated at 37°C for 24, 48 and 72 h. Cells were harvested, fixed in 70% ethanol at 4°C overnight, then washed with PBS. After centrifugation, the cells were incubated in 0.5 ml phosphate-citric acid buffer (0.2 M NaHPO₄, 0.1 M citric acid, pH 7.8) at room temperature for 30 min. The cells were then centrifuged and re-suspended in 1 ml of PI solution (40 μg/ml). The DNA content was analyzed using flow cytometry and Cell Quest software (FACS Calibur™; Becton Dickinson, NJ, USA).

DAPI staining. The U937 cells were incubated with 10 μM pipoxolan for 24 h or in DMSO (solvent) alone as the control, and were then stained with DAPI and photographed under a fluorescence microscope as described previously (17).

Cells were harvested then washed with PBS and fixed with 10% formalin at room temperature. Fixed cells were washed with PBS and stained with DAPI. The percentage of cells showing nuclear condensation and fragmentation, which are characteristics of apoptosis, was determined using fluorescence microscopy.

Detection of reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi_m$). Cells were harvested then washed twice and re-suspended in 10 μM of the ROS dye DCFH-DA, or re-suspended in 4 mol/l of the mitochondrial membrane potential detection dye DiOC6. Subsequently, the cells were incubated at 37°C for 30 min and analyzed by flow cytometry (FACS Calibur™; Becton Dickinson).

Caspase-3 and caspase-9 activity assay. Caspase-3 and -9 activity was assessed using a caspase colorimetric assay kit

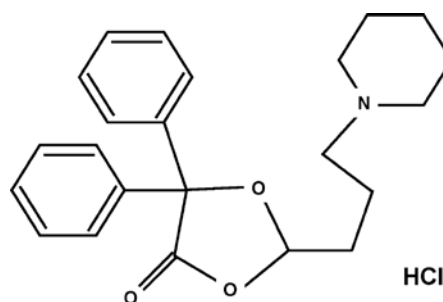


Figure 1. The structure of pipoxolan (5,5-diphenyl-2-(2'-piperidino-ethyl)-1,3-dioxolan-4-one) hydrochloride.

according to the manufacturer's instructions (R&D Systems Inc., MN, USA). Cells were harvested and measured with an ELISA reader (Dynex Tech. Triad LT, MA, USA) at a wavelength of 405 nm.

Western blot analysis. Total protein was prepared with RIPA protein lysis buffer. The concentration of protein was determined by the Bradford method using Bio-Rad protein assay dye reagent (Amresco, OH, USA). The cell lysates containing 20 μg of protein were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, MA, USA). Non-specific binding sites were blocked with 5% non-fat milk in PBST buffer. The PVDF membranes were incubated overnight at 4°C with specific primary antibodies for Bcl-2, Bax, Bcl-xL and GAPDH (Santa Cruz Biotechnology, CA, USA). The membranes were then washed with PBST buffer and incubated with horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology). Immunoreactive proteins were detected using a Western Blotting Chemiluminescence Reagent Plus kit (Millipore) and exposed to X-ray film (Kodak BioMax light film; Sigma-Aldrich, MO, USA).

Tumor xenografts implantation. The protocol for measuring anti-tumor activity using tumor xenografts was performed as previously described (18). Male BALB/c^{nu-/nu-} mice (20 g, 4 weeks of age) were obtained from the National Laboratory Animal Center and allowed to acclimate to laboratory conditions for 1 week before tumor implantation. BALB/c^{nu-/nu-} mice were maintained in accordance with the institutional Animal Care and Use Committee procedures and guidelines. U937 cells (1x10⁷) were injected subcutaneously into the flank of each animal. When a tumor reached an approximate volume of 80 mm³, mice bearing tumors with acceptable morphology and of similar size range were selected and distributed for drug studies. U937 tumors were measured every 3 days using a caliper, and the body weight of the mice was monitored to detect toxicity. Tumor volumes were determined by measuring the length (l) and the width (w) of the tumor, and the volume was calculated as (V = lw²/2) (18). The mice were sacrificed when the tumor burden was >1,000 mm³.

