

Induction of apoptosis by shikonin through a ROS/JNK-mediated process in Bcr/Abl-positive chronic myelogenous leukemia (CML) cells

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This study examined the signaling events induced by shikonin that lead to the induction of apoptosis in Bcr/Abl-positive chronic myelogenous leukemia (CML) cells (e.g., K562, LAMA84). Treatment of K562 cells with shikonin (e.g., 0.5 μ M) resulted in profound induction of apoptosis accompanied by rapid generation of reactive oxygen species (ROS), striking activation of c-Jun-N-terminal kinase (JNK) and p38, marked release of the mitochondrial proteins cytochrome *c* and Smac/DIABLO, activation of caspase-9 and -3, and cleavage of PARP. Scavenging of ROS completely blocked all of the above-mentioned events (i.e., JNK and p38 phosphorylation, cytochrome *c* and Smac/DIABLO release, caspase and PARP cleavage, as well as the induction of apoptosis) following shikonin treatment. Inhibition of JNK and knock-down of JNK1 significantly attenuated cytochrome *c* release, caspase cleavage and apoptosis, but did not affect shikonin-mediated ROS production. Additionally, inhibition of caspase activation completely blocked shikonin-induced apoptosis, but did not appreciably modify shikonin-mediated cytochrome *c* release or ROS generation. Altogether, these findings demonstrate that shikonin-induced oxidative injury operates at a proximal point in apoptotic signaling cascades, and subsequently activates the stress-related JNK pathway, triggers mitochondrial dysfunction, cytochrome *c* release, and caspase activation, and leads to apoptosis. Our data also suggest that shikonin may be a promising agent for the treatment of CML, as a generator of ROS.

Keywords: shikonin, apoptosis, ROS, JNK, cytochrome *c*

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Introduction

Shikonin (C₁₆H₁₆O₅) is a natural naphthoquinone derivative compound that is present in the root tissues of the traditional Chinese medical herb *Lithospermum erythrorhizon*. This herb is found throughout China, including in Southern Yun Nan, Northern Dong Bei, Western Xin Jiang and Tibet. *Lithospermum erythrorhizon* was first recorded in a 2 000-year-old Chinese medical material dictionary “*Materia Medica of Deity of Agriculture*”, also called “*Shen Nong Ben*

Cao Jing” [1]. This dictionary was compiled around the time of the Qin (225 BC–206 BC) and Han (206 BC–220 AD) dynasties. In addition, *Lithospermum erythrorhizon* was described as a herbal medicine in Shi-Zhen Li’s (1518–1593) “*Compendium of Materia Medica*”, which is also called “*Ben Cao Gang Mu*” [2], and was written during the Ming dynasty (1368–1644). *Lithospermum erythrorhizon* has been broadly applied as a traditional Chinese medicine (TCM) for thousands of years in China to treat burns and to promote wound healing; it also has anti-bacterial and anti-inflammatory properties. Later, it was found that shikonins, including the individual compounds shikonin, isobutyryl shikonin, acetyl shikonin, dimethylacryl shikonin and isovaleryl shikonin, are responsible for the anti-bacterial and anti-inflammatory activities of this TCM [3–6].

Recently, it was reported that shikonin induces apoptosis of various cancer cells and performs anti-

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cancer activities [7-10]. Studies suggest that the anti-cancer activities of shikonin derivatives may be attributed to their ability to down-regulate anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL, generate reactive oxygen species (ROS), activate caspases [11, 12], inhibit EGFR phosphorylation [13] and arrest the cell cycle through p53 upregulation [14]. However, the precise mechanisms employed by shikonin to repress cancer growth remain to be defined.

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by increased proliferation of granulocytic cells without loss in their capacity to differentiate. This abnormality results from a reciprocal translocation between the *abl* oncogene on the long arm of chromosome 9 and the *bcr* region on the long arm of chromosome 22. This translocation, designated Bcr/Abl [9], results in lengthening of an arm of chromosome 9 and shortening of an arm of chromosome 22. The latter chromosome is termed the Philadelphia chromosome (Ph¹), named after the city where it was discovered. The resulting *bcr-abl* fusion gene encodes a chimeric protein with strong tyrosine kinase activity. This cytogenetic hallmark (*bcr/abl*) is present in more than 90% of CML cases [15, 16]. CML is the first described malignancy associated with a gene translocation. Presence of the Bcr/Abl tyrosine kinase renders CML resistant to traditional therapeutic approaches, which makes Bcr/Abl an important therapeutic target. The first Bcr/Abl tyrosine kinase inhibitor, STI571 (Gleevec, imatinib mesylate) [17], was recently presented as a revolutionary approach to CML treatment. STI571 is now the first-choice treatment for all newly diagnosed CML patients. Oral administration of STI571 results in a clinical response in more than 90% of CML patients. Importantly, the striking initial efficacy of this drug has been overshadowed by the development of clinical resistance. The mechanisms of resistance to STI571 include Bcr/Abl amplification, mutations in the Bcr/Abl kinase domain and diminished drug uptake, among others [18, 19]. Thus, it is important to search for additional approaches to treat this malignancy.

