

## Original Article

# Icaritin induces cellular senescence by accumulating the ROS production and regulation of the Jak2/Stat3/p21 pathway in imatinib-resistant, chronic myeloid leukemia cells

Shicong Zhu<sup>1</sup>, Cheng Xing<sup>2,3</sup>, Guangsen Zhang<sup>2,3</sup>, Hongling Peng<sup>2,3</sup>, Zhihua Wang<sup>2,3</sup>

<sup>1</sup>Department of Geriatrics, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China;

<sup>2</sup>Department of Hematology, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China;

<sup>3</sup>Institute of Molecular Hematology, Central South University, Changsha, Hunan, China

Received November 25, 2020; Accepted June 18, 2021; Epub August 15, 2021; Published August 30, 2021

**Abstract:** In patients with chronic myelogenous leukemia (CML), resistance to tyrosine kinase inhibitor (TKI) therapy, like imatinib, can cause death, progression to accelerated phase or blast crises, and the need for maintenance treatment. Icaritin is an active component of the genus *Epimedium*, a traditional Chinese herbal medicine. Icaritin has been shown to notably inhibit the growth of CML cells. To explore the potential mechanisms of inhibiting growth and inducing cell senescence in imatinib-resistant CML cells by icaritin, MTT assays were used to assess the cell viability. The apoptosis and cell cycle arrest were evaluated using flow cytometry. The SA- $\beta$ -Gal staining and the intracellular reactive oxygen species (ROS) production were measured using flow cytometry to detect the senescent cells. qRT-PCR was conducted to assess the expression of the cell cycle-associated proteins, and western blotting was used to analyze the expressions of the JAK2 and STAT3 phosphorylation proteins. The results showed that icaritin inhibited cell growth and induced cell senescence in imatinib-resistant CML cells, which is associated with the regulation of the JAK2/STAT3/P21 axis and accompanied by the accumulation of ROS. Our data suggest that icaritin is a promising therapeutic strategy for the treatment of imatinib-resistant patients with CML.

**Keywords:** Icaritin, senescence, reactive oxygen species, Jak2/Stat3/p21 pathway, imatinib-resistant chronic myeloid leukemia

## Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by the expression of the BCR-ABL oncoprotein caused by the reciprocal translocation t(9; 22), which is a major driving force for the evolution of the disease. The suppression of the dysregulated tyrosine kinase activity using BCR-ABL tyrosine kinase inhibitors (TKIs), such as imatinib, dasatinib, and nilotinib, leads to persistent and frequent remissions of the disease from the hematologic, cytogenetic, and molecular points of view [1]. However, resistance to TKIs, especially imatinib, occurs in many patients. Several molecular mechanisms, including BCR-ABL mutations, BCR-ABL overexpression, and BCR-ABL-independent resistance, may contribute to TKI resistance in

patients with CML [2, 3]. Indeed, *BCR-ABL* mutations are found in more than 50% of all resistant patients [4, 5]. The *T315I* mutation has been determined to have the highest level of imatinib resistance and the worst clinical outcomes compared to other mutations [6]. The treatment failure with TKIs suggests that novel molecular therapeutic strategies are required to selectively target TKI-refractory CML cells.

Icaritin is the hydrolysate of icariin and is one of the main components extracted from the plants from the genus *Epimedium*, used in traditional Chinese herbal medicine. Icaritin has been demonstrated to have a multitude of pharmacological and biological activities in various studies. Icaritin induces neuronal and cardiac differentiation [7, 8], promotes the

activity of osteoblasts, and inhibits the activity and differentiation of osteoclasts [9], as well as the progression of hematological malignancies [10, 11]. Our previous studies showed that icaritin can significantly suppress CML cell progression and promote CML cell apoptosis via the MAPK/ERK/JNK and JAK2/STAT3/AKT signaling pathways [11]. We also showed that the anti-multiple myeloma (MM) activity of icaritin is mainly related to the suppression of the JAK2/STAT3 signaling pathway mediated by IL-6 *in vitro* and *in vivo* [12].

Cellular senescence can be induced by various stresses, including oxidative stress, ionizing radiation, and environmental toxins. Senescence is an irreversible cell growth arrest characterized by noticeable changes in cell function and morphology [13]. Senescence has been linked to multiple physiological processes, such as tumor growth suppression, wound healing, tissue repair, and embryonic development [14-16]. Recent evidence suggests that cellular senescence usually expresses multiple biomarkers, e.g., plasminogen activator inhibitor 1 (PAI-1), senescence-associated heterochromatin foci (SAHF), and senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal), together with an increased cell body size [17, 18]. Studies have shown that mitochondrial-derived reactive oxygen species (ROS) are released when the senescence signaling pathways, for instance, Ras, p53, p21 and p16 are activated [19-23].

The specific objective of this study was to investigate the function of icaritin in TKI-resistant CML using imatinib-resistant CML cells (K562R and BaF3/T315I) as *in vitro* models. We also assessed whether icaritin regulates cellular senescence. Our research found icaritin suppressed the proliferation of imatinib-resistant CML cells and induced cellular senescence, which is related to the accumulation of production of reactive oxygen species (ROS), the arrestation of the cell cycle, and the regulation of the JAK2/STAT3/p21 pathway. Our data suggested icaritin could be a promising drug for overcoming imatinib-resistant chronic myeloid leukemia therapy.

### Materials and methods

#### *Cell culture and reagents*

Imatinib-sensitive CML cells (K562 cells and BaF3/P210 cells) and imatinib-resistant CML

cells (K562R cells and BaF3/T315I cells) were stored in an RPMI-1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine (100 U/mL), and penicillin-streptomycin (100 U/mL), provided by the American Type Culture Collection (Manassas, VA, USA). Bone marrow mononuclear cells (BMMNCs) were extracted from the primary CML cells collected from the bone marrow samples of patients with BCR-ABL T315I CML using the Ficoll-Paque isolation solution. A total of six CML patients were enrolled in the study, including three patients in the chronic phase and three patients with blast crisis. All the patients signed the written informed consent. This study was conducted according to the Declaration of Helsinki and was approved by the Institutional Review Board of the Second Xiang-Ya Hospital, Central South University.

Icaritin with a purity of 99.5% was provided by Dr. Kun Meng (Shenogen Pharma Group, Beijing, China). Icaritin was dissolved in DMSO (Sigma, St. Louis, MO, USA) to prepare a stock solution (32 mM) and stored at -20°C. The (4, 5-Dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide (MTT), and 5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactoside (X-gal) were procured from Sigma. The STAT3, phospho-STAT3, and  $\beta$ -actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The JAK2, Phosphor-JAK2, and P21 antibodies, and the AG490 were obtained from Abcam Ltd. (Hong Kong, China).

#### *Assessment of the cell viability*

CML cell lines (K562, BaF3/P210, K562R, BaF3/T315I) and primary CML cells were treated with icaritin at concentrations of 0, 2, 4, 8, 16, and 32  $\mu$ M for 48 h, and the cell viability was assessed with MTT. Additionally, we evaluated the viability of the CML cells treated with icaritin combined with imatinib compared to icaritin or imatinib alone.

#### *Cell apoptosis analysis*

Imatinib-resistant CML cells ( $5 \times 10^5$  cells/mL; K562R, BaF3/T315I) were incubated for 48 h with icaritin at the concentrations indicated above and seeded in a 6-well plate. Annexin V-FITC/propidium iodide (PI) apoptosis detection kits (Becton Dickinson, BD, USA) were used in combination with flow cytometry (FACSCalibur, BD, USA) to evaluate the early apoptosis.

