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Kirenol, a compound from *Herba Siegesbeckiae,* induces apoptosis in human chronic myeloid leukemia K562 cells

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Kirenol is a biologically active substance isolated from *Herba Siegesbeckiae*. In the experiments, we explored a novel antitumor activity of kirenol. The data demonstrated that kirenol had strong cytotoxic effects to human chronic myeloid leukemia K562 cells, the 50% inhibitory concentration (IC_{50}) for kirenol was 53.05 μg/ml, 18.19 μg/ml and 15.08 μg/ml for 24, 48 and 72 h, determined using the MTT assay. Further studies showed that kirenol treatment caused externalization of phosphatidylserine, accumulation of ROS (reactive oxygen species), alteration of mitochondrial membrane potential, release of cytochrome *c*, reduction in the level of the Bcl-2 protein and upregulation of Bax and tBid, kirenol induced cell apoptosis in a caspase-independent manner. Further studies indicated that kirenol treatment triggered the arrest of cell cycle S period which might resulted from the up-regulation of phosphorylation of p53 (Ser 6 and Ser 37) and expression of p21 protein. Our results indicated that kirenol possesses antitumor action on human chronic myeloid leukemia K562 cells *in vitro*. Kirenol may have therapeutic potential for the treatment of cancer that deserves further investigation.

1. Introduction

Cancer develops when the balance between cell proliferation and cell death is disrupted, and the ensuing aberrant proliferation leads to tumor growth (Gartel and Tyner 2002). Mitochondrial damage pathway plays a crucial role in the induction of apoptosis. Mitochondrial membrane permeabilization (MMP) and the subsequent release of mitochondrial death effectors (e.g., cytochrome *c*) are key events in apoptosis (Li et al. 1997), which can be triggered by high level of ROS (Fulda et al. 2010), then all these stimuli reinforce each other, leading to apoptosis (Brookes et al. 2004). Besides, a number of gene products are known to regulate apoptosis, such as the Bcl-2 family members, p53 and p21.

Chronic myeloid leukemia (CML) is a clonal myeloproliferative expansion of transformed, primitive hematopoietic progenitor cells, which involves myeloid, monocytic, erythroid, megakaryocytic, B-lymphoid, and occasionally T-lymphoid lineages (Faderl et al. 1999). CML is a rare disease with an incidence of 1 or 2 cases per 100,000 people every year, and is most common in older people, with a median age at diagnosis of around 65 years (Hehlmann et al. 2007). Therapeutic strategies such stem cell transplantation and tyrosine-kinase inhibitors are used. Molecular-targeted therapy is now available (Hehlmann et al. 2007; Deininger et al. 2005), however, due to problems like rapid progression of disease and therapy resistance, new treatments are still urgently needed.

Kirenol, a main diterpenoid component isolated from a Chinese herb, *Herba Siegesbeckiae*, possesses anti-inflammatory and immunosuppressive properties and has a long history of use as a traditional medicine for the treatment of arthritis. Our recent studies suggested that kirenol could inhibit proliferation and induce apoptosis of type II collagen-specific lymphocytes from rats *ex vivo* and *in vitro*. So far there is not any report about antitumor action of kirenol. In the present study, we demonstrated for the first time that kirenol inhibited proliferation, induced apoptosis and cell cycle S phase arrest in human chronic myeloid leukemia K562 cells, and analyzed a series of apoptosis-related proteins to investigate the anti-carcinogenetic mechanism of kirenol.

2. Investigations and results

2.1. Kirenol inhibited the growth of K562 cells

The inhibitory effect of kirenol on different cell lines was initially determined by the MTT viability assay. From Fig. 1B, it can be seen that kirenol suppressed the proliferation of K562 cells significantly in a dose-dependent manner. Kirenol weakly inhibited the cell viability of Raji, HL-60, THP-1 and Jurkat cell lines, and kirenol exerted little suppression on Molt-4 cells. As kirenol played a significant suppressive role on K562 cells, we consequently focused on the effect of kirenol on K562