Apoptosis was originally defined by Kerr [20] as the orderly and characteristic sequence of structural changes resulting in the programmed death of the cell. Apoptosis is cell suicide achieved through well-characterized morphological and biochemical changes. Apoptosis appears to be a common mechanism of biological activity for cytotoxic agents employed in chemotherapy. There are two well-studied pathways that result in apoptosis: the cell surface death receptor pathway and the mitochondria-initiated pathway. In the cell surface death receptor pathway, death receptors detect the

presence of extracellular death signals and rapidly ignite the cell's intrinsic apoptosis machinery [21-23]. These death receptors include CD95, TNFR1, avian CAR1, DR3, DR4 and DR5. In the mitochondria-initiated pathway, caspase activation is triggered by formation of the multimeric Apaf-1/cytochrome *c* complex, which recruits and activates procaspase-9. Activated caspase-9 cleaves and activates downstream caspases, such as caspase-3, -6 and -7. The release of mitochondrial cytochrome *c* is regulated by Bcl-2 family proteins, including the anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1 and the pro-apoptotic proteins Bax, Bad, Bak and Bid [24-29].

ROS are continually generated and eliminated in biological systems. They play important roles in a variety of normal biochemical functions and abnormal pathological processes. Excessive production of ROS in the cell is known to induce apoptosis [30, 31]. The ability of ROS to inflict severe cellular damage and cause cell death has been exploited as an approach to kill cancer cells. ROS generation has also been shown to play an important role in apoptosis induced by treatment with cisplatin, bleomycin, UV irradiation and Bortezomib (PS341), a proteasome inhibitor recently approved by the FDA for the treatment of multiple myeloma [32].

c-Jun-N-terminal kinase (JNK) is a member of the mitogen-activated protein (MAP) kinase family. Increasing evidence indicates a crucial role of JNK in mitochondrial dysfunction and the subsequent initiation of apoptosis. More specifically, apoptosis is achieved by JNK-initiated release of apoptosis-regulating factors, such as cytochrome *c* and Smac/DIABLO [33, 34], JNK-mediated inactivation of anti-apoptotic proteins, JNK-mediated activation of pro-apoptotic Bcl-2 family proteins [35, 36] and JNK-induced expression of pro-apoptotic proteins, such as Bim [37].

Although the efficacy of shikonin and its derivatives has been broadly studied, their precise mode of action and the molecular basis of their anti-cancer activities remain to be elucidated. Here, we report that a shikonin induces apoptosis of CML cells by the following processes: ROS generation, JNK/c-JUN activation, mitochondrial dysfunction, cytochrome *c* release and caspase activation. The present data also suggest that shikonin may serve as a novel agent for the treatment of CML as it induces excessive ROS production.

Results and Discussion

Shikonin induces apoptosis of CML cells in a dose- and time-dependent manner

Shikonin, the chemical structure [3, 11] of which is shown in Figure 1A, is a natural naphthoquinone-derived

compound present in the root tissues of the Chinese herb *Lithospermum erythrorhizon*. Shikonin induces apoptosis in various cancer cell lines and performs anti-tumor activity *in vivo* [7-10]. To determine whether shikonin mediates apoptosis of CML cells, Bcr/Abl-positive K562 cells were treated with 0.5 μM shikonin for 24 h. A high percentage ($\sim 85\%$) of cells underwent apoptosis, as detected by Annexin V/propidium iodide (PI) analysis (Figure 1B). To further explore shikonin-induced apoptosis, K562 cells were exposed to increasing concentrations of shikonin for varying lengths of time, as indicated (Figure 1C). A significant percentage of cells ($\sim 30\%$) underwent apoptosis following exposure to 300 nM shikonin for 16 h. The percentage of apoptotic cells dramatically increased with increasing concentrations of shikonin, from 200 to 500 nM, when incubated for 12-16 h. A parallel study was performed in another Bcr/Abl-positive cell line, LAMA 84 (Figure 1D). A significant

percentage of these cells ($\sim 40\%$) underwent apoptosis following treatment with 200 nM shikonin for 16 h. Comparatively, LAMA 84 cells were more sensitive to shikonin than K562 cells.

Shikonin-induced apoptosis of CML cells involves both mitochondria and caspases

Shikonin has been reported to induce apoptosis through a mitochondria- and caspase-mediated pathway in human colorectal carcinoma cells [11], and through a mitochondria-independent pathway in human SK-Hep-1 hepatoma cells [12]. To investigate whether mitochondria are involved in shikonin-induced apoptosis of CML cells, the percentage of cells that exhibit “low” 3,3-dihexyloxacarbocynine (DiOC₆) uptake, which reflects a loss of mitochondrial membrane potential ($\Delta\Psi_m$), was examined using the DiOC₆ staining assay after shikonin treatment. The data obtained from flow cytometry experiments