## Icaritin induces cellular senescence

**Table 1.** Sequences of the oligonucleotide primers used for the RT-qPCR in K562R cells

Gene	Sequence
Cyclin D1	fwd: 5'-AGGAACAGAAGTGCAGGAGG-3' rev: 5'-GGATGGAGTTGTCGGTGTAGATG-3'
Cyclin E	fwd: 5'-TGCTGGACAAAGCCCGAGCAAAGA-3' rev: 5'-CGCTGCTCTGCTTCTTACCGCTCT-3'
Cyclin A	fwd: 5'-TCCAAGAGGACCAGGAGAATATCA-3' rev: 5'-GTGCAACCCGTCTCGTCTTC-3'
CDK4	fwd: 5'-GTGTCGGTGCCTATGGGACAGTGT-3' rev: 5'-AGCCTCCAGTCGCCTCAGTAAAGC-3'
CDK2	fwd: 5'-CCAGAAACAAGTTGACGGGAGAGG-3' rev: 5'-CCAGTGAGAGCAGAGGCATCCA-3'
P53	fwd: 5'-CATCTACAAGCAGTCACAGCACAT-3' rev: 5'-CCAACCTCAGGCGGCTCATA-3'
P21	fwd: 5'-TGTCACCGAGACACCACTGGAG-3' rev: 5'-AGCGAGGCACAAGGGTACAAGA-3'
$\beta$ -actin	fwd: 5'-TTCCAGCCTTCTTCTCTGGG-3' rev: 5'-TTGCGCTCAGGAGGAGCAAT-3'

CDK4, cyclin-dependent kinase 4; CDK2, cyclin-dependent kinase 2; fwd, forward; rev, reverse.

**Table 2.** Sequences of the oligonucleotide primers used for the RT-qPCR in BaF3/T315I cells

Gene	Sequence
Cyclin D1	fwd: 5'-GCAGACCATCCGCAAGCAT-3' rev: 5'-GGTAGCAGGAGAGGAAGTTGTTG-3'
Cyclin E	fwd: 5'-CTCAGGTTATCAGTGGTGCACAT-3' rev: 5'-TGGCTTTCTTTGCTTGGGCTTTG-3'
Cyclin A	fwd: 5'-GCATCAGGAAGACCAAGAGAA-3' rev: 5'-TGAAGGACCAGCAGTGACA-3'
CDK4	fwd: 5'-TTGGTGTGGTGCCTATGG-3' rev: 5'-GCCTCCAGCCTCCTTAACAA-3'
CDK2	fwd: 5'-TTCATGGATGCCTCTGCTCTCA-3' rev: 5'-GGCTCTTGCTAGTCCAAAGTCTG-3'
P53	fwd: 5'-CAAGTGAAGCCCTCCGAGTGT-3' rev: 5'-GCCAGGTGGAAGCCATAGTT-3'
P21	fwd: 5'-CCGTGGACAGTGAGCAGTT-3' rev: 5'-CAGACGAAGTTGCCCTCCA-3'
$\beta$ -actin	fwd: 5'-CCTGTATGCCTCTGGTCTGTA-3' rev: 5'-CTGTGGTGGTGAAGCTGTAG-3'

CDK4, cyclin-dependent kinase 4; CDK2, cyclin-dependent kinase 2; fwd, forward; rev, reverse.

### RT-qPCR assays

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the concentration of the extracted RNA

was determined using Nanodrop2000 (OD260) (Thermo Fisher Scientific). RNA (1  $\mu$ g) was reverse transcribed to complementary DNA (cDNA) using PrimeScript RT kits (Takara Bio Inc., Japan) under standard conditions with random primers. SYBR PreMix Ex Taq (Takara Bio Inc., Japan) kits and the LightCycler 96 Detection System (Roche, Sweden) were used for the PCR measurement. The reaction system of the reverse transcription kit was 20  $\mu$ L, including 10  $\mu$ L of SYBR premix (2  $\times$ ), 0.5  $\mu$ L each of the forward and reverse primers of the target genes, 2.0  $\mu$ L of the cDNA template and 7  $\mu$ L of the ddH<sub>2</sub>O. The reaction conditions were set to pre-denaturation for 30 s at 95°C, 1 cycle, then denaturation for 10 s at 95°C, annealing for 10 s at 58°C, extension for 10 s at 72°C, for 40 cycles in total. The relative expression levels were quantified using the 2<sup>- $\Delta\Delta$ CT</sup> method and using  $\beta$ -actin as a reference. The primer sequences are listed in **Tables 1** and **2**.

### Determination of the SA- $\beta$ -galactosidase positive cells

The imatinib-resistant CML cells (K562R, BaF3/T315I) were treated with icaritin (16  $\mu$ M) for 48 h or AG490 (25  $\mu$ M), a specific JAK2 inhibitor, for 1 h and plated in a 6-well plate. After being washed twice with phosphate-buffered saline (PBS), the cells were fixed in formaldehyde for 30 min. The staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl  $\beta$ -D galactopyranoside (X-gal), 5 mM K<sub>3</sub>Fe [CN]<sub>6</sub>, 2 mM MgCl<sub>2</sub> in PBS, pH 7.2) and incubated in the dark at 37°C for 4 h. At least 300 cells were counted in the phase-contrast photomicrographs of the selected fields, and we scored the percentages of the SA- $\beta$ -Gal.

### Measurement of the ROS production

The ROS activity was measured after the acetate groups of membrane-permeable 2,7-dichlorofluorescein diacetate (H2DCFDA) were cleaved by the intracellular esterase and oxidized to fluorescent 2,7-dichlorofluorescein (DCF) by ROS. Briefly, the K562R and BaF3/T315I cells were incubated with icaritin (16  $\mu$ M) for 48 h or AG490 (25  $\mu$ M) for 1 h in a 6-well plate. Then, the collected cells were washed with PBS. Following the application of the DCFDA dye solution (10  $\mu$ M), the cells were incubated for 30 min. Next, the cells were

## Icaritin induces cellular senescence

washed exhaustively with PBS and analyzed using flow cytometry (FACSCalibur, BD).

### *Cell cycle analysis*

The harvested K562R and BaF3/T315I cells were treated with 0, 8, and 16  $\mu\text{M}$  icaritin for 48 h and washed with ice-cold PBS. The cells were fixed overnight with 70% cold ethanol and preconditioned with 10  $\mu\text{g}/\text{mL}$  RNase for 30 min. Furthermore, the cells were marked with propidium iodide (Sigma) and the ModFit LT 3.0 software package was used to determine the cell-cycle profiles on the FACS-Calibur flow cytometry.

### *Western blotting analysis*

The K562R and BaF3/T315I cells were treated with different concentrations of icaritin (0, 4, 8, 16, and 32  $\mu\text{M}$ ) for 48 h. After a thorough washing, the cells were lysed with a lysis buffer (Pierce® IP lysis buffer, Thermo, USA) containing 2 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride. After quantifying the protein concentrations, equal amounts of protein (approximately 30  $\mu\text{g}/\text{well}$ ) were separated on 8%-15% SDS-PAGE and then transferred onto nitrocellulose membranes. Finally, we used several primary antibodies and HRP-conjugated secondary antibodies to label the membranes, which were visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech).

### *Statistical analysis*

For the indicated number of separate experiments, all the values were calculated as the mean  $\pm$  SEM. One-way analyses of variance (ANOVA) followed by post hoc Dunnett's tests were carried out to compare three or more groups. Two-tailed Student's t-tests were used to compare the two groups. All the data were statistically analyzed using GraphPad Prism 5 software (GraphPad, USA). Statistical significance was set at  $P < 0.05$ .

