



# The Endoplasmic Reticulum Stress Response Mediates Shikonin-Induced Apoptosis of 5-Fluorouracil-Resistant Colorectal Cancer Cells

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## Abstract

Resistance to chemotherapeutic drugs is a significant problem in the treatment of colorectal cancer, resulting in low response rates and decreased survival. Recent studies have shown that shikonin, a naphthoquinone derivative, promotes apoptosis in colon cancer cells and cisplatin-resistant ovarian cells, raising the possibility that this compound may be effective in drug-resistant colorectal cancer. The aim of this study was to characterize the molecular mechanisms underpinning shikonin-induced apoptosis, with a focus on endoplasmic reticulum (ER) stress, in a 5-fluorouracil-resistant colorectal cancer cell line, SNU-C5/5-FUR. Our results showed that shikonin significantly increased the proportion of sub-G<sub>1</sub> cells and DNA fragmentation and that shikonin-induced apoptosis is mediated by mitochondrial Ca<sup>2+</sup> accumulation. Shikonin treatment also increased the expression of ER-related proteins, such as glucose regulatory protein 78 (GRP78), phospho-protein kinase RNA-like ER kinase (PERK), phospho-eukaryotic initiation factor 2 (eIF2 $\alpha$ ), phospho-phosphoinositol-requiring protein-1 (IRE1), spliced X-box-binding protein-1 (XBP-1), cleaved caspase-12, and C/EBP-homologous protein (CHOP). In addition, siRNA-mediated knockdown of CHOP attenuated shikonin-induced apoptosis, as did the ER stress inhibitor TUDCA. These data suggest that ER stress is a key factor mediating the cytotoxic effect of shikonin in SNU-C5/5-FUR cells. Our findings provide an evidence for a mechanism in which ER stress leads to apoptosis in shikonin-treated SNU-C5/5-FUR cells. Our study provides evidence to support further investigations on shikonin as a therapeutic option for 5-fluorouracil-resistant colorectal cancer.

**Key Words:** Naphthoquinone, 5-Fluorouracil-resistant colorectal cancer, Apoptosis, Endoplasmic reticulum stress

## INTRODUCTION

Drug resistance is a significant obstacle for successful chemotherapy of colorectal cancer. 5-Fluorouracil (5-FU) is widely used for the treatment of numerous cancers, including colon cancer, despite the low response rate of approximately 20% in this cancer (Kim *et al.*, 2015). Patients with colorectal cancer also exhibit variable response rates to other anticancer drugs (Mohelnikova-Duchonova *et al.*, 2014).

The endoplasmic reticulum (ER) synthesizes lipids involved in the production of the plasma membrane and provides a non-vascular pathway for lipid transport, as well as enzymes for lipid metabolism reactions (Salvador-Gallego *et al.*, 2017). The functions of the ER also comprise protein folding and as-

sembly, vesicle transport, and cellular calcium storage. Therefore, the ER is sensitive to changes in cell homeostasis. Since ER function is essential for cell survival, functional disturbances in this compartment can cause cellular damage and lead to apoptosis (Zhang *et al.*, 2012). For example, disruptions of ER function that alter ER homeostasis lead to the inhibition of protein folding and the accumulation of unfolded proteins. Such disruptions can be caused by modifications in Ca<sup>2+</sup> homeostasis or increased demand for protein folding, for example, due to elevated synthesis of proteins in specialized secretory cells or the expression of a misfolded mutant protein (Basseri and Austin, 2012; Dastghaib *et al.*, 2021). Such dysfunction causes various types of proteotoxicities in the ER, collectively referred to as ER stress (Pluquet *et al.*, 2015).

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To resolve ER stress, a three-pronged signal transduction cascade is activated. The upstream components of the three branches are double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) (Kim *et al.*, 2016). Once these pathways are activated, phosphorylation of eukaryotic translation initiation factor-2 $\alpha$  (eIF2 $\alpha$ ) results in the downregulation of protein synthesis, while ER functional capacity is improved by upregulating the transcription of genes encoding ER chaperones, protein folding enzymes, and components of the ER-associated degradation system.

*Lithospermum erythrorhizon* is a medicinal plant that is widely used in traditional oriental medicine (Han *et al.*, 2015; Prasad *et al.*, 2015). The bioactive component in *L. erythrorhizon* root extract is a naphthoquinone derivative called shikonin, which has been shown to trigger apoptosis in the SNU-407 colon cancer cells (Han *et al.*, 2019). Shikonin also has a significant apoptotic effect on cisplatin-resistant human ovarian cells (Shilnikova *et al.*, 2018). While shikonin is known to exert apoptotic effects in cancer cells, including drug-resistant cancers, little is known about the molecular mechanisms underlying these effects. In light of findings, which suggest that ER stress may be involved in drug resistance in colorectal cancer cells, we hypothesized that the functional effects of shikonin in cancer may involve the ER stress response. In this study, we investigated whether shikonin induces apoptosis in colorectal cancer 5-FU-resistant SNU-C5 (SNU-C5/5-FUR) cells via activating the ER stress.

## MATERIALS AND METHODS

### Reagents

Shikonin and antibodies against GRP78, phospho-eIF2 $\alpha$ , phospho-IRE1, and XBP-1 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Thiazolyl blue tetrazolium bromide (MTT), ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), Hoechst 33342, propidium iodide (PI), tauroursodeoxycholic acid (TUDCA), and actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rhod-2 acetoxymethyl ester (Rhod-2, AM) and ER-Tracer Blue-White DPX were purchased from Molecular Probes (Eugene, OR, USA). Antibodies against phospho-PERK, caspase-12, and C/EBP-homologous protein (CHOP) were purchased from Cell Signaling Technology (Beverly, MA, USA). All other chemicals and reagents used were of analytical grade.

