Isoferulic acid inhibits human leukemia cell growth through induction of G2/M-phase arrest and inhibition of Akt/mTOR signaling

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Abstract. Hematologic malignancy is a serious disease that develops quickly and aggressively, severely threatening human health owing to its high mortality. The current study aimed to evaluate the antitumor effects of isoferulic acid (IFA) on leukemia cells and investigate the possible molecular mechanisms. Hematologic cancer cell lines (Raji, K562 and Jurkat) were treated with IFA in a dose-dependent manner and proliferation was measured by a cell proliferation assay. Cell cycle arrest was detected via flow cytometry using propidium iodide (PI) staining. Cell apoptosis and apoptosis-associated signal pathways were analyzed via Annexin V/PI staining and western blot assays, respectively. IFA inhibited cell viability, induced cell apoptosis and triggered cell cycle arrest in G2/M phase in Raji, K562, and Jurkat cells in a dose-dependent manner. In response to IFA treatment, the levels of cleaved poly(ADP-ribose) polymerase and cleaved caspase-3 were increased in Jurkat and K562 cells, which was associated with increased phosphorylation of Cdc2 and reduction of Cyclin B1 levels. IFA remarkably attenuated the phosphorylation of mTOR and Akt in Jurkat cells. Collectively, the present data suggested that IFA had therapeutic effects on Jurkat, K562, and Raji cells, indicating it as a promising candidate for the treatment of hematologic malignancy.

Introduction

Hematologic malignancy is a serious disease that develops quickly and aggressively, and severely threatens human health due to its high mortality (1-3). The incidence of complete remission of hematologic malignancy has increased recently, with new therapeutic strategies (4). For instance, chimeric antigen receptor (CAR) T-cells targeting CD19 and therapeutic antibodies targeting CD20 have been developed and tested in patients with B-cell lymphoma in preclinical and clinical trials (5). However, there is no effective treatment for T-cell lymphoma, and the treatment of hematologic malignancy is relatively limited. Since the 5-year survival rate of patients with leukemia is between 45-55% (6-8), developing new therapeutic strategies and finding new agents for curing hematologic malignancy has become urgent.

Isoferulic acid (IFA), also known as 3-(3-hydroxy-4-methoxyphenyl)-2-propenoic acid, is a natural compound extracted from *Cimicifuga heracleifolia* (CH), which is frequently used in traditional medicine in Asian countries for treating inflammatory diseases and specific cancers (9,10). As one of the important active ingredients in CH, IFA has several therapeutic effects. These include the inhibition of several inflammatory diseases (11), elimination of viral infections (12), clearance of reactive oxygen species (ROS) (13), alleviation of metabolic diseases (14) and the reduction of glucose-induced glycation of bovine serum albumin (11,15). Although IFA affects cell cycle arrest (16), inhibits tumor cell proliferation and prompts cell apoptosis (17-19), whether it inhibits leukemia cells remains to be clarified. *In vitro* and *in vivo* experiments should be carried out to show whether IFA could become a potential candidate for treating leukemia.

Leukemia is a hematologic malignancy that generally originates in the bone marrow, and develops numerous abnormal leukocytes (20). Abnormal undifferentiated leukocytes dramatically proliferate, expand and resist cell apoptosis, resulting in immature cells in the bone marrow and peripheral blood (21). Inhibition of tumor cell growth and promotion of cell apoptosis are two frequent intervention strategies for eliminating cancer cells (22). Protein kinase B (Akt), a main downstream signal of PI3K, is an important protein in promoting cell proliferation, differentiation, migration and angiogenesis, while also protecting cancer cells against apoptosis (23-25). Activated Akt promotes cell proliferation by activating ribosomal protein S6 kinase and eukaryotic initiation factor 4E (26). It also modulates the cell cycle and drives the cells to go through both G1/S and G2/M cell cycle checkpoints (27). Cyclin B-Cdc2 (also known as Cdk1) is an important complex for the regulation of G2/M transition; it is negatively modulated by Wee1 and myelin transcription factor

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1, and positively regulated by Cdc25B. Both modulatory cell signaling pathways are precisely controlled by Akt (28-30). Therefore, interventions that target Akt-mediated cell signals may be able to inhibit cancer.

In the present study, IFA was found to inhibit cell growth and promote cell apoptosis in Jurkat, K562 and Raji cell lines. Leukemia cells were significantly arrested in G2/M phase, due to the increased phosphorylation of Cdc2 and reduced expression of Cyclin B1 after treatment with IFA. Furthermore, the latter was identified to attenuate the phosphorylation of mTOR and Akt. The results indicated that IFA has an impact on leukemia *in vitro* and may be a promising candidate for treating hematologic malignancy.