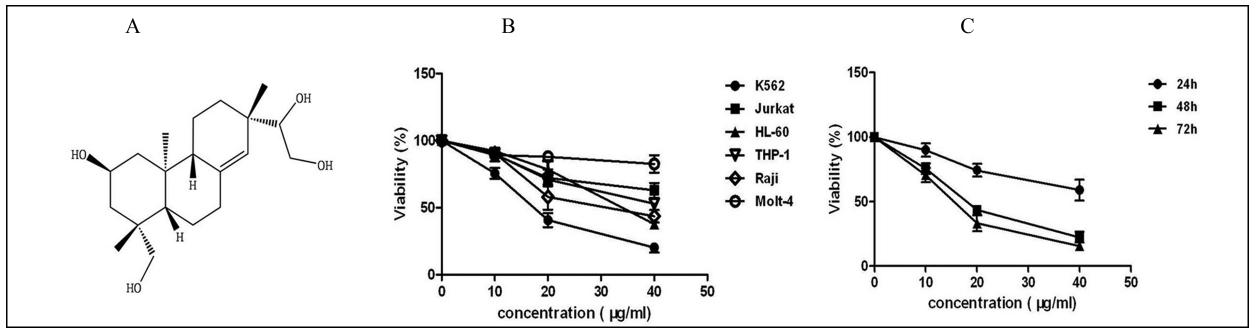


Fig. 1: (A) Chemical structure of kirenol. (B) Different cell lines were treated with various concentrations of 0–40 μg/ml kirenol for 48 h, the effect of kirenol on cell viability was measured using the MTT assay. (C) K562 cells were treated with various concentrations of 0–40 μg/ml kirenol for 24–72 h. The effect of kirenol on cell viability was measured using the MTT assay. Data represented the mean ± SD of at least three independent experiments.

cells. Fig. 1C shows the dose-dependent effect of kirenol in the range of 10–40 μg/ml, the IC_{50} of kirenol on K562 cells was 53.05 μg/ml, 18.19 μg/ml and 15.08 μg/ml for 24, 48 and 72 h, respectively. With prolonging of reactive time, the rate of cell proliferation decreased in a time-dependent manner as well.

2.2. Kirenol induced cell apoptosis of K562 cells in a caspase-independent manner

To determine whether the growth inhibitory effect of kirenol on K562 cell line was related to the induction of cell apoptosis, several apoptotic parameters were measured. Fluorescence microscopic examination indicated that K562 cells displayed marked nuclear condensation and fragmentation after kirenol treatment, as shown in Fig. 2A.

After kirenol treatment (10, 20 and 40 μg/ml) for 24 h, the percentage of cell apoptosis was 8.29 ± 0.53%, 13.89 ± 1.36%, and 51.85 ± 6.25%, respectively, which was statistically different from the control (Fig. 2C).

To investigate whether the cell apoptosis induced by kirenol is dependent on caspase activation, caspase 3 and caspases 9 activity was measured at 24 h after treatment with 40 μg/ml of kirenol. As shown in Fig. 2D, no significant increase in the levels of caspase 3 and caspases 9 activity was observed after kirenol treatment. Moreover, western blot analysis revealed that pro-caspase 3, pro-caspase 8 and PARP were not cleaved into their active form at different times (Fig. 2E, 2F).

2.3. Kirenol induced K562 cells apoptosis via the mitochondrial pathway

There is increasing evidence that an altered mitochondrial function is linked to apoptosis and a decrease in membrane potential, mediated by a polyprotein channel called the permeability transition pore including the voltage-dependent anion channel (Shimizu et al. 1999), which can be induced by increased level of ROS (Fulda et al. 2010). To investigate the underlying mitochondrial mechanism of apoptosis in K562 cells, we analyzed the mitochondrial membrane potential and level of ROS after kirenol treatment. Kirenol induced the time-dependent loss of mitochondrial membrane potential, as measured by flow cytometry (Fig. 3A, 3B). Besides, kirenol promoted the accumulation of ROS at different times (Fig. 3C, 3D).

It has been suggested that mitochondrial dysfunction is responsible for cytochrome *c* release (Kantrow and Piantadosi 1997). So we analyzed the effect of kirenol on release of mitochondrial cytochrome *c* into cytosol, cellular subfractions were prepared after kirenol (40 μg/ml) treatment for 12, 24 and 48 h. As shown in Fig. 3E, the level of mitochondrial cytochrome *c* was decreased significantly after treatment for 12 and 24 h, correspondingly, the level of cytosolic cytochrome *c* was markedly increased in cells which treated with kirenol for 24 h. These results suggested that the mitochondrial pathway might be involved in cell apoptosis induced by kirenol.