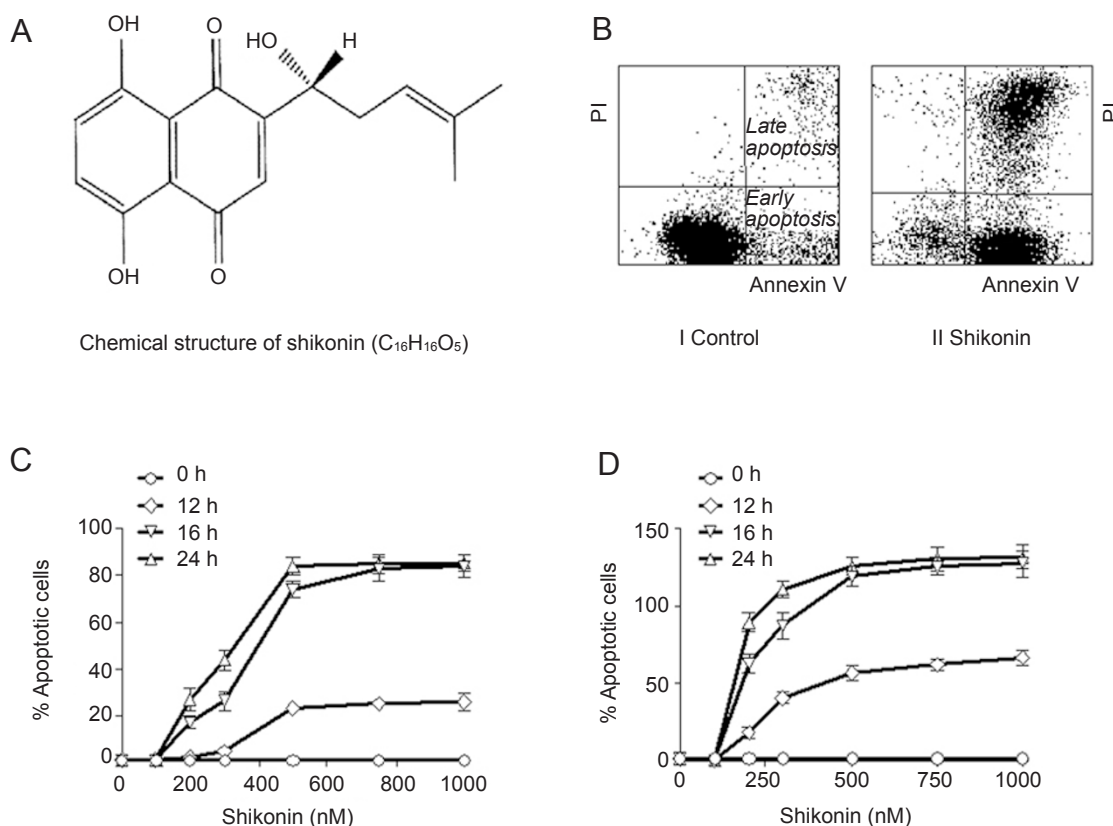


Figure 1 Apoptosis induced by shikonin treatment of CML cells. **(A)** The chemical structure of shikonin. **(B)** After K562 cells were treated with 0.5 μM shikonin for 24 h, the percentage of cells undergoing apoptosis was determined by flow cytometry following Annexin V/PI staining. Cells that were positive for Annexin V but not PI, which are present in the lower right quadrant, are early apoptotic cells. Cells that were both Annexin V- and PI-positive, which are present in the upper right quadrant, are late apoptotic cells. After K562 **(C)** and LAMA 84 **(D)** cells were exposed to increasing concentrations of shikonin for the designed time intervals, apoptosis was revealed by DAPI staining.

demonstrate that shikonin mediates a mitochondrial permeability transition (loss of $\Delta\psi_m$) in K562 cells (Figure 2A), which suggests that mitochondria are involved in shikonin-induced apoptosis. To determine the contribution of caspases to this process, K562 cells were treated with shikonin in the absence or presence of a broad-spectrum caspase inhibitor, BOC-fmk, for 24 h. Shikonin-induced apoptosis was completely blocked by BOC-fmk treatment (Figure 2B). Furthermore, western blotting analysis indicated that shikonin treatment resulted in a profound mitochondrial release of cytochrome *c* and Smac/DIABLO, cleavage of myeloid cell leukemia-1 (Mcl-1, an anti-apoptotic protein in the Bcl-2 family), caspase-3 and -9 and PARP. The release of

Smac/DIABLO and cleavage of Mcl-1, caspase-3 and -9 and PARP were blocked by BOC-fmk, while the release of cytochrome *c* was not affected by BOC-fmk (Figure 2C). Altogether, these results indicate that cytochrome *c* release, which is an indicator of mitochondrial injury, is upstream of caspase activation and that Mcl-1 attenuation is secondary to caspase activation, as it is a caspase-mediated event. In this case, cleavage of Mcl-1 may amplify the extent of apoptosis induced by shikonin. This hypothesis is based on previous studies that demonstrated that caspase-mediated cleavage of Mcl-1 inhibits its anti-apoptotic function [38]. In addition, we noted that BOC-fmk did not completely block cleavage of caspase-9. This is expected since this caspase is autocatalytic. Together,