## Results

### *Icaritin suppresses the proliferation of both the CML cell lines and the primary CML cells*

To study the effect of icaritin on the growth of the CML cells, imatinib-sensitive cells (K562

cells and BaF3/P210 cells) and imatinib-resistant cells (K562R cells and BaF3/T315I cells) were treated with various concentrations of icaritin for 48 h, and the inhibitory effect on cell proliferation was evaluated using MTT assays. Similarly, we tested the inhibitory effects on the proliferation of the primary BCR-ABL T315I-CML BMMNCs treated with different concentrations of icaritin. Moreover, we compared the inhibitory effects of icaritin, imatinib, and icaritin in combination with imatinib on the K562R cells and the BaF3/T315I cells. Our data showed that the effect of icaritin on the suppression of the CML cell proliferation was dose-dependent. The IC<sub>50</sub> values of icaritin were  $6.474 \pm 1.11 \mu\text{M}$  (K562 cells),  $7.628 \pm 1.34 \mu\text{M}$  (K562R cells),  $6.926 \pm 1.44 \mu\text{M}$  (BaF3/P210 cells), and  $4.580 \pm 0.88 \mu\text{M}$  (BaF3/T315I cells) (**Figure 1A**). The growth of the primary BCR-ABL T315I-CML BMMNCs was significantly suppressed by the icaritin (**Figure 1B**). Additionally, we confirmed that the K562R and BaF3/T315I cells were resistant to imatinib but were sensitive to icaritin (**Figure 1C** and **1D**). Our results showed that the icaritin had excellent anti-CML activity, particularly in imatinib-resistant CML cells *in vitro*.

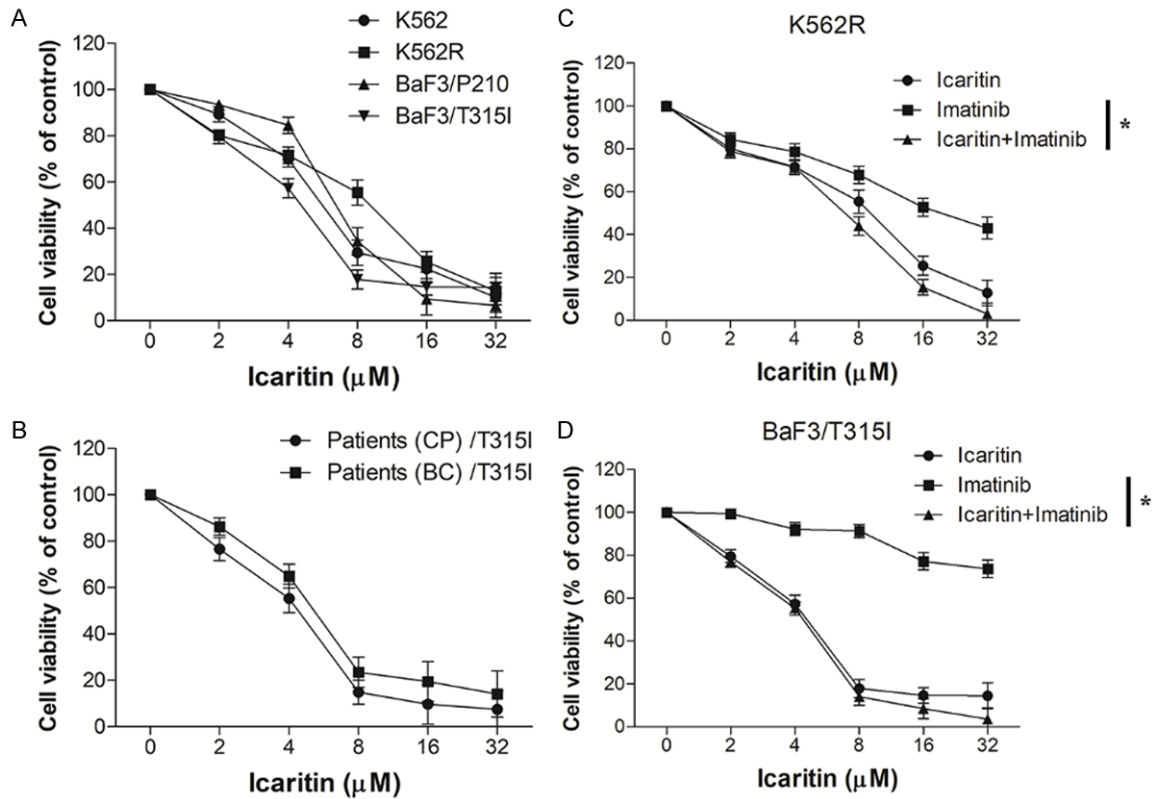
### *Icaritin induces apoptosis in BaF3/T315I cells but not in K562R cells*

To determine whether the effect of icaritin is related to apoptosis in the imatinib-resistant CML cells, we treated the K562R and BaF3/T315I cells with diverse concentrations of icaritin for 48 h, and the ratio of the apoptosis was evaluated through flow cytometry using the Annexin V-FITC/PI assay. Interestingly, the icaritin did not induce apoptosis in the K562R cells, but it induced apoptosis in the BaF3/T315I cells at high concentrations ( $\geq 16 \mu\text{M}$ ) (**Figure 2A, 2B**).

### *Icaritin induces imatinib-resistant CML cell senescence and increases the ROS levels*

To determine the activity of the SA- $\beta$ -Gal, a biomarker of aging and senescence extensively used in mammalian cells, the K562R and BaF3/T315I cells were stained with a SA- $\beta$ -Gal staining kit after the icaritin (8, 16  $\mu\text{M}$ ) treatment for 48 h or the AG490 (25  $\mu\text{M}$ ) treatment for 1 h. As shown in **Figure 3A**, the percentage of the senescent SA- $\beta$ -Gal-positive cells incre-

## Icaritin induces cellular senescence



**Figure 1.** The effects of icaritin and imatinib on the growth inhibition of CML cells. A. The dose-response curve for the effects of icaritin in the cell survival of imatinib-sensitive cells (K562 cells and BaF3/P210 cells) and imatinib-resistant cells (K562R cells and BaF3/T315I cells). The values represent the mean  $\pm$  SEM of triplicate cultures. B. The effect of icaritin on the growth inhibition on primary BCR-ABL T315I-CML BMMNCs (n = 6) from three patients in the chronic phase and three patients with blast crisis. Mean  $\pm$  SEM. C and D. Both the K562R cells and the BaF3/T315I cells were treated with icaritin, imatinib, or icaritin combined with imatinib and the cell viability was analyzed using MTT. The data represent the percentages of surviving cells. Mean  $\pm$  SEM. Asterisks indicate statistically significant differences compared with imatinib treatment using 2-tailed Student's t-tests. \* $P < 0.05$ .