Materials and methods

Reagents and antibodies. IFA was ordered from TargetMol. Cell Counting Kit-8 (CCK-8) and trypan blue staining cell viability assay kits were ordered from Beyotime Institute of Biotechnology. An Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was purchased from BestBio Biotechnology. Cleaved poly(ADP-ribose) polymerase (PARP cat. no. 5625), cleaved caspase-3 (cat. no. 9661), b-actin (cat. no. 3700), phosphorylated (p)-Cdc2 (Tyr15) (cat. no. 4539), total-Cdc2 (cat. no. 9116), Cyclin B1 (cat. no. 12231), p-Akt (Thr308) (cat. no. 13038), total-Akt (cat. no. 4685), p-mTOR (Ser2448) (cat. no. 5536) and total-mTOR (cat. no. 2983) were ordered from Cell Signaling Technology, Inc. Horseradish peroxidase (HRP)-conjugated anti-mouse/rabbit IgG antibody was ordered from Jackson ImmunoResearch (cat. no. 111-035-003). Other chemical reagents were purchased from Sigma-Aldrich; Merck KGaA.

Cells and cell culture. Jurkat (acute lymphoid leukemic T cells), K562 (chronic myeloid leukemia), and Raji (Burkitt's lymphoma) cells were purchased from American Type Culture Collection and maintained in RPMI-1640 medium with 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator containing 5% CO_2.

Cell viability assay. CCK-8 assay was applied to detect the cell viability. Briefly, cells were seeded into 96-well plates at $2x10^4$ cells/well for 24 h. IFA at 5, 15 and 45 μM was added for 12, 24 and 48 h. CCK-8 (10 μl) was added and the absorbance at 450 nm was measured after incubation for 2 h. In addition, the trypan blue staining cell viability assay kit was used to detect cell proliferation. Raji, K562 and Jurkat cells were planted into 10-cm dishes at $1x10^6$ cells/dish. After cell culture for 5 days, at the point of cell treatment, the cells were collected, stained with trypan blue within 2 min and counted by a hemocytometer at room temperature. The IFA-untreated cell group was normalized to 100% cell viability.

Cell cycle assay. Cell cycle was determined by using PI staining. Briefly, cells were seeded at a density of $5x10^5$ cells/ml in 12-well plates for 12 h. Then, cells were treated with IFA at 5, 15 and 45 μM. After incubation for 24 h, cells were collected and fixed with 70% ethanol overnight at 4˚C. After removing ethanol and neutralizing RNA, PI was used to stain DNA at 4˚C. Afterwards, flow cytometry was used to analyze cell cycle distribution (FACSCalibur, BD Biosciences; FlowJo 7.6, FlowJo LLC).