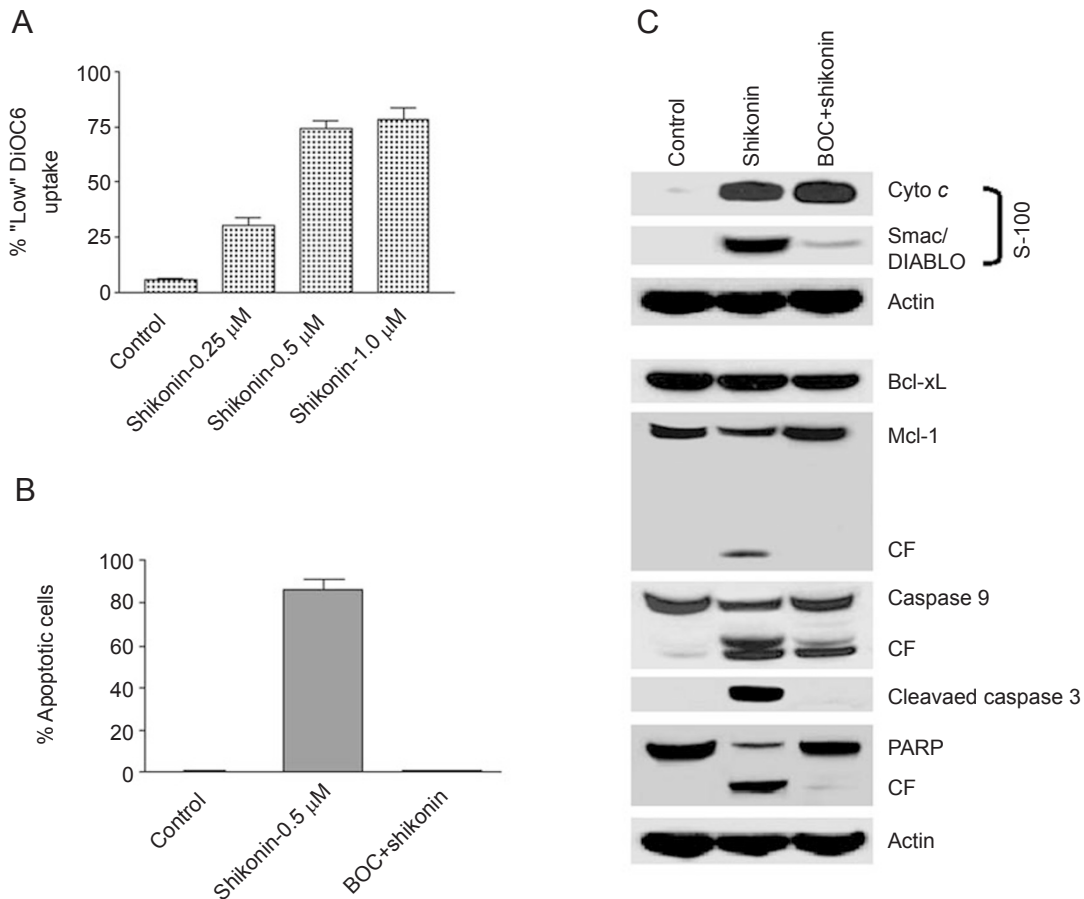


Figure 2 Apoptosis is induced by shikonin through a mitochondria and caspase-mediated pathway in CML cells. **(A)** After K562 cells were treated with the indicated concentrations of shikonin for 24 h, DiOC₆ uptake was examined by flow cytometry. **(B)** After K562 cells were treated with 0.5 μ M shikonin in the absence or presence of 50 μ M BOC-fmk for 24 h, apoptosis was quantified under a microscope by DAPI staining. **(C)** Western blot analyses of Bcl-xL, Mcl-1, caspase-9, cleaved caspase-3 and PARP from whole cell lysates were performed using treatment regimens similar to those described in **(B)**. In addition, S-100 fractions were obtained and submitted to Western blot analyses of cytochrome *c* (cyto *c*) and Smac/DIABLO. Actin served as loading control. CF: cleaved fragment. After repeating the experiment three times, similar results/patterns were obtained. Thus, representative Western blots are shown in the figure.

these results suggest that shikonin-induced apoptosis in K562 cells is mediated by a pathway that involves mitochondria injury, cytochrome *c* release and caspase activation.