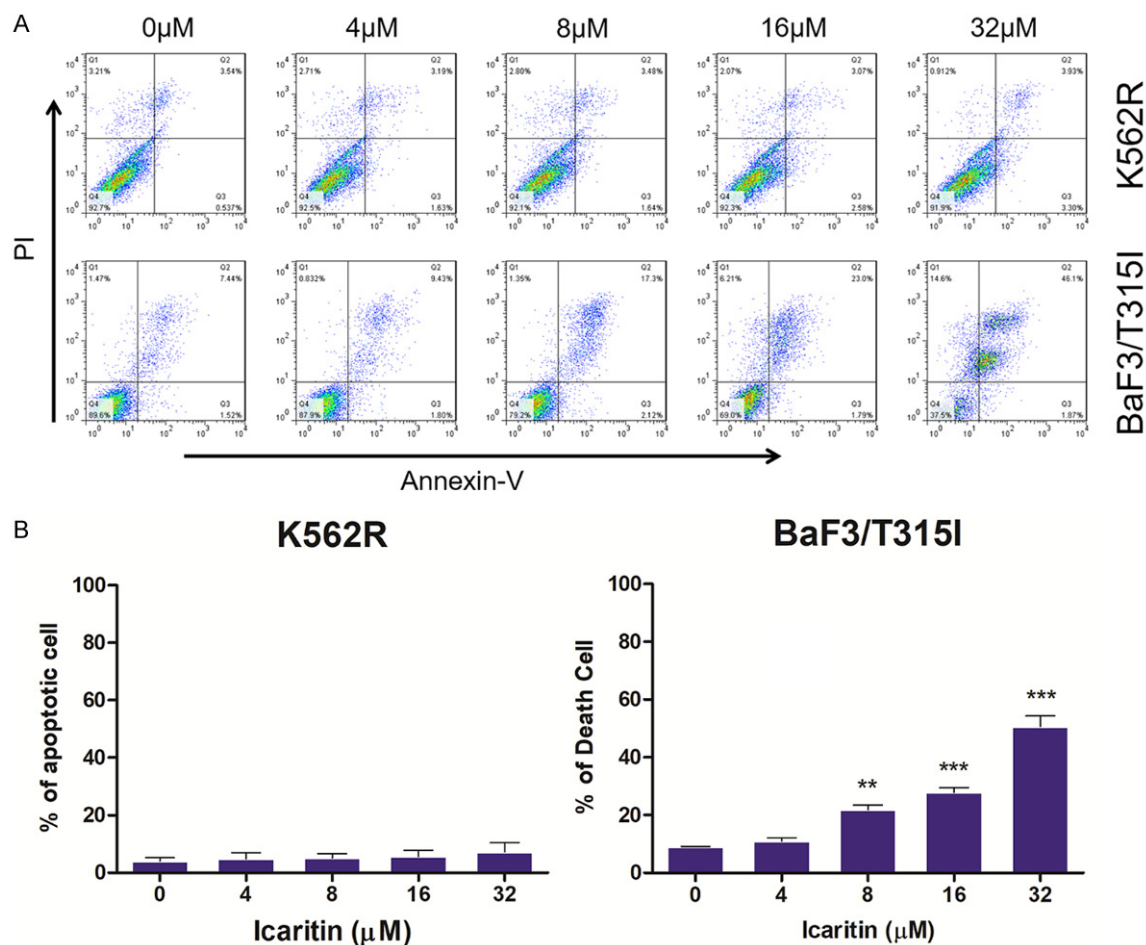
used after the treatment with icaritin or AG490 in a dose-dependent manner (Figure 3B).

As a secondary messenger in various cellular signaling pathways, ROS are end products of metabolism, and they cause senescence as a result of inordinate ROS accumulation [24]. To further explore the function of ROS in icaritin-induced senescence, the DCF biomarker was used to estimate the production of intracellular ROS using flow cytometry. Our results indicated that intracellular ROS levels were notably increased in cells treated with icaritin and AG490, compared with the control cells, while there was no significant difference between the two treatments. These data suggested that icaritin induced senescence associated with the accumulation of ROS in the K562R and BaF3/T315I cells (Figure 3C, 3D).

*Icaritin arrests the cell cycle of the imatinib-resistant CML cells at the G1 phase*

To further investigate the role of icaritin in imatinib-resistant cells, we analyzed whether growth inhibition was linked with cell cycle arrest by evaluating the cell cycle distribution of the K562R and BaF3/T315I cells. We found that icaritin significantly blocked the cell cycle at G1 phase in a concentration-dependent manner (Figure 4A, 4B). Subsequently, we measured the expressions of the cell cycle-associated proteins using qRT-PCR and found that icaritin downregulated the cyclin D1, cyclin E, cyclin A, CDK2, and CDK4 expressions in the BaF3/T315I cells. Similarly, icaritin downregulated the cyclin D1, cyclin A, and CDK4 levels, but it did not affect the cyclin E and CDK2 expressions in the K562R cells (Figure 4C).

## Icaritin induces cellular senescence



**Figure 2.** Icaritin induced apoptosis in the BaF3/T315I cells but not in the K562R cells. A. The K562R cells and BaF3/T315I cells were treated with different concentrations of icaritin for 48 h. The early apoptosis was assessed through flow cytometry using an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit. B. The columns represent the average percentage of the Annexin V positive cells from more than three independent experiments, which are shown as the mean  $\pm$  SEM. The asterisks indicate statistically significant differences compared with the controls (0  $\mu$ M of icaritin) using one-way ANOVA followed by post hoc Dunnett's tests. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

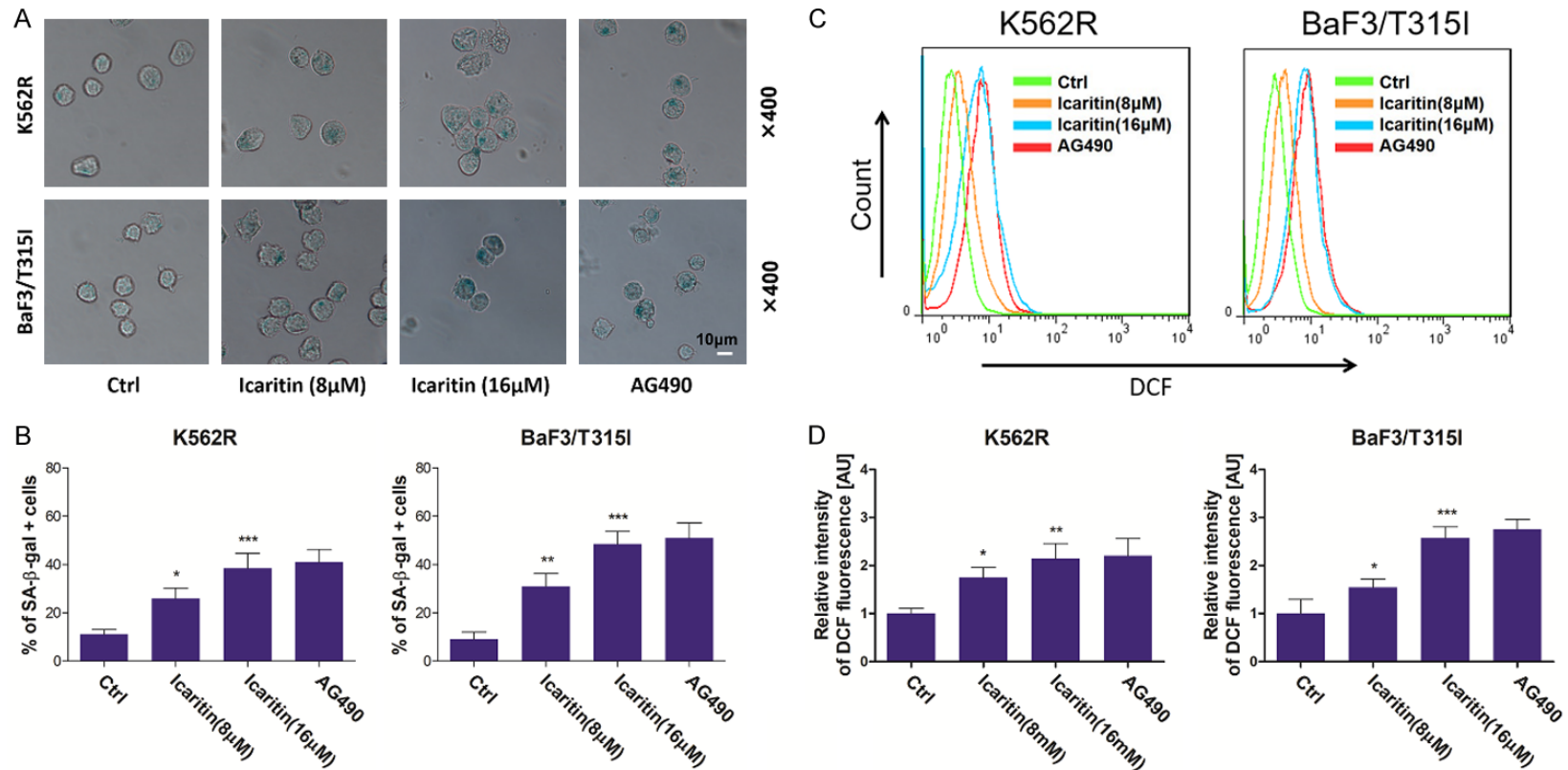
### Induction of cellular senescence via the JAK2/STAT3/p21 pathways