2.4. Kirenol modified the expression level of Bcl-2 family proteins in K562 cells

The proteins of the Bcl-2 family play critical roles in the regulation of apoptosis by functioning as promoters (e.g., Bax, Bid) or inhibitors (e.g.,Bcl-2) of cell apoptosis (Reed 1998). In order to investigate the mechanism how kirenol altered the mitochondrial functions, we further tested the expression level of the Bcl-2, Bax and Bid. As shown in Fig. 4A, western blot analysis revealed that Kirenol decreased the level of Bcl-2 protein after treatment for 24 and 48 h. Moreover, Kirenol upregulated the level of Bax and tBid (cleaved Bid) at different times.

2.5. Kirenol induced the arrest of cell cycle S phase

In order to further determine the role of kirenol in the control of cell cycle in K562 cells, cell cycle distribution analysis was conducted. As shown in Fig. 5B, K562 cells without drug exposure presented 48.99 ± 1.76 % population in the S-phase. However, kirenol treatment resulted in an evident accumulation of the S fraction, under 10 and 20 μg/ml kirenol, the S fraction rose to 52.89 ± 1.67 % and 56.05 ± 0.42 %, respectively. When cells were treated with 40 μg/ml kirenol, approximately 70.04 ± 1.10 % of the cells were arrested at the S phase. The results suggest that inhibition of cell proliferation and induction of cell apoptosis in K562 cells may be associated with the induction of arrest of cell cycle S phase.

2.6. Kirenol increased the phosphorylation of p53 and expression of p21 protein

The tumor suppressor p53 is a crucial transcriptional factor and plays a central role in the regulation of cell cycle, DNA repair, and apoptosis (Mihara et al. 2003). The phosphorylation of p53 is related to activate the expression of downstream target genes, which can lead to different cellular outcomes such as cell cycle arrest and apoptosis (Brady et al. 2011). To investigate the effect of kirenol on p53 and phospho-p53, we measured the expression level of p53, phospho-p53 (Ser 6 and Ser 37). As shown in Fig. 5C and Fig. 5D, kirenol increased significantly the level of phospho-p53 (Ser 6) and phospho-p53 (Ser 37) after treatment for 24 and 48 h. However, the level of total p53 did not change after kirenol treatment.

We next examined whether kirenol could affect p53 downstream target gene, p21, which involved in cell cycle arrest in response to DNA-damaging agents (Kang et al. 1999). As shown in Fig. 5C