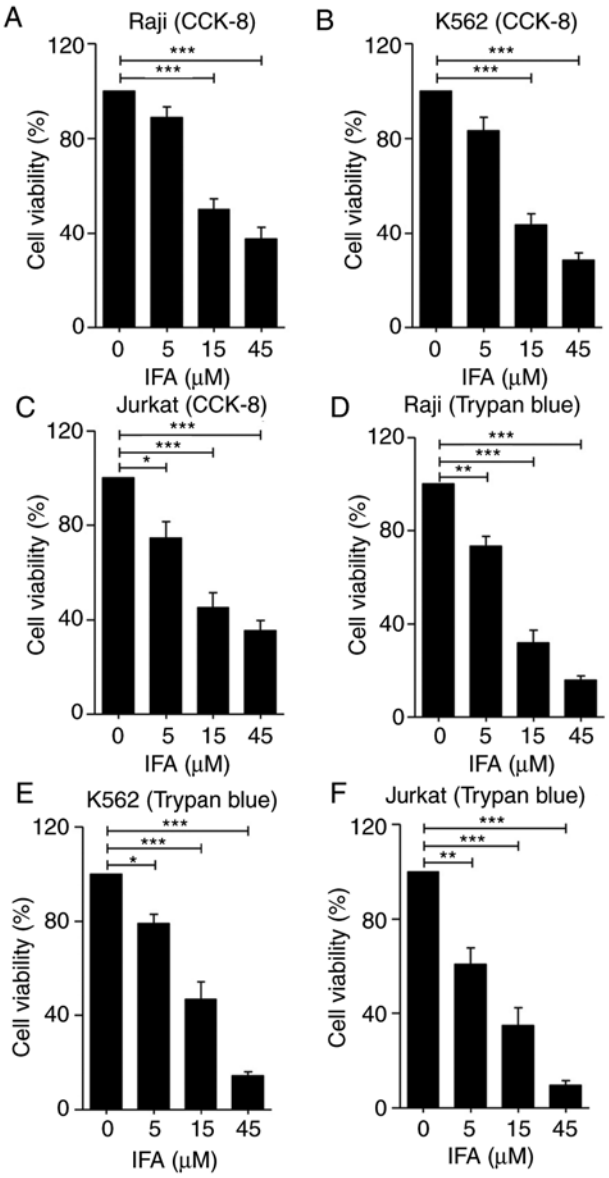

Figure 1. IFA inhibits leukemia cell proliferation in dose-dependent manner. Raji, K562 and Jurkat cells were incubated with IFA at 0, 5, 15, and 45 μM for 24 h. (A-C) CCK-8 and (D-F) trypan blue assays were performed to evaluate the anti-proliferation effect of IFA on Raji, K562 and Jurkat cells. Data are expressed as mean ± S.D. of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. control group. CCK-8, Cell Counting Kit-8; IFA, isoferulic acid.

Cell apoptosis analysis. Annexin V-FITC/PI analysis (Qiaoxin) was applied to detect cell apoptosis. Briefly, cells were seeded onto 12-well plates at $5x10^5$ cells overnight. After treatment with 0, 5, 15 and 45 μM of IFA for 24 h, cells were harvested and rinsed with PBS. Then, cells were resuspended with binding buffer. The Annexin V-FITC and PI were incubated with cells for 15 min and 5 min at 4˚C, respectively. Finally, flow cytometry was used to analyze the percentages of apoptotic cells (FACSCalibur; BD Biosciences; FlowJo 7.6, FlowJo LLC).