As a tumor suppressor, p53 is not only related to cell cycle arrest, apoptosis, and the induction of cellular senescence, but it is also a target of ROS. Additionally, p21, which is a cyclin-dependent kinase inhibitor and a downstream effector of p53, plays a significant role in aging [20, 23]. We further examined the p53 and p21 levels using qRT-PCR and determined that icaritin induced the expression of p21 in a dose-dependent manner in K562R and BaF3/T315I cells. The p53 levels were upregulated in the K562R cells, which accompany the p53 mutations, and the p53 levels were downregulated in the BaF3/T315I cells (Figure 5A). This

evidence indicates that p21 plays a critical role in cell senescence induced by icaritin but is independent of p53. Moreover, we explored the p21 expressions in K562R and BaF3/T315I cells treated with AG490 and found that AG490 also increased the expression of p21 (Figure 5B).

Multiple transcription factor binding sites are present in the p21 promoter, especially the common binding sites for the signal transducer and the activator of transcription (STAT) proteins and SIE sequences [25]. To better understand the mechanism by which icaritin induces the upregulation of p21, we investigated the role of the JAK2/STAT3 pathway and evaluated the expressions of the phosphorylated JAK2

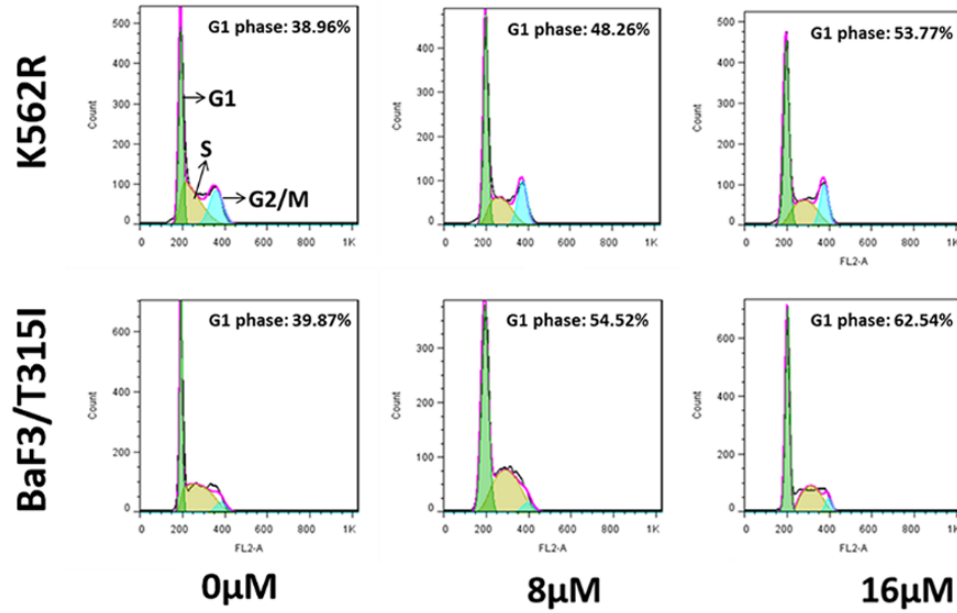
## Icaritin induces cellular senescence



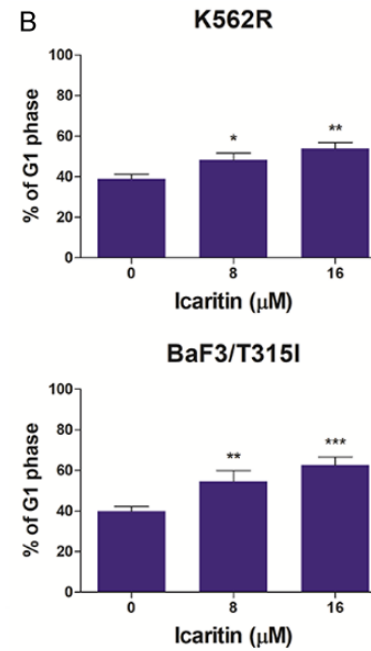
**Figure 3.** Icaritin induced senescence in the K562R cells and the BaF3/T3151 cells. **A.** Representative photomicrographs of SA-β-Gal staining in the control cells or the cells after being treated with icaritin (8, 16 μM) for 48 h or AG490 (25 μM) for 1 h. The cells stained in blue represent the SA-β-Gal-positive senescent cells. The original magnification was 400 (objective lens 40) under a lighted microscope (Olympus BX-50 microscope), and the images were captured using DP Controller software (Olympus) at room temperature. Scale bars, 10 μm. **B.** The percentage of the SA-β-Gal-positive cells was analyzed and scored by counting at least 300 cells in phase contrast photomicrographs of representative fields. The data (mean ± SEM) are based on three different cell cultures. Asterisks represent statistically significant differences ( $P < 0.05$ ). **C and D.** The K562R and BaF3/T3151 cells were treated with icaritin (8, 16 μM) or AG490 (25 μM). Intracellular ROS production was assessed using DCF biomarkers and analyzed using flow cytometry. The percentage (%) of the positive cells refers to the fraction of the fluorescent cells, indicating an ROS presence. A typical result from three independent experiments is shown. Columns represent the relative intensity of DCF fluorescence from more than three independent experiments, which are shown as the mean ± SEM. Asterisks indicate statistically significant differences compared with the controls using one-way ANOVA followed by post hoc Dunnett's test. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