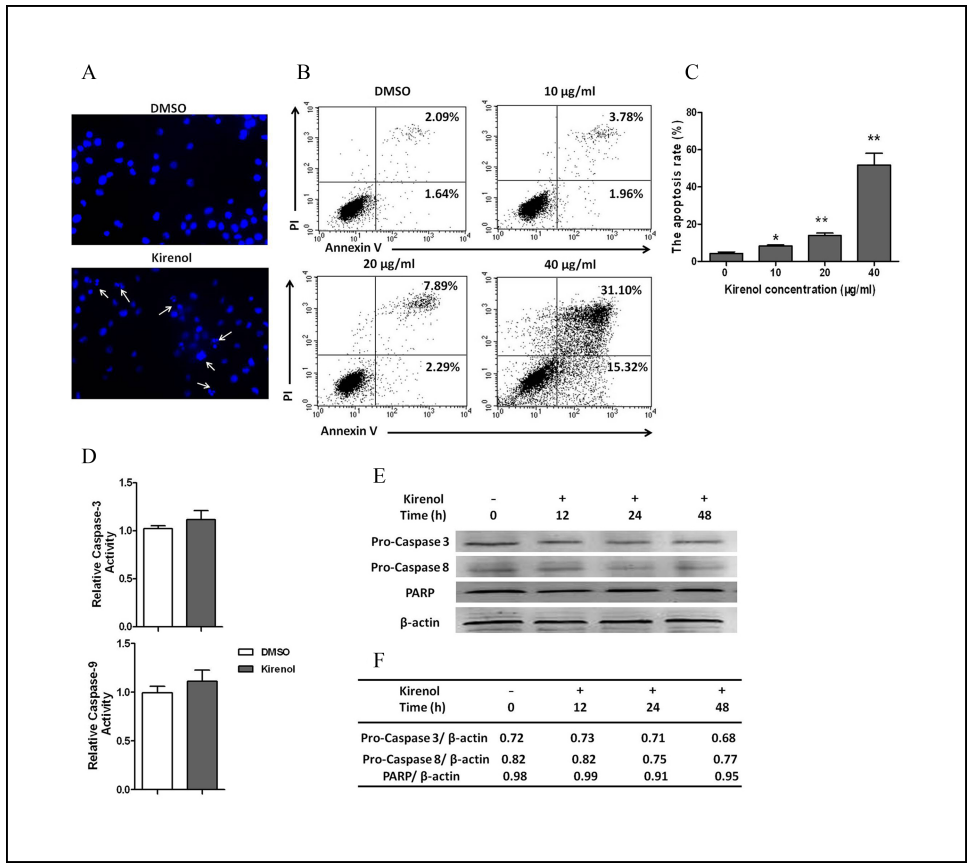


Fig. 2: Kirenol caused K562 cells apoptosis. (A) K562 cells were incubated in the medium without or with 40 μg/ml kirenol for 24 h, then stained with Hoechst 33342 and observed using a fluorescence microscope. Apoptotic cells were indicated by the arrows (×200 magnification). (B) Cells were treated with 0, 10, 20 and 40 μg/ml kirenol for 24 h. The induction of apoptosis was determined by Annexin V-FITC/PI staining assay. (C) Quantification of the number of apoptotic cells (*$P < 0.05$, **$P < 0.01$). (D) The panels showed caspase 3 and caspase 9 activity of K562 cells. (E) Representative western blot of pro-caspase 3, pro-caspase 8 and PARP. (F) The expression of pro-caspase 3, pro-caspase 8 and PARP in kirenol-treated and controlled cells was quantified by densitometry. Data represented the mean ± SD of at least three independent experiments.

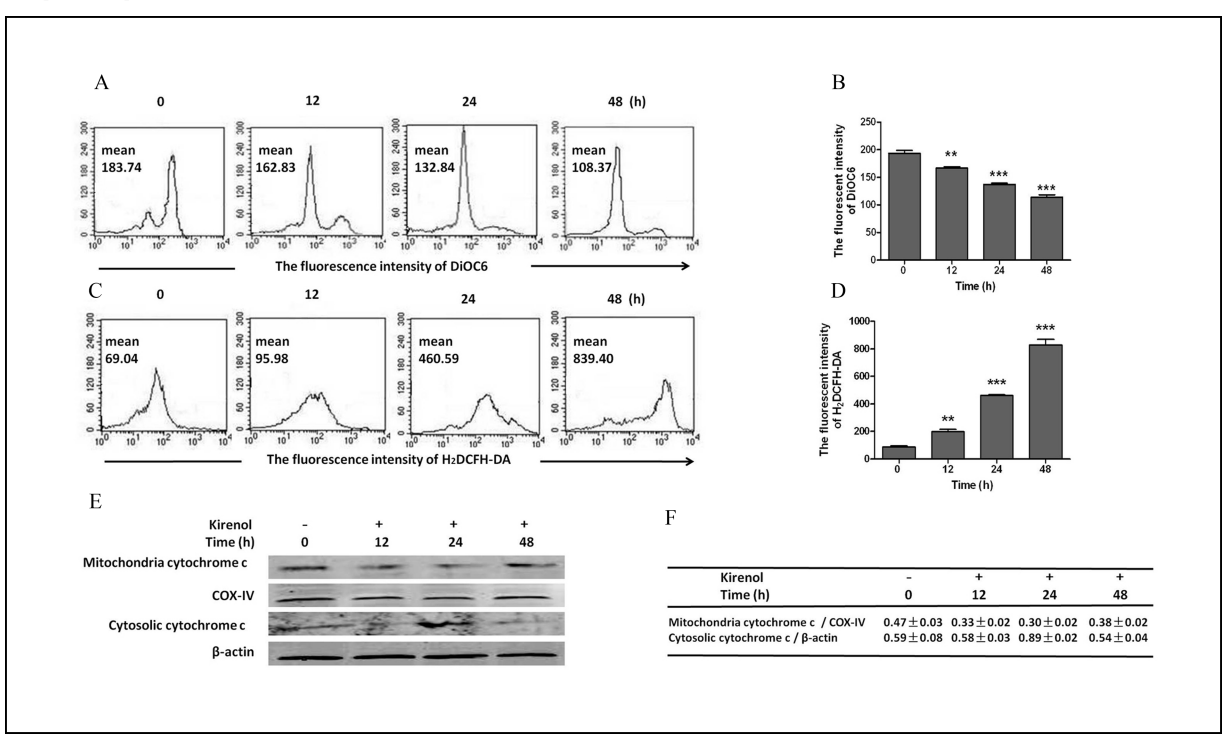
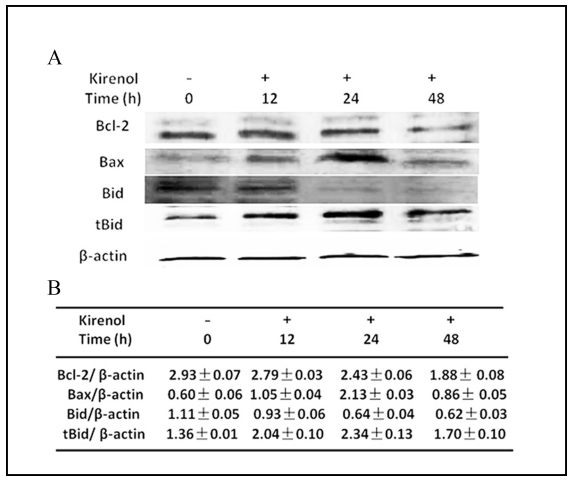