Statistical analysis. Results are presented as the mean ± SD. Differences between treatment groups, which consisted of matched samples, were assessed by the Student's t-test. A confidence level of 1% (*P<0.01) was considered to be significant.

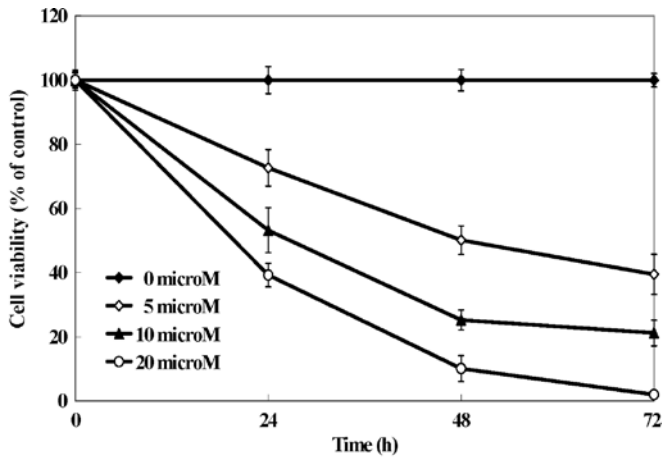


Figure 2. Percentage of viable U937 leukemia cells after 10 μ M pipoxolan treatment. After harvesting, the number of viable cells was determined by PI exclusion and flow cytometry. Each point is the mean \pm SD of 3 experiments.

Results

Cytotoxic effects of pipoxolan on U937 leukemia cells. To study the cytotoxic effects of pipoxolan on U937 leukemia cells, we determined cell viability using the PI-exclusion assay. Pipoxolan inhibited U937 cell proliferation in a dose- and time-dependent manner (Fig. 2).

Effects of pipoxolan on the cell cycle of U937 leukemia cells. After U937 cells were treated with 10 μ M pipoxolan for 24 h, the cell cycle was examined and analyzed by flow cytometry. Following pipoxolan treatment, there was a significant number of U937 cells in the sub-G1 apoptotic phase (Fig. 3A). DAPI staining of the pipoxolan-treated cells revealed cell shrinkage and chromatin condensation and fragmentation characteristic of apoptosis (Fig. 3B).

Effects of Pipoxolan on mitochondrial membrane potential ($\Delta\Psi_m$) and ROS production in U937 cells. U937 cells treated with 10 μ M pipoxolan for various time periods were analyzed for mitochondrial membrane potential and ROS production by flow cytometry. There was a decrease in the mitochondrial membrane potential in the treated U937 cells over time (Fig. 4A). Pipoxolan induced ROS production in a time-dependent manner (Fig. 4B). With pre-treatment of the pipoxolan-treated U937 cells with N-acetyl-L-cysteine, a ROS chelator, the induction of ROS production was reversed (Fig. 4B).

Effects of Pipoxolan on caspase-9 and caspase-3 activity in U937 leukemia cells. Treatment with 10 μ M pipoxolan caused the activation of caspases-9 and -3 in a time- and dose-dependent manner (Fig. 5A and B); however, there was no detected activation of caspase-8 (data not shown). In order to demonstrate that activation of caspases-9 or -3 is essential for pipoxolan-induced apoptosis, caspase-9 and -3 inhibitors were used to block pipoxolan-induced apoptosis. U937 cells were pre-treated with Z-LEHD-FMK (a caspase-9 inhibitor) (Fig. 5A) or Z-DEVD-FMK (a caspase-3 inhibitor) (Fig. 5B) for 1 h and then exposed to 10 μ M pipoxolan for various time