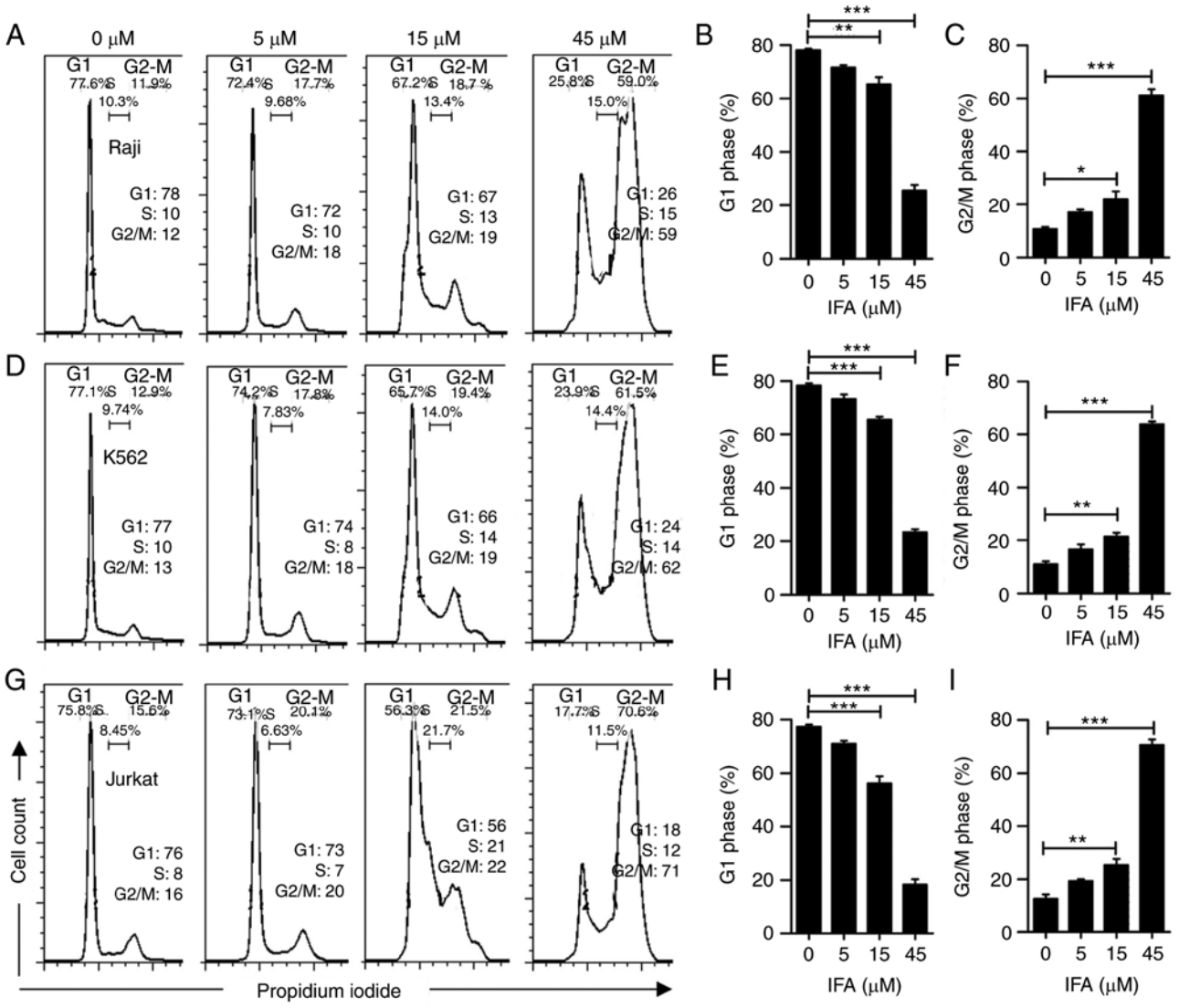

Figure 2. IFA treatment increases cell cycle arrest at G2/M phase and reduces the proportions of cells in G1 and S phase in Raji, K562 and Jurkat cells. (A-C) Raji, (D-F) K562 and (G-I) Jurkat cells were incubated with IFA at 0, 5, 15 and 45 μM for 24 h. Then, cells were harvested and fixed overnight. Flow cytometry were used to monitor and analyze cell distribution after staining with propidium iodide. Percentage of Raji, K562 and Jurkat cells in different cell cycles was quantified. Data are expressed as mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. control group. IFA, isoferulic acid.

Western blot analysis. Cells were treated with IFA at 5, 15 and 45 μM for 24 h. Then, cells were lysed with RIPA Lysis Buffer and the protein concentration were determined by BCA protein assay and the protein lysis mixed with loading control (all from Beyotime Institute of Biotechnology) and heated at 100˚C for 5 min. Samples (20 μg) were loaded on 10% of SDS-PAGE gels and transferred to nitrocellulose membranes. Before incubation with different antibodies (1:1,000) overnight at 4˚C, the membranes were blocked with 5% non-fat milk for 2 h at room temperature. After being washed with TBS-Tween 20 (TBST containing 0.1% Tween 20) five times every 5 min, the membranes were incubated with HRP-conjugated secondary antibodies for another 2 h at room temperature and then washed with TBST three times. The protein bands were visualized using chemiluminescent substrate reagent (Shanghai Shenger Biotechnology Co., Ltd.). The semi-quantitative analysis was performed by using ImageJ software (v1.48U; National Institute of Health).

Statistical analysis. The data were presented as means ± SD based on at least three independent experiments. The statistical analysis was performed using Prism 5 (GraphPad Software, Inc). One-way ANOVA followed by Bonferroni post hoc test were used to determine the difference between groups. P<0.05 was considered a statistically significant difference.

Results

IFA displays anti-proliferation activity in Raji, K562 and Jurkat cell lines. To determine the effect of IFA on proliferation of leukemia cells, Raji, K562 and Jurkat cells were treated with IFA for 24 h with 0, 5, 15 and 45 μM, and cell viability was investigated using CCK-8 and trypan blue staining assays. As presented in Fig. 1A, IFA showed a significant inhibitory effect on Raji cell growth in a dose-dependent manner. After 24 h, the viable cells decreased by 30(±7)%-65(±9)% in the presence of 5-45 μM, compared with that of the control group. Furthermore, similar inhibitory effects of IFA on K562