Icaritin induces cellular senescence

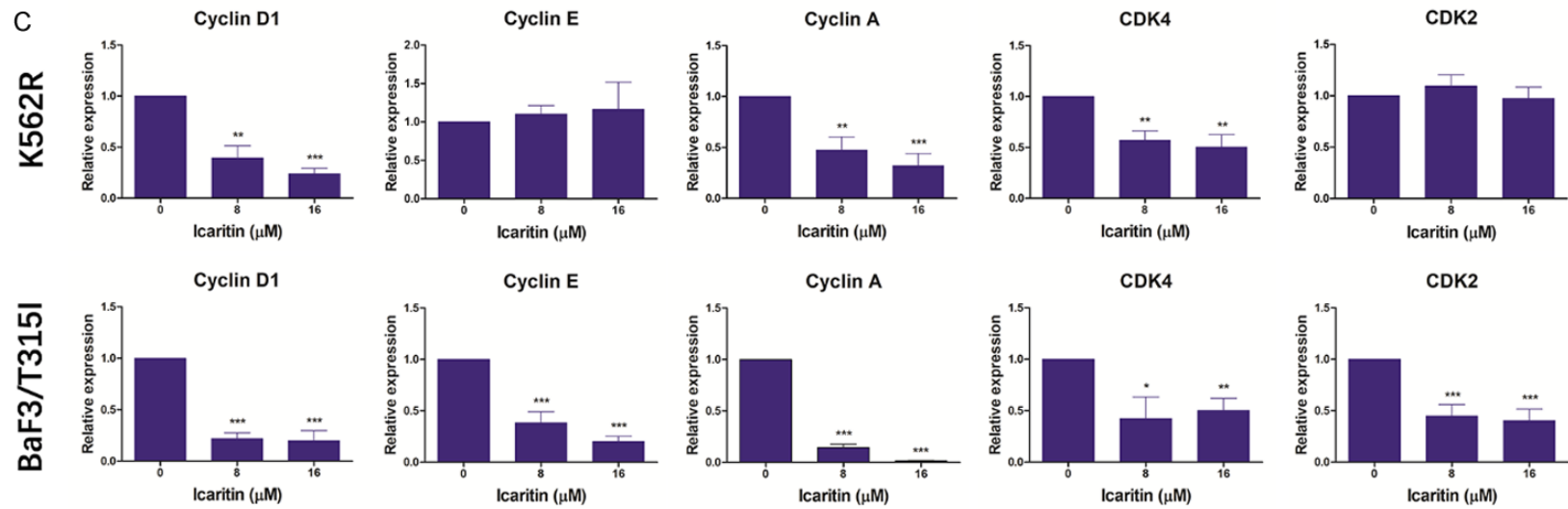
A



B



C





## Icaritin induces cellular senescence

**Figure 4.** Icaritin induced imatinib-resistant CML cell cycle arrest at the G1 phase. A and B. The K562R cells and BaF3/T315I cells were treated with different concentrations of icaritin for 48 h and measured using flow cytometry. The columns represent the average percentages of the G1 phase arrested cells. Mean  $\pm$  SEM. C. The expressions of Cyclin D1, Cyclin E, Cyclin A, CDK2, and CDK4 were downregulated in the BaF3/T315I cells after being exposed to icaritin, but in the K562R cells, only the Cyclin D1, Cyclin A, and CDK4 levels were downregulated by icaritin. The data are shown as the mean  $\pm$  SEM. Asterisks indicate statistically significant differences compared with controls (0  $\mu$ M of icaritin) using one-way ANOVA followed by post hoc Dunnett's test. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

and STAT3 proteins. Icaritin notably inhibited the p-JAK2 and p-STA3 expressions (**Figure 5C**). In addition, treatment with the JAK2 inhibitor AG490 suppressed the phosphorylation of JAK2 and STAT3, but it increased the p21 levels (**Figure 5D**). Altogether, these findings indicate that icaritin induces cell senescence through the inhibition of the JAK2/STAT3/p21 axis.

### Discussion

CML patient outcomes have dramatically improved, and survival rates have been upgraded to a practically normal lifespan after the advent of TKIs. However, more than a quarter of patients fail to respond to the first-line TKI therapy, and many of these patients develop an accelerated phase or a blast crisis [26]. Resistance to imatinib is the most common form of TKI resistance. T315I, a common mutation, is found in approximately 20% of imatinib-resistant patients. Therefore, new treatment options for TKI-refractory CML cells are needed to improve CML prognosis. In this study, we observed that the growth of the CML cells resistant to imatinib, such as the K562R and BaF3/T315I cells, was inhibited by the icaritin treatment. Additionally, we controlled the primary BCR-ABL T315I-CML BMMNCs of six CML patients with different concentrations of icaritin, showing that it remarkably suppressed the primary CML cell proliferation. Furthermore, we demonstrated that high concentrations of icaritin treatment induced the apoptosis of the BaF3/T315I and K562R cells, but the effect was stronger in the K562R cells than it was in the BaF3/T315I cells.

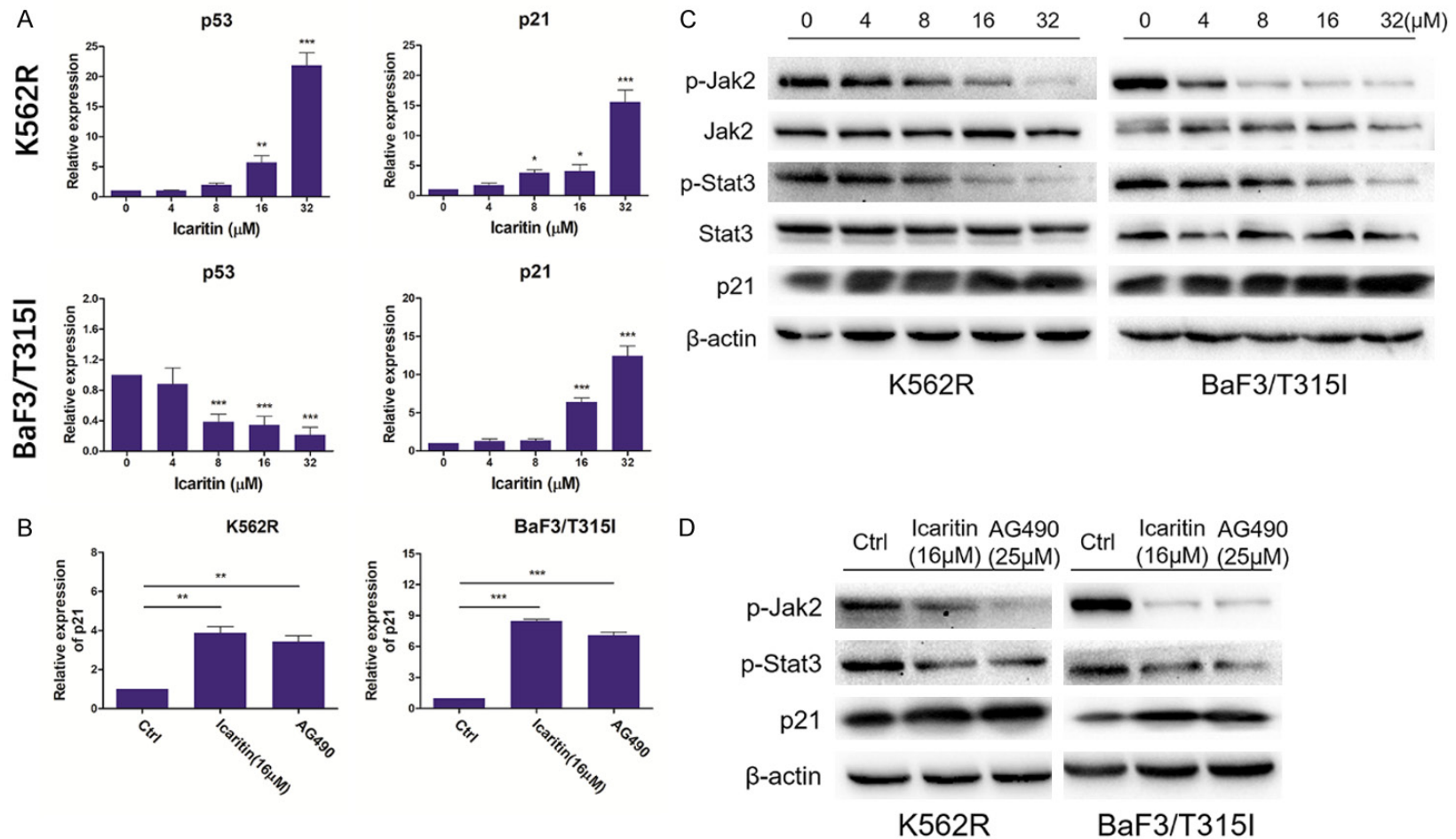
Currently, there are drugs that are capable of inducing senescence in cancer cells. In this study, icaritin treatment in K562R and BaF3/T315I cells induced senescence and caused very distinctive changes in morphology, such as an enlargement of cell volume and an increase in the label for  $\beta$ -galactosidase, a biomarker of senescence. In addition to maintaining the steady state of cell growth, ROS is essential in

the regulation of cell senescence, which is induced by ROS accumulated in the mitochondria or other intracellular compartments [27, 28]. We showed that icaritin stimulates the K562R and BaF3/T315I cells to generate ROS. These findings suggest that icaritin induces cellular senescence in imatinib-resistant CML cells, which is associated with the accumulation of ROS.