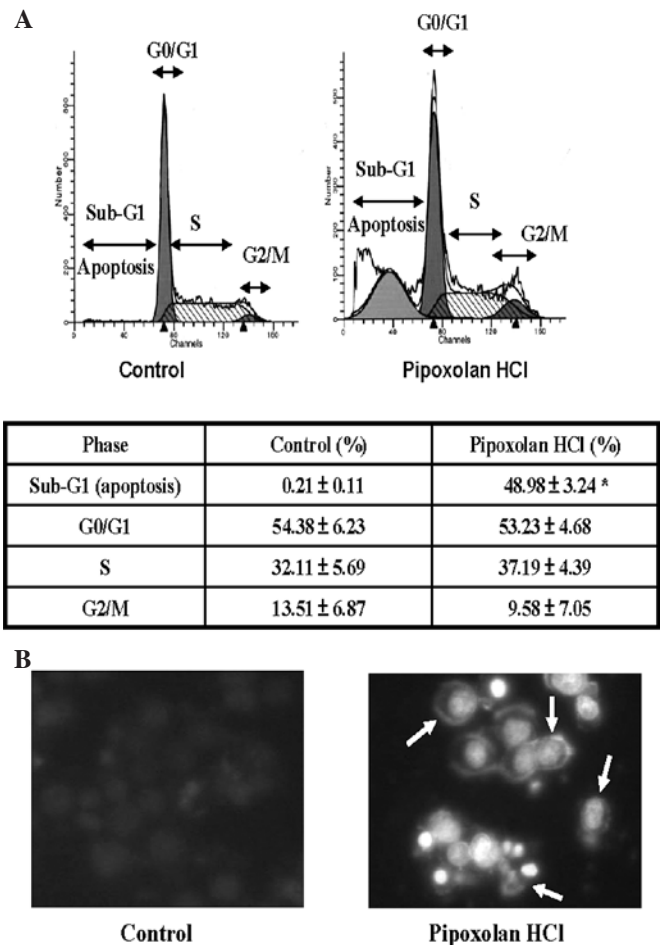


Figure 3. (A) Flow cytometric analysis of the cell cycle after pipoxolan treatment of U937 leukemia cells. (B) DAPI staining of U937 leukemia cells after pipoxolan treatment. Data represent the mean \pm SD of three experiments. * P <0.01, differences between pipoxolan and control.

periods. As shown in Fig. 5A and B, the inhibitors blocked pipoxolan-induced caspase-9 and -3 activity, respectively.

Effects of Pipoxolan on Bcl-2, Bcl-xL and Bax protein expression in U937 leukemia cells. The expression of Bax, Bcl-2 and Bcl-xL in the treated U937 leukemia cells was analyzed by Western blotting. Pipoxolan down-regulated Bcl-2 and Bcl-xL and up-regulated Bax protein expression in a time-dependent manner (Fig. 6).

Effects of pipoxolan on U937 tumor xenografts. On the basis of the significant growth-inhibitory effect of pipoxolan *in vitro*, we investigated whether pipoxolan possessed anti-cancer cell growth activity *in vivo*. Pipoxolan (10 and 30 mg/kg) inhibited tumor growth over a period of 20-45 days (Fig. 7A) and inhibited tumor weight (Fig. 7B).

Discussion

Pipoxolan is a spasmolytic agent used in alleviating smooth muscle spasms (19). In animal experiments, pipoxolan inhibited the acetylcholine- or histamine-induced contraction of isolated guinea-pig jejunum, rabbit ileum and guinea-pig tracheal smooth muscle (19), but did not affect the pulse rate