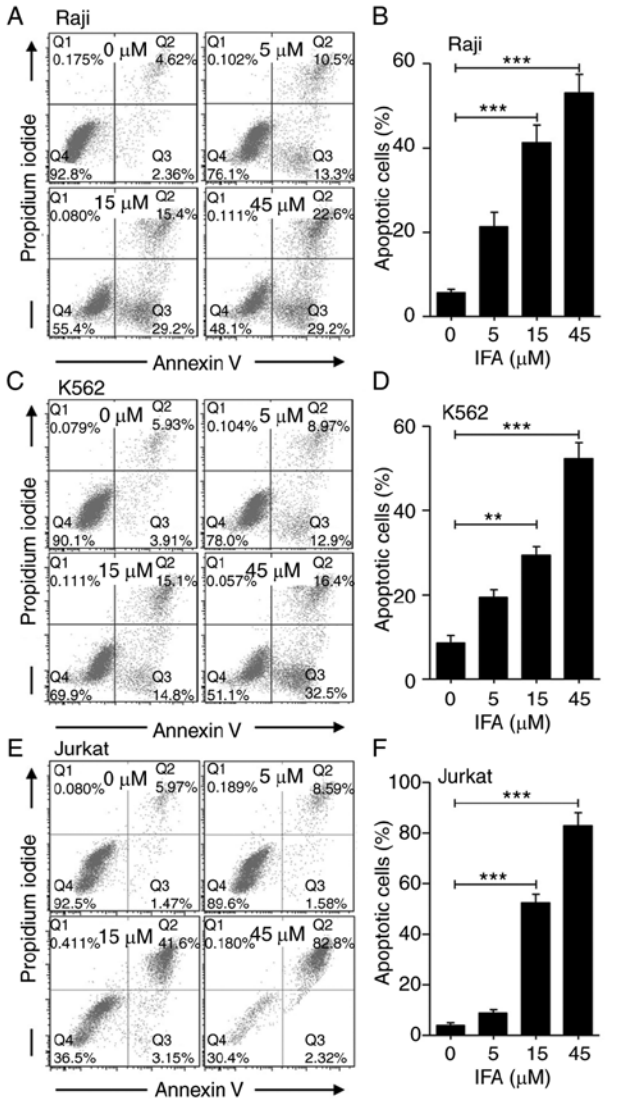

Figure 3. IFA prominently induces cell apoptosis in a dose-dependent manner. (A) Raji, (C) K562 and (E) Jurkat cells were treated with IFA at the indicated concentration for 24 h and analyzed by flow cytometry following Annexin-FITC and propidium iodide double-staining. The percentages of apoptotic (B) Raji, (D) K562 and (F) Jurkat cells were evaluated in three separate experiments. Data are expressed as mean ± SD of three independent experiments. **P<0.01, ***P<0.001 vs. control group. IFA, isoferulic acid.

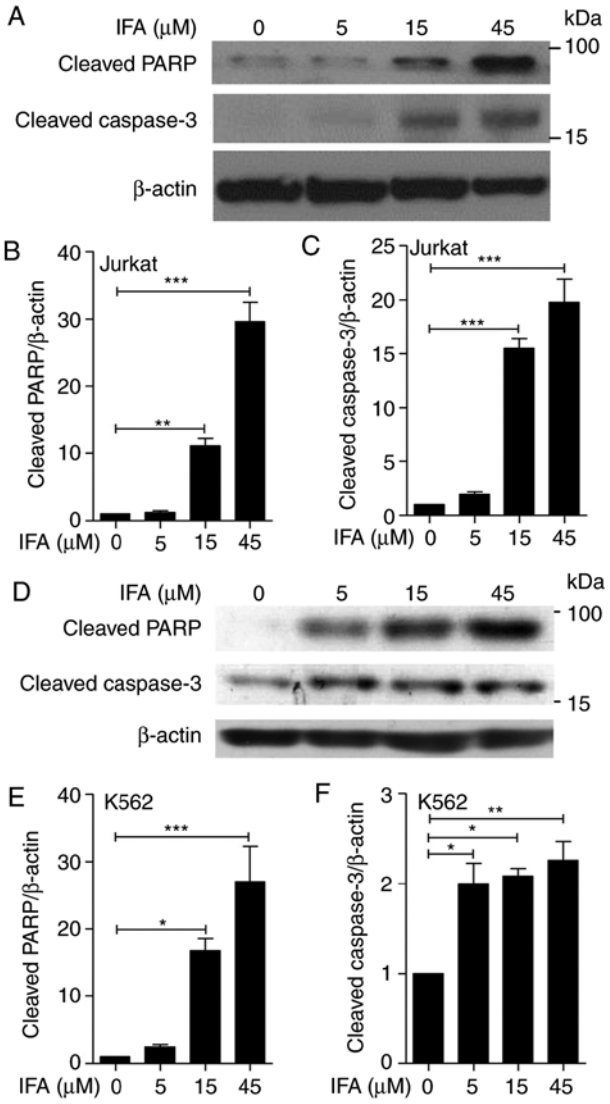

Figure 4. IFA enhances the expression of cleaved PARP and cleaved caspase-3 in Jurkat and K562 cells. (A) Jurkat and (D) K562 cells were incubated with IFA at 5, 15 and 45 μM for 24 h. Western blotting was performed to detect the expression of cleaved PARP and cleaved caspase-3 in (A) Jurkat and (B) K562 cells. The ratios of (B and E) cleaved PARP and (C and F) cleaved caspase-3 to β-actin in (B and C) Jurkat and (E and F) cells were quantified using ImageJ software. Data are expressed as mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. control group. IFA, isoferulic acid; PARP, poly(ADP-ribose) polymerase.