Fig. 3: Kirenol induced disruption of the mitochondrial membrane potential, increased the level of ROS and induced the mitochondrial cytochrome *c* release in K562 cells. Mitochondrial membrane potential changes were measured using DiOC6 staining, and the accumulation of ROS was measured by H_2DCFH-DA staining. (A) Representative image of DiOC6 staining by flow cytometry. (B) Quantification of fluorescence intensity of DiOC6 (**$P < 0.01$, ***$P < 0.001$). (C) Representative image of H_2DCFH-DA staining by flow cytometry. (D) Quantification of fluorescence intensity of H_2DCFH-DA (**$P < 0.01$, ***$P < 0.001$). (E) K562 cells were treated with 40 μg/ml Kirenol for the indicated times. The cytosolic fraction was separated, and western blot analysis was performed. Anti-COX-IV and anti-β-actin antibodies were reprobed to confirm equal protein loading in mitochondria and cytosol, respectively. (F) The expression of cytochrome *c* in kirenol-treated and controlled cells was quantified by densitometry. Data represented the mean ± SD of at least three independent experiments.



B

Kirenol	-	+	+	+
Time (h)	0	12	24	48
Bcl-2/ β-actin	2.93 ± 0.07	2.79 ± 0.03	2.43 ± 0.06	1.88 ± 0.08
Bax/β-actin	0.60 ± 0.06	1.05 ± 0.04	2.13 ± 0.03	0.86 ± 0.05
Bid/β-actin	1.11 ± 0.05	0.93 ± 0.06	0.64 ± 0.04	0.62 ± 0.03
tBid/ β-actin	1.36 ± 0.01	2.04 ± 0.10	2.34 ± 0.13	1.70 ± 0.10

Fig. 4: Kirenol modified the expression of Bcl-2, Bax, Bid and tBid. K562 cells were treated with 40 μg/ml kirenol for different times as indicated. The whole cell lysates were prepared, and western blot analysis was performed. (A) Representative western blot of Bcl-2, Bax, Bid and tBid. (B) The expression of Bcl-2, Bax, Bid and tBid in kirenol-treated and controlled cells was quantified by densitometry. Data represented the mean ± SD of at least three independent experiments.

and Fig. 5D, kirenol significantly increased the level of p21 protein after treatment for 12, 24 and 48 h. Thus, kirenol treatment can up-regulate the level of phosphorylation of p53 and p21, which may contribute to kirenol-induced cell cycle arrest.