Senescence is a stress response that is initiated by multiple mechanisms. Senescent cells retreat from the cell cycle promoted by the growth factors or the mitogens and lose the ability to proliferate [29]. Recent studies have found that cellular senescence is associated with some signaling pathways, including the p53/p21 [30], p16-pRb [31], and p53/p16-independent signaling pathways [32]. Hence, we evaluated the expressions of p53, p21, and p16 and found that p21 was increased in both types of cells, and p53 was upregulated in K562R cells but downregulated in BaF3/T315I cells. Interestingly, p16 was not detected in either of the two cell types (data not shown). Notably, p21 plays an important role through a p53-independent pathway in the cellular senescence induced by icaritin in K562R and BaF3/T315I cells.

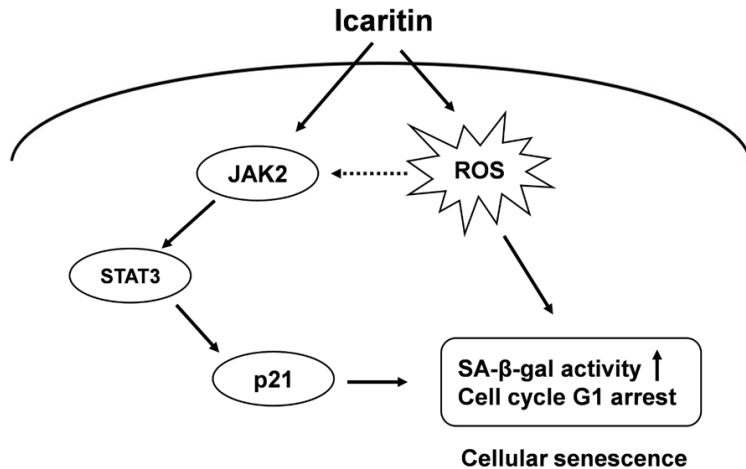
P21 functions as a cell cycle inhibitor, inhibiting the cyclin kinase complex, proliferating cell nuclear antigens, and inhibiting the transcription factors and coactivators, which results in tumor growth arrest [33]. Icaritin promotes cell cycle arrest at the G1 phase and decreases the expressions of cyclin D1, cyclin A, CDK2, and CDK4, suggesting that the effect of CML cell cycle arrest by icaritin is associated with the upregulation of p21 expression and decreased cell cycle-related proteins. The p21 gene appears to be directly regulated by STAT, and three sequences in this promoter contain potential STAT-binding sites [25]. The expressions of multiple cancer-related genes, such as tumor angiogenesis, oncogenic cell signaling, tumor immune surveillance, and metastasis,

## Icaritin induces cellular senescence



**Figure 5.** Icaritin induced cellular senescence via the JAK2/STAT3/p21 pathway. A. Icaritin induced the expression of p21 in a dose-dependent manner in K562R and BaF3/T315I cells, and the P53 levels were upregulated in the K562R cells and downregulated in the BaF3/T315I cells. The data represent mean  $\pm$  SEM. Asterisks indicate statistically significant differences compared with the controls (0  $\mu$ M of icaritin) using one-way ANOVA followed by post hoc Dunnett's tests. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . B. The K562R and BaF3/T315I cells were treated with icaritin (16  $\mu$ M) and AG490 (25  $\mu$ M) for 48 h. The expression of p21 was analyzed using qRT-PCR. The results showed that AG490 also increased the p21 expression. The asterisks indicate statistically significant differences compared with the controls determined using 2-tailed Student's t-test. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . C. The K562R and BaF3/T315I cells were treated with different concentrations of icaritin; the cell lysates were analyzed by western blotting. D. AG490, a specific JAK2 inhibitor, also suppressed the phosphorylation of JAK2 and STAT3 and increased the p21 expression; these figures are from three separate experiments.

## Icaritin induces cellular senescence



**Figure 6.** A schematic summary of the signaling pathway underlying the senescence of the imatinib-resistant CML cells induced by icaritin. Icaritin can induce the senescence of imatinib-resistant K562R and BaF3/T315I cells, which is related to the accumulation of production of ROS and the inhibition of the JAK2/STAT3/p21 pathway.

are regulated by phosphorylated STAT3, which has transcriptional activity [34]. Based on this evidence, we proposed that icaritin regulates p21 expression by suppressing the activation of JAK2/STAT3 signaling. To validate this hypothesis, we examined the phosphorylation of JAK2 and STAT3. In addition, AG490 was used to treat the K562R and BaF3/T315I cells. We found that icaritin inhibits the phosphorylation of JAK2 and STAT3. Consistently, the JAK2 inhibitor, AG490, also represses JAK2/STAT3 activation and increased p21 expression.

Nevertheless, our study has certain limitations. First, whether there is a relationship between ROS accumulation and the activation of the JAK2/STAT3/P21 pathway needs to be investigated. Additionally, icaritin inhibits the proliferation of imatinib-resistant CML cell lines by inducing cellular senescence, which needs to be further validated on primary cells. Finally, the antitumor effect of icaritin and whether cellular senescence is involved in this effect need to be further verified in animal models.

In conclusion, icaritin plays a vital role as an anti-tumor agent in imatinib-resistant CML cells, inducing the inhibition of proliferation, the induction of apoptosis, cell cycle arrest, and cellular senescence induction. More importantly, we revealed the effect of icaritin on the induction of senescence in CML cells, which is associated with the regulation of the JAK2/STAT3/P21 axis and accompanied by the accu-

mulation of ROS (Figure 6). These results suggest that icaritin is a potential option as a promising therapeutic strategy for imatinib-resistant chronic myeloid leukemia.

### Acknowledgements

We thank Dr. Kun Meng (Shenogen Pharma Group, Beijing, China) for providing icaritin. This work was supported by the National Natural Science Foundation, P. R. China (grant nos. 81600183 and 8207-0175).

### Disclosure of conflict of interest

None.

**Address correspondence to:** Zihua Wang and Hongling Peng, Department of Hematology, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China; Institution of Molecular Hematology, The Second XiangYa Hospital, Central South University, No. 139 Middle Renmin Road, Changsha 410011, Hunan, China. E-mail: wangzhihuazsc@csu.edu.cn (ZHW); penghongling@csu.edu.cn (HLP)

### References

- [1] An X, Tiwari AK, Sun Y, Ding PR, Ashby CR Jr and Chen ZS. BCR-ABL tyrosine kinase inhibitors in the treatment of Philadelphia chromosome positive chronic myeloid leukemia: a review. *Leuk Res* 2010; 34: 1255-1268.
- [2] Kantarjian HM, Talpaz M, Giles F, O'Brien S and Cortes J. New insights into the pathophysiology of chronic myeloid leukemia and imatinib resistance. *Ann Intern Med* 2006; 145: 913-923.
- [3] Yaghmaie M and Yeung CC. Molecular mechanisms of resistance to tyrosine kinase inhibitors. *Curr Hematol Malig Rep* 2019; 14: 395-404.
- [4] Jabbour E, Kantarjian H, Jones D, Talpaz M, Bekele N, O'Brien S, Zhou X, Luthra R, Garcia-Manero G, Giles F, Rios MB, Verstovsek S and Cortes J. Frequency and clinical significance of BCR-ABL mutations in patients with chronic myeloid leukemia treated with imatinib mesylate. *Leukemia* 2006; 20: 1767-1773.
- [5] Lahaye T, Riehm B, Berger U, Paschka P, Muller MC, Kreil S, Merx K, Schwindel U, Schoch C, Hehlmann R and Hochhaus A. Response and