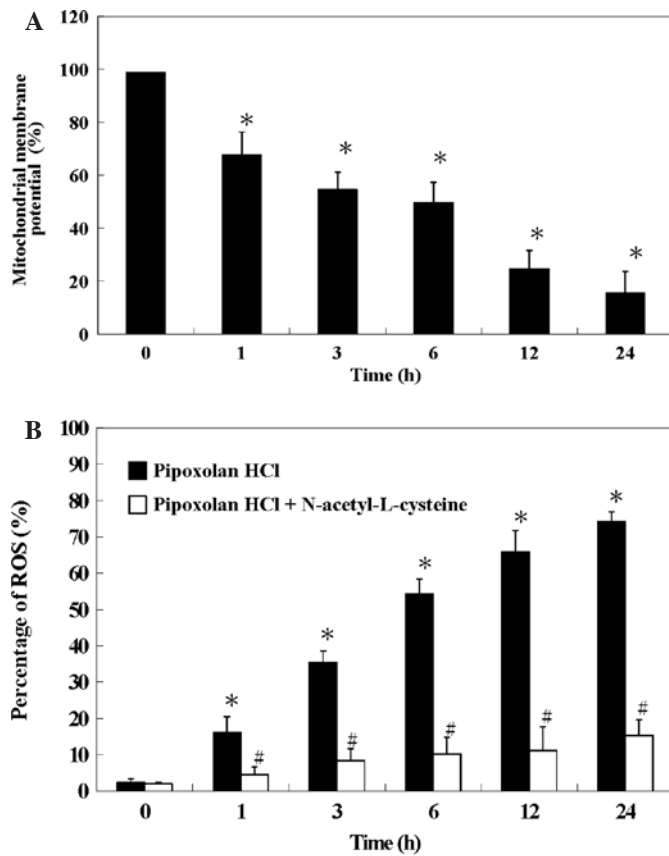


Figure 4. Flow cytometric analysis of mitochondrial membrane potential ($\Delta\Psi_m$) (A) and reactive oxygen species (ROS) (B) in U937 leukemia cells incubated with $10\ \mu\text{M}$ pipoxolan for various time periods. The mitochondrial membrane potential ($\Delta\Psi_m$) and ROS were respectively determined by flow cytometry using DiOC6 (40 nM), a mitochondrial membrane potential dye or DCFH-DA ($5\ \mu\text{M}$), a ROS dye. U937 cells were also pre-treated with N-acetyl-L-cysteine ($100\ \mu\text{M}$), a ROS chelator, for 1 h, then treated with pipoxolan ($10\ \mu\text{M}$) for the indicated times. The zero concentration was defined as the control. * $P < 0.01$, pipoxolan compared to control.

or breathing (20). In humans, pipoxolan is reported to alleviate dysmenorrhea, renal colic, bilateral urinary lithiasis, cholelithiasis, chronic gastritis, post-natal uterine pain, urolithiasis, hydronephrosis and migraines (15,16,21). However, it remains unclear whether pipoxolan effectively induces the elimination of pre-malignant cells *in vivo*.

It is well known that mitochondria play a key role in the regulation of apoptosis (22). Mitochondrial dysfunction, including the loss of mitochondrial membrane potential, increases the generation of ROS, permeability transition and release of cytochrome c into the cytosol, and is associated with apoptosis (23,24). Thus, we hypothesized that a mitochondrial-dependent pathway is involved in the pipoxolan-induced apoptosis observed in U937 leukemia cells.

Caspases are well known to play important roles in apoptosis. According to the substrate specificity, caspases are grouped into 'apoptotic initiators', such as caspases-8 and -9, and 'apoptotic effectors', such as caspase-3 (25-27). Our data demonstrated that pipoxolan-induced apoptosis was observed at 24 h. Pipoxolan promoted the activation of both caspase-9, an apoptotic initiator, and caspase-3, an apoptotic effector, in the U937 cells in our study, suggesting that pipoxolan-induced apoptosis involves a caspase-dependent mitochondria pathway.