(Fig. 1B) and Jurkat cells (Fig. 1C) were also detected. To further confirm the anti-proliferative effect of IFA on leukemia cells, a trypan blue staining cell viability assay kit was used to detect the amount of live cells. Raji, K562 and Jurkat cells were incubated with IFA at 0, 5, 15 and 45 μM for 5 days. The total amount of cells was calculated and the percentage of cell viability was presented in Fig. 1D-F. Similarly, IFA dose-dependently inhibited the proliferation of Raji, K562 and Jurkat cells (Fig. 1D-F). These results indicated that IFA inhibited leukemia cell proliferation.

IFA induces cell cycle arrest at G2/M of Raji, K562 and Jurkat cells. The effect of IFA on cell cycle arrest in Raji, K562 and Jurkat cells was measured by flow cytometry. Raji, K562 and Jurkat cells were treated with IFA at 0, 5, 15 and 45 μM for 24 h. As presented in Fig. 2A, IFA treatment increased the percentage of Raji cells in G2/M phase in a dose-dependent manner, and decreased the proportions of Raji cells in G1 and S phase . Meanwhile, the percentage in G2/M phase increased from 12% to 59% upon treatment with 45 μM of IFA . Fig. 2B and C show the quantification of cell arrest at G1, S and G2/M. It was also found that IFA had a similar effect on K562 (Fig. 2D-F) and Jurkat cells (Fig. 2G-I). These results revealed that IFA induced cell cycle arrest at G2/M phase in Raji, K562 and Jurkat cells.

IFA enhances apoptosis of Raji, K562 and Jurkat cells. It was further determined that significant G2/M cell cycle arrest led to cell apoptosis in Raji, K562 and Jurkat cells. As indicated in Fig. 3, using Annexin V/PI staining showed that IFA significantly triggered cell apoptosis in a dose-dependent manner in Raji, K562 and Jurkat cells,. The proportion of apoptotic

cells increased from 7% to 24, 44 and 52% (Fig. 3A and B), from 10% to 22, 30 and 49% (Fig. 3C and D), and from 7% to 11, 45 and 85% (Fig. 3E and F) after treatment with IFA for 24 h at 5, 15 and 45 μM in Raji, K562 and Jurkat cells, respectively. These results suggested that IFA could promote the apoptosis of leukemia cells compared with control treatment.

IFA induces the expression of apoptotic proteins. Given that the mitochondrial-related apoptotic pathway is one of the main pathways of apoptosis (31), the effect of IFA was detected on the expression of cleaved PARP and cleaved caspase-3, two major mitochondrial apoptosis-associated proteins. Because IFA displayed more of an inhibitory effect on Jurkat cells, Jurkat cells were treated with IFA at 5, 15 and 45 μM for 24 h (Fig. 4A). The expression of cleaved PARP and cleaved caspase-3 dramatically augmented after incubation with the indicated concentrations of IFA. Statistical analysis indicated a dose-dependent manner in IFA-induced expression of cleaved PARP and cleaved caspase-3 (Fig. 4B and C). To further confirm the effect of IFA on cell apoptosis, K562 cells were treated with IFA at 5, 15 and 45 μM for 24 h; in these cells the cleavage of PARP and cleavage of caspase-3 was detected (Fig. 4D). Similarly, IFA dose-dependently increased cleaved PARP and cleaved caspase-3 levels (Fig. 4E and F). These data showed that IFA enhanced cell apoptosis by inducing the cleavage of PARP and caspase-3.

IFA modulates the phosphorylation of Cdc2 and expression of Cyclin B1. To further verify the effect of IFA-induced cell cycle arrest, the expression of cell cycle associated protein Cdc2 and Cyclin B1 were detected. In Fig. 5A, there was a significant increase in Cdc2 phosphorylation (Tyr15) and a decrease in the expression of Cyclin B1 after IFA treatment, compared with the control group in Jurkat cells. Fig. 5B and C showed that IFA significantly induced the phosphorylation of Cdc2 and attenuated the expression of Cyclin B1 in a dose-dependent manner. These results indicated that G2/M phase arrest induced by IFA was associated with increased phosphorylation of Cdc2 and the reduction of Cyclin B1 in Jurkat cells.