ROS trigger multiple signaling cascades involved in shikonin-induced apoptosis

A previous study reported that shikonin induces ROS formation, but that it does not induce a mitochondrial permeability transition in the course of inducing apoptosis of human SK-Hep-1 hepatoma cells [12]. However, the results shown in Figure 2A demonstrate that mitochondria are involved in shikonin-induced apoptosis of K562 cells. In order to test whether shikonin

leads to ROS generation in CML cells, K562 cells were treated with shikonin, as indicated in Figure 3A. Rapid generation of ROS was detected at 1 h following shikonin treatment. Importantly, ROS production was markedly blocked by LNAC treatment (*L-N*-acetylcysteine, free radical scavenger) ($P < 0.001$) (Figure 3A). Consequently, apoptosis induced by shikonin was also completely abolished by LNAC treatment (Figure 3B). It is known that ROS activates multiple signaling pathways, including MAPK and Akt signal transduction cascades [30]. Western blot analysis was employed to evaluate the activity of MAPK and Akt signal transduction pathways after shikonin treatment (Figure 3C). The results show that shikonin-induced ROS generation was

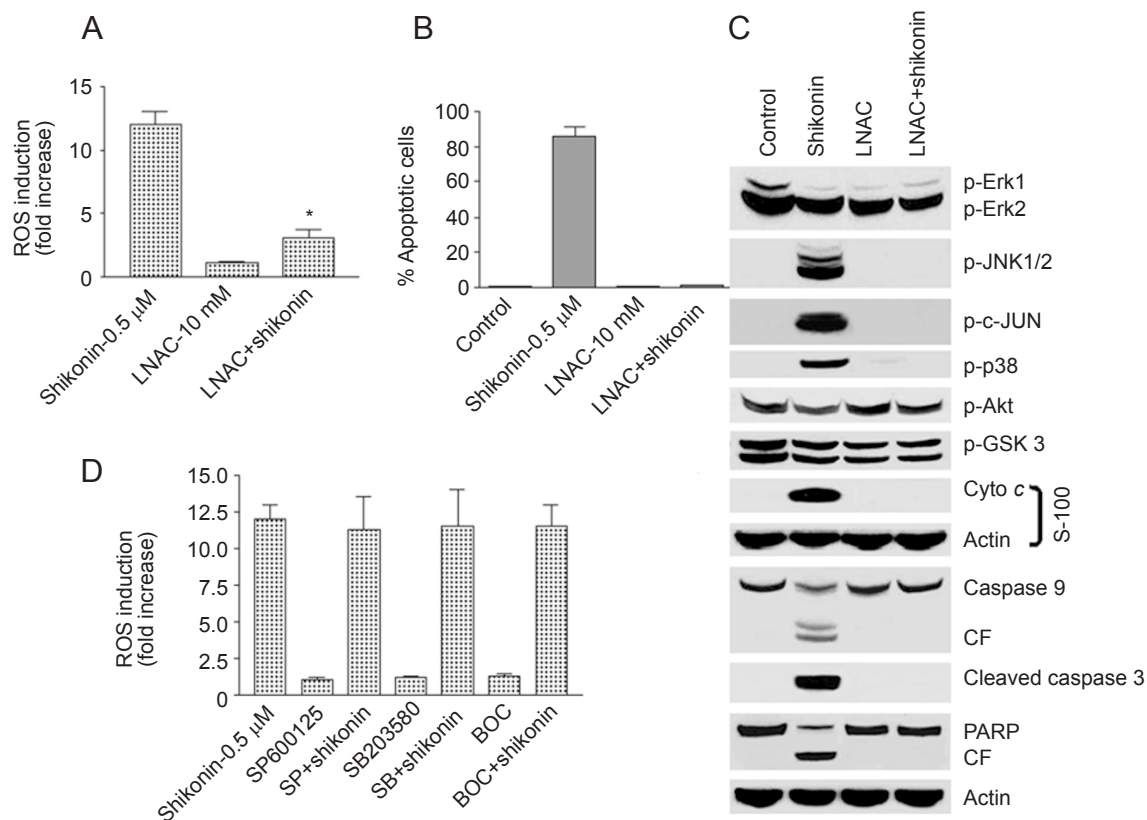


Figure 3 Shikonin-induced ROS generation in CML cells. **(A)** K562 cells were treated with 0.5 μ M shikonin in the absence or presence of 10 mM LNAC for 1 h and ROS generation was examined by flow cytometry. * $P < 0.001$. **(B)** After treatment for 24 h, apoptosis was quantified by DAPI staining. **(C)** Western blot analyses of phospho-Erk1/2, phospho-JNK, phospho-c-JUN, phospho-p38, phospho-Akt, phospho-GSK 3, caspase-9, cleaved caspase-3 and PARP from the whole cell lysates were performed after shikonin treatment for 24 h of p-JNK (top, middle and bottom bands in the blots are JNK2, JNK3 and JNK1, respectively) and p-GSK3 (top and bottom bands are GSK3 α and GSK3 β , respectively). In addition, S-100 fractions were isolated for Western blot analysis of cytochrome *c* (cyto *c*). **(D)** ROS generation was examined by flow cytometry after K562 cells were treated with 0.5 μ M shikonin in the absence or presence of 30 μ M SP600125, 10 μ M SB203580 and 50 mM BOC-fmk for 1 h. CF: cleaved fragment. After repeating the experiment three times, similar results/patterns were obtained. Thus, representative Western blots are shown in the figure.

followed by striking increases in the levels of phospho-JNK, phospho-c-JUN and phospho-p38, a significant decrease in the level of phospho-Erk1 (44 kDa), as well as a profound release of cytochrome *c*, and cleavage of caspase-3 and -9 and PARP. LNAC treatment completely reversed JNK and p38 phosphorylation, cytochrome *c* release and caspase cleavage, suggesting that ROS generation is upstream of the examined mediators. Moreover, LNAC completely blocked shikonin-mediated Smac/DIABLO release (data not shown). However, there was no appreciable modification of phosphorylation of Erk2 (42 kDa), Akt or GSK 3 after LNAC treatment.