3. Discussion

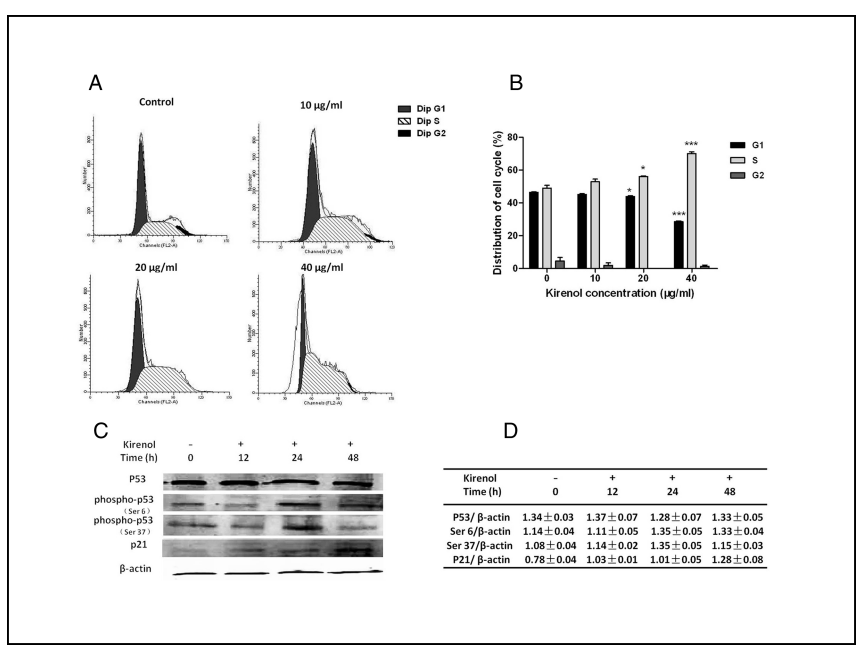
Kirenol, one of the major active ingredients of *Herba Siegesbeckiae*, is a diterpenoid component and has been found to have potent anti-inflammatory and immunosuppressive properties. However, no reports on antitumor properties of kirenol were found. Thus, in the present experiments, we explored the effects of kirenol on the proliferation, apoptosis and cell cycle of human leukemia cell line K562.

Emerging evidence has demonstrated that the anticancer activity of certain chemotherapeutic agents is involved in the induction of apoptosis and cell cycle arrest. Our results indicated that kirenol strongly inhibited K562 cell proliferation in a time- and dose-dependent manner. Furthermore, kirenol induced cells apoptosis which was demonstrated by several lines of evidence. Kirenol treatment induced nuclear changes by Hoechst 33342 staining, enhanced the loss of plasma membrane polarity as assayed by Annexin V staining, increased ROS level, disrupted the mitochondrial membrane potential and induced cytochrome *c* release. Since the caspase activity was not changed in kirenol-treated K562 cells, we speculated that kirenol-mediated apoptosis can occur through a caspase-independent pathway.

Apoptosis is tightly regulated by anti-apoptotic and pro-apoptotic molecules, including proteins of the Bcl-2 family, and can be mediated by several different pathways. The ratio of Bax/Bcl-2 is critical in determining whether cells will undergo apoptosis. We found that kirenol treatment increased the expression of Bax protein and the cleavage of Bid into tBid, and down-regulated the level of Bcl-2. Additionally, kirenol treatment produced high level of ROS which could cause disorders in cellular functions (Kang and Pervaiz 2012). These effects may be responsible for leading to disruption of mitochondrial membrane potential, and increasing the release of cytochrome *c* from mitochondria to the cytosol. Since kirenol induced cell apoptosis was not associated with the activation of caspase 3, caspase 9 and caspase 8, it is supposed that the caspase-independent death factors released from mitochondria, such as apoptosis-inducing factor (AIF) (Donovan and Cotter 2004) and endonuclease-G (Cande et al. 2004; Hollstein et al. 1991) may play an important role in the kirenol-induce apoptotic process.

Controlling cell cycle progression in cancer cells is a potential effective strategy to inhibit tumor growth (Pavletich 1999). Our data showed that kirenol-treated K562 cells showed a significant increase in S-phase arrest, indicating that kirenol can arrest cell cycle progression.

As a central tumor suppressor, p53 mainly functions by inducing transcription of many different downstream genes, such as p21, which are controlled by post-translational modifications, such as phosphorylation (Laptenko and Prives 2006; Hanahan and Weinberg 2000; van Loo et al. 2002). The phosphorylation



Kirenol	-	+	+	+
Time (h)	0	12	24	48
P53/ β-actin	1.34 ± 0.03	1.37 ± 0.07	1.28 ± 0.07	1.33 ± 0.05
Ser 6/β-actin	1.14 ± 0.04	1.11 ± 0.05	1.35 ± 0.05	1.33 ± 0.04
Ser 37/β-actin	1.08 ± 0.04	1.14 ± 0.02	1.35 ± 0.05	1.15 ± 0.03
P21/ β-actin	0.78 ± 0.04	1.03 ± 0.01	1.01 ± 0.05	1.28 ± 0.08