IFA attenuates Akt/mTOR signaling. Since the Akt/mTOR signaling pathway plays a crucial role in cancer cell survival, it was further explored whether IFA induced apoptosis and G2/M phase arrest via the modulation of Akt/mTOR signaling (28,29). Jurkat cells were treated with the indicated concentrations of IFA for 24 h. Treatment of IFA remarkably suppressed the phosphorylation of both Akt (Thr308) and mTOR (Ser2448; Fig. 5D). Additionally, a dose-dependent manner was found in the phosphorylation of Akt and mTOR after IFA treatment (Fig. 5E and F). These results indicated that IFA inhibited the Akt/mTOR signaling pathway in Jurkat cells.

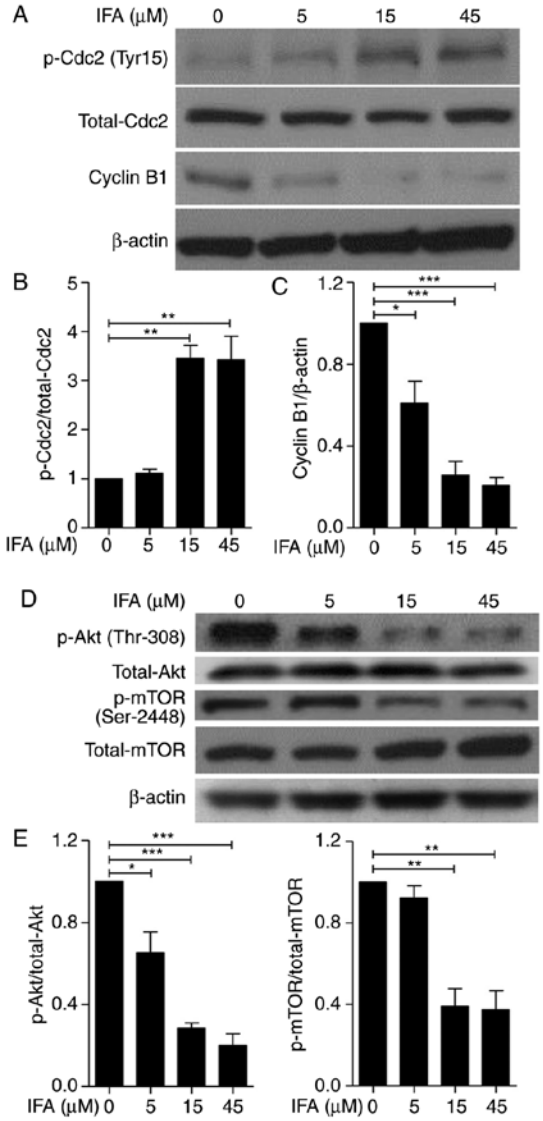

Figure 5. IFA induces the phosphorylation of Cdc2, and reduces the expression of Cyclin B1, as well as phosphorylation of Akt and mTOR in Jurkat cells. Jurkat cells were incubated with 5, 15 and 45 μM of IFA for 24 h. (A) Phosphorylation of Cdc2, the expression of Cyclin B1, and (D) the phosphorylation of Akt and mTOR were measured by western blotting. (B) The ratios of phosphorylation of Cdc2 to total Cdc2, (C) the expression of Cyclin B to β-actin, (E) phosphorylation of Akt to total Akt, and (F) phosphorylation of mTOR to total mTOR were quantified using ImageJ software. The expression of β-actin was detected as loading control. Data are expressed as mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. control group. IFA, isoferulic acid; p, phosphorylated.

Discussion

The presence of IFA leads to the dose-dependent inhibition of Raji, K562, and Jurkat cell proliferation. Microtubules are considered to be important targets for cancer treatment, as disruption of microtubule dynamics would result in cell cycle arrest, followed by cell apoptosis (32). Paclitaxel is an effective chemotherapy drug that could inhibit microtubule formation, resulting in G2/M cell cycle arrest (33). Cell cycle analysis demonstrated that the induction of G2/M phase arrest by IFA was concomitant with a decrease in G0/G1 and S phase in Raji, K562 and Jurkat cells. As is well known, activation of Cdc2, driven by Cyclin B1 binding and dephosphorylation at Tyr15 by Cdc25C, is an important stage in G2/M phase transition. Cyclin B1 accumulation is regarded as a marker of G2/M phase arrest, since it generally accumulates in G2 phase, reaches its peak at metaphase of cell division and decreases during anaphase (34). These results indicated that IFA could

induce G2/M phase arrest in Jurkat cells through Cyclin B1 accumulation and the inhibition of Cdc2 phosphorylation.