If ROS generation is the proximal event of JNK and p38 activation in shikonin-treated K562 cells, inhibition of JNK, p38 and caspase activity would not influence ROS generation. To test this hypothesis, K562 cells were exposed to shikonin in the absence or presence of JNK, p38 and caspase inhibitors, independently. As shown in Figure 3D, ROS generation was not appreciably modified after addition of the inhibitors (SP600125, a specific JNK inhibitor; SB203580, a specific p38 inhibitor; and BOC-fmk, a pan-caspase inhibitor). These results are consistent with the prediction that ROS generation is the initial event and that it precedes activation of the MAPK and Akt signaling cascades. Results from parallel experiments performed in LAMA84 cells showed that a significant amount of ROS (e.g., 9-fold increase) was generated after only 1 h of exposure to shikonin, which was blocked by LNAC (data not shown).

JNK activation contributes to shikonin-induced apoptosis of CML cells

The preceding results indicated that shikonin activated JNK, c-JUN and p38 signaling cascades (Figure 3C). We next tested the functional roles of these signals in drug-induced apoptosis. K562 cells were treated with shikonin in the absence or presence of pharmacological inhibitors of JNK and p38. A time-course study showed that JNK activation persists after treatment with 0.5 μ M shikonin (Figure 4A). SP600125, a specific JNK inhibitor, markedly blocked shikonin-induced apoptosis ($P < 0.001$). However, SB203580, a specific p38 inhibitor, did not appreciably modify drug-induced apoptosis (Figure 4B). Western blotting analysis showed that treatment with SP600125 markedly blocked shikonin-induced c-JUN phosphorylation, cytochrome *c* release, and cleavage of caspase-3 and -9 and PARP, while SB203580 had no effect on any of these events (Figure 4C).

In order to determine whether JNK activation contributes to shikonin-induced apoptosis, K562 cells were transfected with a JNK1 short interfering RNA (siRNA) and then treated with shikonin. Western blotting analysis indicated that knock-down of JNK1 markedly

blocked cytochrome *c* release and PARP cleavage (Figure 4D) as well as the induction of apoptosis (Figure 4E). In addition, decreased JNK1 activity, achieved by JNK siRNA-mediated knock-down or JNK inhibition by SP600125, did not affect shikonin-mediated ROS generation (data not shown). Together, these data suggest that activation of JNK, and not p38, plays a functional role in shikonin-induced apoptosis. These results also indicate that JNK activation is upstream of cytochrome *c* release, caspase activation and PARP cleavage.

Putative model of signaling cascades in shikonin-induced apoptosis of CML cells

The above results suggest a model in which shikonin induces apoptosis by inducing production of ROS, which subsequently leads to activation of JNK, release of cytochrome *c*, and cleavage of caspases and PARP. Blockage of ROS generation, JNK activation and caspase activation affected the corresponding downstream cascades (Figure 5). ROS generation by shikonin represents the proximal and initiating event in drug-induced apoptosis.

In summary, our results indicate that apoptosis induced by shikonin treatment of CML cells is caused by the rapid generation of ROS, which triggers JNK phosphorylation, mitochondrial injury and caspase activation. ROS generation and JNK activation play critical roles in shikonin-induced apoptosis. This result also suggests that shikonin may serve as a novel agent for CML treatment by inducing excessive production of ROS.

Materials and Methods

Cells

The K562 cell line was purchased from American Type Culture Collection, Rockville, MD. The LAMA84 cell line was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Sub-confluent, logarithmically growing cells were placed in sterile plastic T-flasks, to which the designated drugs were added. The flasks were then incubated in a humidified incubator with 5% CO₂ at 37 °C for various lengths of time, as indicated.

Materials

Shikonin was obtained from Shanghai TCI Chemical Co. (Shanghai, China); this chemical had a purity of >97%. BOC-D-fmk was purchased from Enzyme System Products (Livermore, CA). LNAC, DiOC₆, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), PI and SP600124 were obtained from Sigma Chemical Co. (Beijing, China).