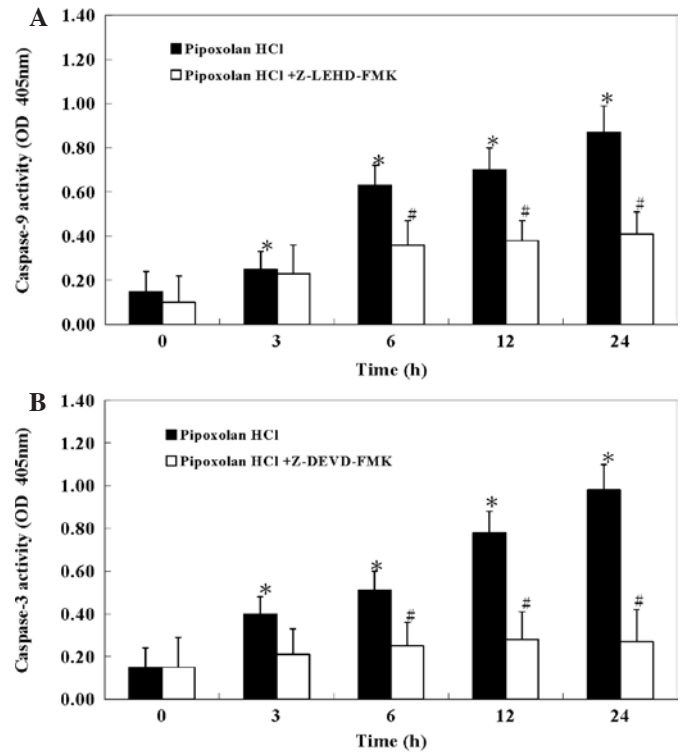


Figure 5. Effects of pipoxolan on the activity of caspase-9 and caspase-3 in U937 leukemia cells. U937 cells were also pre-treated with caspase-9 inhibitors (A, $10\ \mu\text{M}$ Z-LEHD-FMK) or caspase-3 (B, $10\ \mu\text{M}$ Z-DEVD-FMK) for 1 h, then treated with $10\ \mu\text{M}$ pipoxolan for 0 and 3-24 h, and whole-cell lysates were subjected to a caspase activity assay. The 0 h was defined as the control group. Data represent the mean \pm SD of three experiments. * $P < 0.01$, differences between pipoxolan and the control; # $P < 0.01$, differences between pipoxolan + Z-LEHD-FMK (A) and the control or pipoxolan + Z-DEVD-FMK and the control (B).

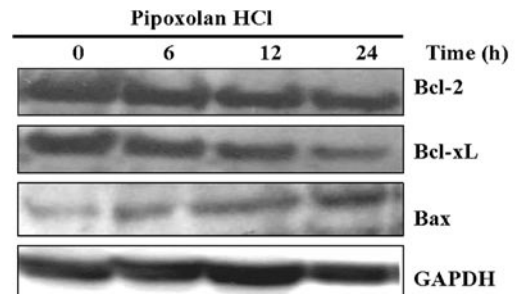


Figure 6. Effects of pipoxolan on the activity of Bcl-2, Bcl-xL and Bax in U937 leukemia cells after treatment with pipoxolan. U937 cells were treated with $10\ \mu\text{M}$ pipoxolan for 6-24 h, then the cytosolic fractions were separated by SDS-PAGE, transferred onto cellulose membrane and blotted with Bcl-2, Bcl-xL and Bax specific antibodies. GAPDH was used as an internal control.

Bcl-2 is recognized as a novel type of multidrug-resistant protein that protects tumor cells from the cytotoxic effects of anti-cancer drugs (28). Bax protein shares considerable amino acid homology with Bcl-2 and is capable of binding to Bcl-2 (28,29). Thus, Bax forms Bcl-2/Bax heterodimers and Bax/Bax homodimers, the latter being preferentially formed when the level of Bax exceeds that of Bcl-2. Formation of Bcl-2/Bax heterodimers appears to inhibit the anti-apoptotic function of Bcl-2, and the Bax/Bax homodimers contribute to the initiation of apoptosis (30,31). Thus, higher levels of Bax relative