## Icaritin induces cellular senescence

- resistance in 300 patients with BCR-ABL-positive leukemias treated with imatinib in a single center: a 4.5-year follow-up. *Cancer* 2005; 103: 1659-1669.
- [6] Etienne G, Dulucq S, Huguet F, Schmitt A, Lascaux A, Hayette S, Fort MP, Sujobert P, Bijou F, Morisset S, Tavitian S, Bidet A, Turcq B, Robbesyn F, Chollet C, Belloc F, Durrieu F, Mahon FX and Nicolini FE. Incidence and outcome of BCR-ABL mutated chronic myeloid leukemia patients who failed to tyrosine kinase inhibitors. *Cancer Med* 2019; 8: 5173-5182.
- [7] Wang Z, Wang H, Wu J, Zhu D, Zhang X, Ou L, Yu Y and Lou Y. Enhanced co-expression of beta-tubulin III and choline acetyltransferase in neurons from mouse embryonic stem cells promoted by icaritin in an estrogen receptor-independent manner. *Chem Biol Interact* 2009; 179: 375-385.
- [8] Wo YB, Zhu DY, Hu Y, Wang ZQ, Liu J and Lou YJ. Reactive oxygen species involved in prenylflavonoids, icariin and icaritin, initiating cardiac differentiation of mouse embryonic stem cells. *J Cell Biochem* 2008; 103: 1536-1550.
- [9] Huang J, Yuan L, Wang X, Zhang TL and Wang K. Icaritin and its glycosides enhance osteoblastic, but suppress osteoclastic, differentiation and activity in vitro. *Life Sci* 2007; 81: 832-840.
- [10] Li Q, Huai L, Zhang C, Wang C, Jia Y, Chen Y, Yu P, Wang H, Rao Q, Wang M and Wang J. Icaritin induces AML cell apoptosis via the MAPK/ERK and PI3K/AKT signal pathways. *Int J Hematol* 2013; 97: 617-623.
- [11] Zhu J, Li Z, Zhang G, Meng K, Kuang W, Li J, Zhou X, Li R, Peng H, Dai C, Shen JK, Gong F, Xu Y and Liu S. Icaritin shows potent anti-leukemia activity on chronic myeloid leukemia in vitro and in vivo by regulating MAPK/ERK/JNK and JAK2/STAT3/AKT signalings. *PLoS One* 2011; 6: e23720.
- [12] Zhu S, Wang Z, Li Z, Peng H, Luo Y, Deng M, Li R, Dai C, Xu Y, Liu S and Zhang G. Icaritin suppresses multiple myeloma, by inhibiting IL-6/JAK2/STAT3. *Oncotarget* 2015; 6: 10460-10472.
- [13] Kuilman T, Michaloglou C, Mooi WJ and Peeper DS. The essence of senescence. *Genes Dev* 2010; 24: 2463-2479.
- [14] Krizhanovsky V, Yon M, Dickins RA, Hearn S, Simon J, Miething C, Yee H, Zender L and Lowe SW. Senescence of activated stellate cells limits liver fibrosis. *Cell* 2008; 134: 657-667.
- [15] Demaria M, Ohtani N, Youssef SA, Rodier F, Toussaint W, Mitchell JR, Laberge RM, Vijg J, Van Steeg H, Dolle ME, Hoeijmakers JH, de Bruin A, Hara E and Campisi J. An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Dev Cell* 2014; 31: 722-733.
- [16] Munoz-Espin D, Canamero M, Maraver A, Gomez-Lopez G, Contreras J, Murillo-Cuesta S, Rodriguez-Baeza A, Varela-Nieto I, Ruberte J, Collado M and Serrano M. Programmed cell senescence during mammalian embryonic development. *Cell* 2013; 155: 1104-1118.
- [17] Kuilman T and Peeper DS. Senescence-messaging secretome: SMS-ing cellular stress. *Nat Rev Cancer* 2009; 9: 81-94.
- [18] Kurz DJ, Decary S, Hong Y and Erusalimsky JD. Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J Cell Sci* 2000; 113: 3613-3622.
- [19] Basu SK, Gonit M, Salotti J, Chen J, Bhat A, Gorospe M, Viollet B, Claffey KP and Johnson PF. A RAS-CaMKKbeta-AMPKalpha2 pathway promotes senescence by licensing post-translational activation of C/EBPbeta through a novel 3'UTR mechanism. *Oncogene* 2018; 37: 3528-3548.
- [20] Kim YY, Jee HJ, Um JH, Kim YM, Bae SS and Yun J. Cooperation between p21 and Akt is required for p53-dependent cellular senescence. *Aging Cell* 2017; 16: 1094-1103.
- [21] van Deursen JM. The role of senescent cells in ageing. *Nature* 2014; 509: 439-446.
- [22] Maria J and Ingrid Z. Effects of bioactive compounds on senescence and components of senescence associated secretory phenotypes in vitro. *Food Funct* 2017; 8: 2394-2418.
- [23] Passos JF, Nelson G, Wang C, Richter T, Simillion C, Proctor CJ, Miwa S, Olijslagers S, Hallinan J, Wipat A, Saretzki G, Rudolph KL, Kirkwood TB and von Zglinicki T. Feedback between p21 and reactive oxygen production is necessary for cell senescence. *Mol Syst Biol* 2010; 6: 347.
- [24] Dimozi A, Mavrogonatou E, Sklirou A and Kletzas D. Oxidative stress inhibits the proliferation, induces premature senescence and promotes a catabolic phenotype in human nucleus pulposus intervertebral disc cells. *Eur Cell Mater* 2015; 30: 89-102.
- [25] Chin YE, Kitagawa M, Su WC, You ZH, Iwamoto Y and Fu XY. Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. *Science* 1996; 272: 719-722.
- [26] Steegmann JL, Baccarani M, Breccia M, Casado LF, Garcia-Gutierrez V, Hochhaus A, Kim DW, Kim TD, Khoury HJ, Le Coutre P, Mayer J, Milojkovic D, Porkka K, Rea D, Rosti G, Saussele S, Hehlmann R and Clark RE. European LeukemiaNet recommendations for the management and avoidance of adverse events of

## Icaritin induces cellular senescence

- treatment in chronic myeloid leukaemia. *Leukemia* 2016; 30: 1648-1671.
- [27] Devasagayam TP, Tilak JC, Boloor KK, Sane KS, Ghaskadbi SS and Lele RD. Free radicals and antioxidants in human health: current status and future prospects. *J Assoc Physicians India* 2004; 52: 794-804.
- [28] Lu T and Finkel T. Free radicals and senescence. *Exp Cell Res* 2008; 314: 1918-1922.
- [29] Rufini A, Tucci P, Celardo I and Melino G. Senescence and aging: the critical roles of p53. *Oncogene* 2013; 32: 5129-5143.
- [30] Young AP, Schlisio S, Minamishima YA, Zhang Q, Li L, Grisanzio C, Signoretti S and Kaelin WG Jr. VHL loss actuates a HIF-independent senescence programme mediated by Rb and p400. *Nat Cell Biol* 2008; 10: 361-369.
- [31] Collado M, Gil J, Efeyan A, Guerra C, Schumacher AJ, Barradas M, Benguria A, Zaballos A, Flores JM, Barbacid M, Beach D and Serrano M. Tumour biology: senescence in premalignant tumours. *Nature* 2005; 436: 642.
- [32] Campisi J and d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 2007; 8: 729-740.
- [33] Abbas T and Dutta A. p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer* 2009; 9: 400-414.
- [34] Timofeeva OA, Tarasova NI, Zhang X, Chasovskikh S, Cheema AK, Wang H, Brown ML and Dritschilo A. STAT3 suppresses transcription of proapoptotic genes in cancer cells with the involvement of its N-terminal domain. *Proc Natl Acad Sci U S A* 2013; 110: 1267-1272.