Assessment of apoptosis

Following drug treatment, apoptotic cells were detected by DAPI staining, which allowed for identification of apoptotic

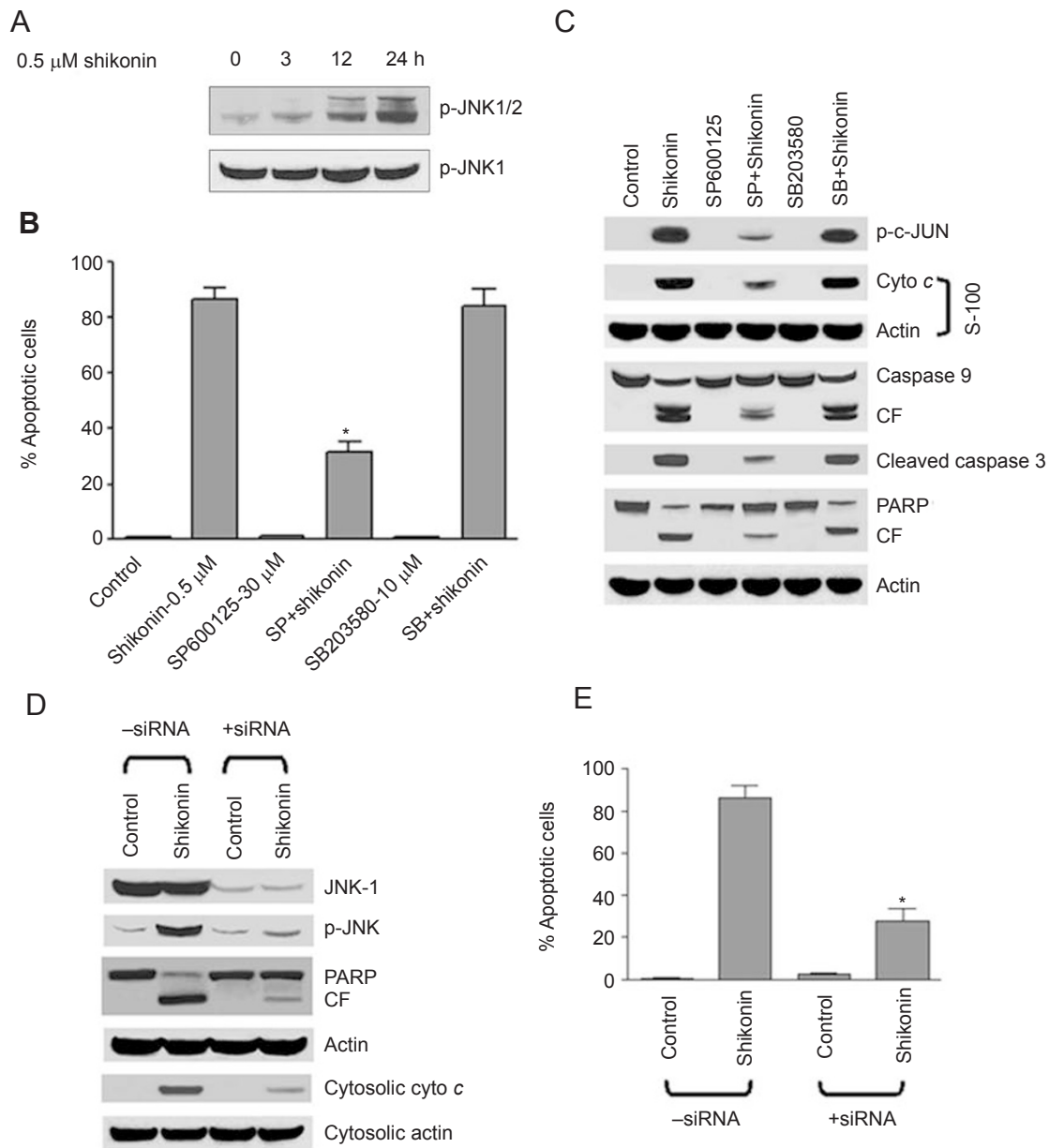


Figure 4 JNK activation by shikonin contributed to drug-induced apoptosis of CML cells. **(A)** The time-course study of JNK activation after 0.5 μ M shikonin treatment in K562 cells. **(B)** After K562 cells were treated with shikonin in the absence or presence of 30 μ M SP600125, or the absence or presence of 10 μ M SB203580, for 24 h apoptosis was examined by DAPI staining. * $P < 0.001$. **(C)** Western blot analyses of phospho-c-JUN, caspase-9, cleaved caspase-3 and PARP from whole cell lysates were performed after treatment for 24 h. In addition, S-100 fractions were isolated for Western blot analysis of cytochrome c. **(D)** After K562 cells were transfected with JNK1-siRNA for 48 h, followed by a 24 h treatment with 0.5 μ M shikonin, Western blot analyses of the indicated proteins were performed, and apoptosis was evaluated by DAPI staining **(E)**. CF: cleaved fragment, cyto c: cytochrome c. After repeating the experiments three times, similar results/patterns were obtained. Thus, representative Western blots are shown in the figure.

nuclear changes as described previously [39]. Briefly, 1×10^5 cells were harvested and washed with phosphate-buffered saline (PBS) and then fixed with 1% glutaraldehyde at room temperature for 30 min. After washing with PBS, cells were re-suspended in 20 μ l of PBS and mixed with 5 μ l of a 10 μ g/ml stock solution of DAPI.

Cell suspensions were mounted on slides. Cells were visualized under microscope with a 360-370 nm excitation light and 420-460 nm emission filter; apoptotic cells appeared with characteristic condensed and fragmented nuclei.