It is commonly known that programmed cell death can regulate cell survival/death balance through blockade of the cell cycle (35). A number of chemotherapeutic agents, such as doxorubicin, cisplatin and etoposide, have been shown to promote cell apoptosis by causing cell cycle arrest (36). In this study, IFA was shown to be capable of substantially increasing the level of apoptosis in Raji, K562 and Jurkat cells. In general, cell apoptosis can be activated through two signaling pathways, namely the mitochondrial pathway and the death receptor pathway (37). The mitochondrial pathway (also called the intrinsic pathway), which is mainly regulated by the Bcl-2 family, is identified as the leading apoptotic signaling pathway (36). The mitochondrial membrane potential decreases following a decrease in the intracellular Bcl-2/Bax ratio and an increase in the permeability of the outer mitochondrial membrane, resulting in the release of cytochrome C (38). The released cytochrome C in the cytosol can trigger the caspase cascade, after which PARP is cleaved as a substrate by caspase-3, leading to cell apoptosis (39). In this study the apoptosis evoked by IFA was shown to be associated with the upregulation of cleaved PARP and cleaved caspase-3. This suggested that IFA could induce apoptosis through the activation of mitochondria-associated intrinsic apoptosis signaling. Future studies will be aimed to determine how IFA modulates mitochondria-mediated apoptosis and which molecule is targeted by IFA.

Akt/mTOR signaling plays a vital role in the regulation of tumor cell survival, growth and apoptosis (23,24). Akt activation could prevent the release of apoptosis-stimulating factors from mitochondria by promoting the activation of antiapoptotic proteins (such as Bcl-2) and inhibiting some proapoptotic proteins (such as Bax and caspase) (40). Rapamycin analogs (rapalogs), the first generation of allosteric mTORC1 inhibitors, selectively bind to FK506 binding protein 12 of mTORC1 and inhibit mTOR signals (41). Rapalogs could reduce the proliferation of acute myeloid leukemia (AML) cells and clonogenicity of leukemic progenitor cells in preclinical and clinical settings (42). However, their effects were mainly cytostatic, partially affecting apoptosis, as confirmed in phase 1 and 2 clinical studies (43). The treatment of AML cells with a combination of PIM inhibitor AZD1897 and Akt inhibitor AZD5363 augmented the blockade of the mTOR axis and led to the induction of apoptosis (44). Since IFA had an impact on the inhibition of phosphorylation in the Akt/mTOR signal pathway, the combination of IFA and Akt inhibitors may be more effective for treatment of leukemia.

Furthermore, IFA displays various biological functions potentially beneficial in the treatment of various different diseases. Pretreatment with IFA could attenuate methylglyoxal (MG)-induced dysfunction and apoptosis in INS-1 pancreatic β-cells through the mitochondrial survival pathway and the upregulation of glyoxalase 1 activity (16). IFA derivatives also display varying degrees of anticancer potency, with some exhibiting excellent histone deacetylase inhibitory activity (45). IFA could prevent DNA damage and MG-induced protein glycation through free radical scavenging activity (46). Dilshara *et al* (9) demonstrated that IFA suppresses the production of not only nitric oxide and prostaglandin E2, but also their regulatory genes in lipopolysaccharide-stimulated BV2 microglial cells by inhibiting PI3K/Akt-dependent NF-κB activity and enhancing nuclear-related factor 2-mediated heme oxygenase-1 expression. In addition, IFA may play an important role in preventing diabetic complications by inhibiting advanced glycation end product formation and oxidation-dependent protein damage (15). The present findings indicated that IFA can induce leukemia cell apoptosis and initiate apoptotic signal pathways, thus suggesting the possibility of IFA having different targets in different diseases.

In the present study, IFA inhibited p-Akt and p-mTOR, suggesting that it might inhibit Raji, K562 and Jurkat cell proliferation and induce cell apoptosis via attenuation of Akt/mTOR signaling pathway. Taken together, this study indicated the therapeutic effect of IFA on Raji, K562 and Jurkat cells, thereby suggesting it as a promising candidate for the treatment of hematologic malignancy. Conduction of *in vivo* experiments will be the next step to determine whether IFA can inhibit leukemia cell proliferation in mouse models, and whether it can induce apoptosis *in vivo*, so as to accentuate the possibility of clinical application of IFA.

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Availability of data and materials

The analyzed datasets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZL and HZ designed research and revised the manuscript; GF and NZ performed the research analysis and wrote the manuscript; ZL GF NZ LW and HZ analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that there is no competing interests.

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