Fig. 5: Kirenol induced cell cycle S-phase arrest. (A) Cells were incubated with various concentrations (0, 10, 20 and 40 μg/ml) of kirenol for 48 h, the distribution of cell cycle was detected by PI staining. The figures were representative of three separate experiments. (B) The cell cycle distribution was quantified in kirenol-treated and controlled cells (*$P < 0.05$, ***$P < 0.001$). (C) Representative western blot of p53, phospho-p53 and p21. (D) The expression of p53, phospho-p53 and p21 in kirenol-treated and controlled cells was quantified by densitometry. Data represented the mean ± SD of at least three independent experiments.

of p53 at the N-terminal residue Ser 6 is related to the response to DNA damage (Higashimoto et al. 2000), and the phosphorylation at Ser 37 is involved in regulating p21 protein expression (Tang et al. 2006). We found that following kirenol treatment, the level of phospho-p53 (Ser 6 and Ser 37) was up-regulated. p21 is the founding member of the Cip/Kip family of CKIs, which can bind and inhibit a broad range of cyclin/Cdk complexes, with a preference for those containing Cdk2 (Harper et al. 1995; Zhang et al. 1993). p21 plays an essential role in growth arrest after DNA damage (Brugarolas et al. 1995), and over-expression leads to G1 and G2 or S-phase arrest (Niculescu et al. 1998; Ogryzko et al. 1997). Our results showed that kirenol treatment could increase the protein expression of p21 in K562 cells, which may contribute to kirenol-induced cell cycle arrest. In conclusion, our results demonstrated that kirenol inhibited proliferation, induced S-phase arrest and apoptosis in human chronic myeloid leukemia K562 cells. Additionally, we found that kirenol-induced growth arrest in K562 cells was mediated through mitochondrial pathway, including the accumulation of ROS, disruption of mitochondrial membrane potential, release of cytochrome *c*, reduced level of Bcl-2, enhanced expression of Bax and tBid. Besides, the level of phospho-p53 and p21 was upregulated by kirenol treatment. Taken together, these findings provide biological evidence that, kirenol is a potential antitumor agent. Further investigation into the mechanism of kirenol on other human cancer cell lines as well as the *in vivo* research is required.

4. Experimental

4.1. Cell lines and reagents

Human chronic myeloid leukemia cell line K562, T-cell leukemia cell line Jurkat, promyelocytic leukemia cell line HL-60, lymphoma cell line Raji, monocyte leukemia cell line THP-1, and lymphoblastic leukemia cell line Molt-4 were obtained from ATCC (Rockville, USA), cell lines were cultured in RPMI1640 (GibcoBRL, USA) containing 10% fetal bovine serum (FBS; GibcoBRL) in 5% CO_2 incubator at 37 °C. Kirenol (purity > 99%) was extracted by the State Key Laboratory of Natural and Biomimetic Drugs at Peking University (Beijing, China). The chemical structure of kirenol is shown in Fig. 1A. Kirenol was initially dissolved in dimethylsulfoxide (DMSO), stored at -20 °C, and then thawed before use. 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Annexin V-FITC/PI was obtained from Baosai Biotechnology (Beijing, China). Caspase 3 assay kit, caspase 9 assay kit and mitochondria/cytosol fractionation kit were purchased from Biovision (Milpitas, CA, USA). The anti-cytochrome *c* antibody was purchased from BD PharMingen (San Diego, CA, USA). The anti-Bcl-2, anti-Bax, anti-Bid, anti-p21 antibodies, and phospho-p53 antibody sampler kit were from Cell Signaling Technology (Beverly, MA, USA). The antibodies against pro-caspase 3, pro-caspase 8 and PARP were purchased from BD Transduction Laboratories (Lexington, KY, USA). 3,3'-Dihexyloxacarbocyanineiodide (DiOC6) and 2',7'-Dichlorodihydrofluorescein diacetate (H_2DCFH-DA) were obtained from Molecular Probes (Eugene, OR, USA). The BCA protein assay reagent was purchased from Pierce Biotechnology (Rockford, IL, USA).

4.2. Cell viability assay

Cell viability was determined by assaying for the reduction of MTT. Cells were plated at a density of 2×10^4 per well in 96-well plates in RPMI1640 + 10% FBS and then treated with 0, 10, 20, 40 μg/ml Kirenol. After exposure to Kirenol for 24, 48 and 72 h, 10 μl MTT solution was added for 4 h. The cells were lysed with 0.04N HCl in isopropyl alcohol, and the absorbance was read at 570 nm. Cell viability was calculated as follows: cell viability (%) = absorbance of test group / absorbance of controlled cell group × 100%.