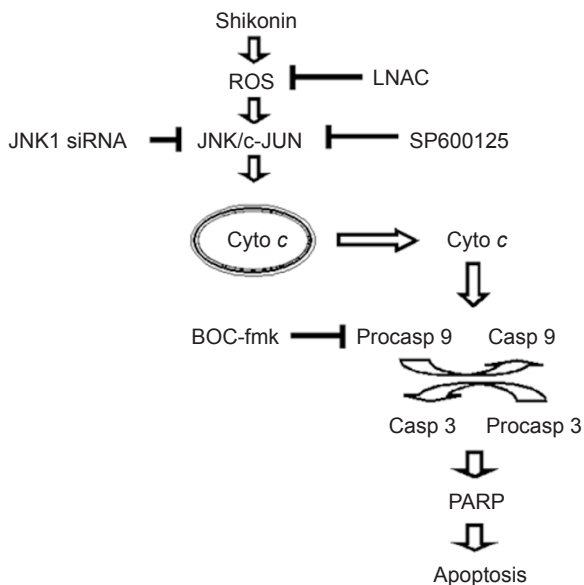


Figure 5 A putative model of the hierarchical signaling events leading to apoptosis of CML cells following treatment with shikonin. Shikonin induces the generation of ROS, activation of JNK, release of cytochrome *c*, and cleavage of caspases and PARP. ROS generation by shikonin is the proximal event that triggers multiple signaling cascades in drug-induced apoptosis.

Annexin V/PI staining

To confirm the results of morphological analysis, Annexin V/PI staining was employed. For Annexin V/PI assays, cells were stained with Annexin V-FITC and PI and then monitored for apoptosis by flow cytometry in accordance with the manufacturer's protocol (BD PharMingen, San Diego, CA, USA). Briefly, 0.5×10^6 cells were washed twice with PBS and stained with 5 μ l of Annexin V-FITC and 10 μ l of PI (5 μ g/ml) in 1 \times binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM CaCl₂) for 15 min at room temperature in the dark. Apoptotic cells were quantified using a FACScan cytofluorometer. Both early apoptotic (Annexin V-positive, PI-negative) and late apoptotic (double positive of Annexin V and PI) cells were detected.

Assessment of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial membrane potential was monitored using the fluorescent dye DiOC₆. For each condition, 4×10^5 cells were incubated for 30 min at 37 °C in 1 ml of 40 nM DiOC₆ and subsequently analyzed using a FACScan cytofluorometer with excitation and emission settings of 488 and 525 nm, respectively. The percentage of cells exhibiting a low level of DiOC₆ uptake exhibited a loss of mitochondrial membrane potential.

Detection of ROS

Generation of ROS was assessed using fluorescent probe dihydroethidium (DHE) staining, as was previously described [40]. In the presence of superoxide (O₂⁻), DHE is oxidized to fluorescent products, which are monitored by flow cytometry. Briefly, cells were incubated with 20 μ M DHE in tissue culture medium for 30 min at 37°C, and then washed, re-suspended in PBS, and

subsequently monitored by flow cytometry.

Western blot analysis

Following drug treatment, cell lysates were prepared and Western blot analysis was performed with primary antibodies, as described previously [40]. The following antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, USA): caspase 9, cleaved caspase 3, cytochrome *c*, phospho-ERK1/2, phospho-JNK, phospho-c-JUN (Ser73), phospho-Akt, phospho-p38 and actin. To ensure equivalent loading and transfer, all blots that had been probed for proteins other than actin were stripped and re-probed with an antibody against actin. The western blots shown in the figures are representative of three independent experiments unless otherwise indicated.

Assessment of cytochrome *c* release from mitochondria

Following drug treatment, the release of cytochrome *c* from mitochondria was analyzed by a selective digitonin permeabilization method. Briefly, 4×10^6 cells per condition were re-suspended in 50 μ l of permeabilization buffer (containing 75 mM NaCl, 8 mM Na₂PO₄, 1 mM NaH₂PO₄, pH 7.4, 250 mM sucrose (added fresh before use), 1 mM EDTA, 700 μ g/ml digitonin) (final concentration of digitonin 35 μ g/ 4×10^6 cells). Cells were incubated in the above permeabilization buffer at room temperature for 1 min, after which the pellet was removed by centrifugation for 3 min at 13 000 \times g, and the supernatant containing cytochrome *c* protein was obtained.

siRNA-mediated knock-down

A JNK1-specific siRNA (5'-GCAGAAGCAAACGTGAC AACAA-3') and a negative control siRNA (Catalog# AM4635) were purchased from Ambion (Austin, TX, USA). Cells were transfected with siRNAs at a final concentration of 30 nM using siPORT™ NeoFX™ Transfection Agent, following the manufacturer's protocol.

Statistical analysis

All of the data are expressed as mean \pm SD of three individual experiments. Differences between groups were determined using the Student's *t* test for unpaired observations. *P* < 0.05 was considered significant.

Acknowledgments

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