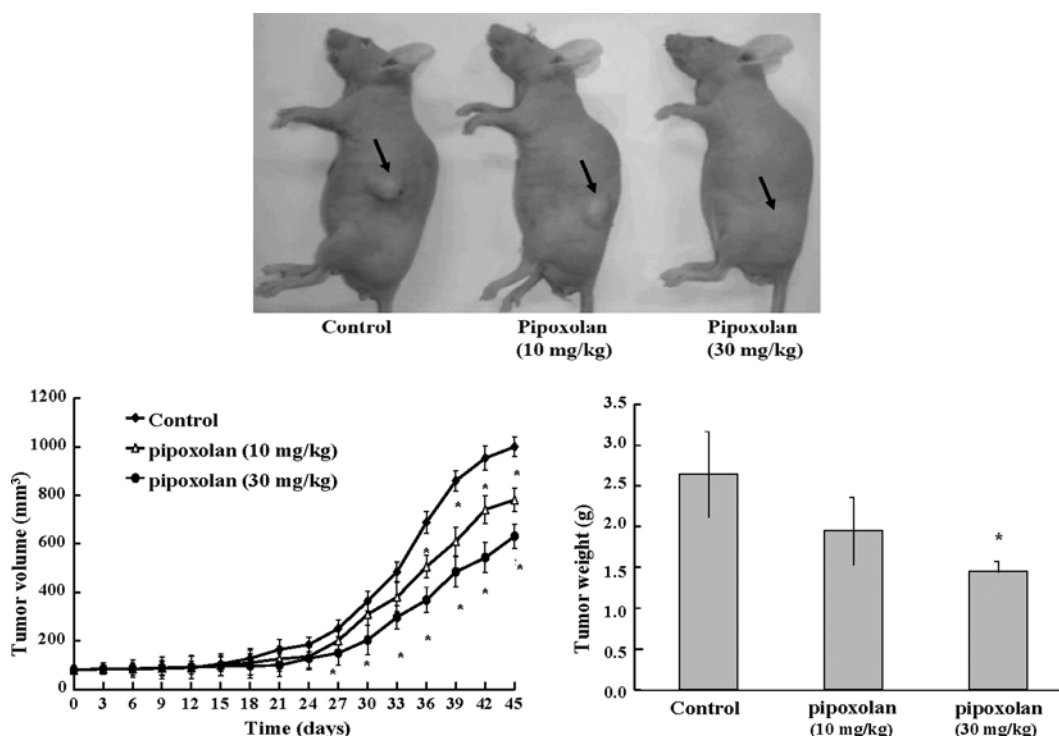


Figure 7. Effect of pipoxolan on U937 cell growth in an *in vivo* model. U937 cells (1×10^7) were subcutaneously injected into the flank of each animal. When a tumor reached an approximate volume of 80 mm³, mice bearing tumors with acceptable morphology and of similar size range were selected and started pipoxolan treatment. A representative photo of male BALB/c^{nu-/nu-} mice showing tumors after treatment with 10 and 30 mg/kg pipoxolan for 45 days (A). Tumor volume (B) and tumor weight (C) were determined. Data are expressed as the mean \pm SD. * $P < 0.01$ represents differences between pipoxolan treatment and control.

to Bcl-2 may increase cell susceptibility to apoptosis (31). In this study, we showed that treatment with pipoxolan modulates the levels of Bcl-2, Bcl-xL and Bax proteins, resulting in apoptosis.

In addition, our study clearly demonstrated that, *in vivo*, pipoxolan acted as an anti-leukemia cell growth agent to markedly inhibit tumor xenograft growth and tumor weight in mice.

In summary, our results indicate that pipoxolan induces the apoptosis of U937 leukemia cells through regulation of Bcl-2 family proteins and caspase-9 and -3 activation *in vitro*. Pipoxolan decreased the percentage of viable cells, promoted ROS production, decreased mitochondria membrane potential, induced caspase-9 and -3 activity and led to apoptosis, as demonstrated by chromatin condensation and the significant number of cells in the sub-G1 apoptotic phase of the cell cycle. Most importantly, we showed that pipoxolan has potent anti-cancer cell growth activity *in vivo*. The findings from this study suggest that pipoxolan has potential as a novel alternative therapeutic agent for the treatment of leukemia and, possibly, of other cancers as well.

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