4.3. Hoechst 33342 staining

For analysis of chromatin condensation or nuclear fragmentation, Kirenol-treated cells were harvested and fixed in 3% paraformaldehyde (PFA), incubated in 0.1% Triton-X100-PBS for 30 min, and then stained in 0.25 μg/ml Hoechst 33342 for 15 min. The cells were observed using the IX70 fluorescence microscope (Olympus, Japan).

4.4. Flow cytometry analysis for apoptosis quantification

For flow cytometry apoptosis assays, Kirenol-treated cells (0, 10, 20, 40 μg/ml) were harvested and washed twice with PBS, followed by resuspending in 200 μl Annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, 2.5 mM $CaCl_2$, PH 7.4), then 10 μl FITC-conjugated Annexin V was added according to the manufacturer's protocol. After incubation for 20 min at room temperature in the dark, 10 μl PI was added and following 200 μl binding buffer was added and samples were immediately analyzed with a FACSCalibur flow cytometer using the CellQuest software (BD Bioscience, USA).

4.5. Assay of mitochondrial membrane potential

For the measurement of mitochondrial membrane potential changes, Kirenol-treated cells (40 μg/ml) and cotrolled cells were harvested and washed twice with PBS, followed by 20 nM of DiOC6 staining, according to the manufacturer's instructions. The mean fluorescence of DiOC6 positive cells was analyzed using a flow cytometer.

4.6. Analysis of ROS

For the assay of level of ROS, kirenol-treated cells (40 μg/ml) and cotrolled cells were harvested and washed twice with PBS, followed by using 2 μM of H_2DCFH-DA staining, according to the manufacturer's instructions. The mean fluorescence of H_2DCFH-DA positive cells was analyzed by a flow cytometer.

4.7. Caspase activity assay

Caspase 3 and caspase 9 activity was detected using caspase 3 and caspase 9 assay kit according to the manufacturer's directions. K562 cells were incubated in 6-well plates for 24 h with kirenol concentration of 40 μg/ml. A control without kirenol treatment was incubated in the mean time. Afterwards, 5×10^6 cells were suspended in 50 μl of chilled cell lysis buffer and incubated cells on ice for 10 min. After protein concentration was measured, 100 μg protein was treated by addition of caspase 3 or caspase 9 substrate solution for assay. Caspase activity was measured using a plate reader using a wavelength excitation of 380 nm and emission of 460 nm in POLARStar (BMG Labtechnology, Germany).

4.8. Assay of cytochrome c release from mitochondria

K562 cells were treated with kirenol (40 μg/ml) for 0, 12, 24, 48 h, then harvested and homogenized with a Dounce homogenizer using mitochondria/cytosol fractionation kit according to the manufacturer's direction. The homogenates were centrifuged at 2000 rpm for 15 min to remove unbroken cells and nuclei. The cytosolic fractions were obtained by further centrifugation at 10,000 g for 30 min. The supernatants were used for identification of cytosolic cytochrome *c* with western blot (Buolamwini 1999).

4.9. Analysis of cell cycle

Cell cycle distribution was determined by DNA staining with PI. Briefly, 1×10^6 cells were incubated with kirenol (0, 10, 20, 40 μg/ml) for 48 h. Cells were washed in PBS and fixed in 70% ethanol overnight, then collected and resuspended in PBS containing 50 μg/ml PI, 0.1 mg/ml RNase, and 5% Triton X-100, and incubated at 37 °C for 30 min. Cells were analyzed with a flow cytometer and the percentage of cells in the different phases of cell cycle was analyzed by using CellQuest software.

4.10. Western blot analysis

Cells were plated in 6-well plates at density of 1×10^6 per well, cells were treated with kirenol (40 μg/ml) for 0, 12, 24, 48 h. After the indicated times, cells were collected and lysed in lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 1 mmol/l phenylmethylsulphonyl fluoride, and protease inhibitors]. Cell debris was removed by centrifugation (15000 rpm, 15 min at 4 °C). After protein concentration was measured, equal amounts of protein were separated by 12.5% SDS-PAGE, then transferred to nitrocellulose membranes. The membranes were blocked and incubated overnight at 4 °C with primary antibodies, followed by incubation for 1 h with the secondary antibodies. Finally, protein expression was detected using the Odyssey Infrared Imaging System (LI-COR Biosciences, USA).

4.11. Statistical analysis

Results were expressed as the mean ± SD. Statistical analysis was carried out by the Student's t-test with $P < 0.05$ representing significance. At least three independent experiments were performed.

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