### Cell culture

SNU-C5/5-FUR cells were obtained from the Resistant Cell Research Center of Chosun University (Gwangju, Korea). The cells were subcultured twice a week in presence of 140  $\mu$ M 5-FU for over 6 months until they were stably drug resistant (Kang *et al.*, 2014). The cells were cultured at 37°C with 5% CO<sub>2</sub> in RPMI-1640 medium (Thermo Fisher Scientific, Grand Island, NY, USA) containing HEPES and supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotic-antimycotic solution. The drug resistance of the SNU-C5/5-FUR cell line was maintained by treatment with 5-FU once a month.

### Cell viability assessment

The cells were seeded into a 24-well plate at a density of  $0.8 \times 10^5$  cells/mL and incubated for 16 h. They were then treated with shikonin at a concentration of 1, 2, 3, 4, 5, 6, 8, 10, or 15  $\mu$ M, or pretreated with an ER stress inhibitor (TUDCA) prior to treatment with shikonin for 30 min. The cells were subsequently incubated at 37°C for 48 h, and then 125  $\mu$ L MTT stock solution (2 mg/mL) was added to each well. After 4 h, the formazan crystals were dissolved in 350  $\mu$ L DMSO, and the absorbance at 540 nm was measured using a SpectraMax i3X multi-detection microplate reader (Molecular Devices, Sunnyvale, CA, USA) (Piao *et al.*, 2019).

### Cell morphology analyses

Cells were seeded into a 60 mm culture dish at a density of  $0.8 \times 10^5$  cells/mL and incubated for 16 h. After treatment with 3.3  $\mu$ M shikonin, cells were observed for 1, 2, and 3 days, and changes in cell morphology were documented using a phase contrast inverted microscope (DP71 digital microscope camera, Olympus, Tokyo, Japan). All images were acquired at 20 $\times$  magnification.

### Colony formation assay

Approximately 100 cells were inoculated into a 35 mm culture dish and allowed to grow for 2 days. The cells were then treated with 3.3  $\mu$ M shikonin and incubated for 10 days until colonies formed. To aid visualization, the colonies were stained using a Diff-Quik kit (Sysmex, Kobe, Japan) according to the manufacturer's instructions.

### Detection of sub-G<sub>1</sub> hypodiploid cells

Flow cytometry analysis was performed after cells were stained with propidium iodide (PI) to assess the proportion of sub-G<sub>1</sub> cells with hypodiploid DNA content, which are considered to represent apoptotic cells. Briefly, cells were seeded in 6-well plates in triplicate for each of the control and shikonin-treated groups. Twenty hours later, cells were pretreated with 3.3  $\mu$ M shikonin (treated). The cells were harvested after 48 h and fixed with 70% ethanol (1 mL) for 30 min at 4°C. Subsequently, cells were washed twice with cold PBS+2 mM EDTA, to prevent aggregation, and incubated in the dark for 30 min at 37°C in PBS/2 mM EDTA containing PI (final concentration, 100  $\mu$ g/mL) and RNase A (final concentration, 100  $\mu$ g/mL). The analysis was performed using a FACSCalibur instrument, and the percentage of sub-G<sub>1</sub> hypodiploid cells was evaluated using CellQuest and ModFit Software (Becton Dickinson, San Jose, CA, USA).

### DNA fragmentation analysis

Cellular DNA fragmentation was assessed by measuring DNA fragments released into the cell cytoplasm. To facilitate detection, DNA was labeled with the non-radioactive thymidine analog, BrdU. DNA fragments were detected immunologically using an ELISA kit from Roche Diagnostics (Mannheim, Germany), according to the manufacturer's instructions.

### Measurement of mitochondrial Ca<sup>2+</sup>

Mitochondrial Ca<sup>2+</sup> levels were monitored using Rhod-2 AM (Mészáros *et al.*, 2012). Cells were treated with shikonin for 48 h; harvested, washed, resuspended in PBS containing 1  $\mu$ M Rhod-2 AM; and incubated for 15 min at 37°C. Subsequently, the cells were washed and suspended in PBS for further anal-

ysis by flow cytometry. To confirm the flow cytometry results, the cells were seeded in 4-well chambers, and image analysis was conducted by loading cells with Rhod-2 AM for 30 min at 37°C. After washing, the stained cells were mounted on microscope slides with mounting medium (DAKO, Carpinteria, CA, USA). Images were captured on a confocal microscope using the Laser Scanning Microscope 5 PASCAL software (Carl Zeiss, Jena, Germany).

### Nuclear fragmentation analysis by Hoechst 33342 staining

The cells were seeded at  $1.0 \times 10^5$  cells/mL in medium with or without EGTA. After 16 h incubation, the cells were treated with shikonin and incubated at 37°C for another 48 h. Alternatively, the cells were seeded in a 24-well plate at  $1.0 \times 10^5$  cells/mL. After 16 h incubation, the cells were treated with TUDCA and incubated for 30 min, followed by treatment with shikonin and incubation at 37°C for 48 h. After staining with Hoechst 33342 cell-permeable nuclear counterstain dye for 10 min, nuclear fragmentation (indicating apoptosis) was determined as previously described (Piao *et al.*, 2019).

### Western blot analysis

The harvested cells were washed once with PBS, lysed with RIPA buffer containing protease inhibitors on ice for 20 min, and centrifuged at  $14,000 \times g$  for 10 min. The supernatant was collected, and the Quant-iT™ protein assay kit was used to determine protein concentrations (Thermo Fisher Scientific). After boiling an aliquot of the lysate (40 µg protein) for 5 min, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) based on their molecular weight. The separated proteins were transferred onto nitrocellulose membranes, which were subsequently incubated with primary antibodies against GRP78, phospho-PERK, phospho-eIF2 $\alpha$ , phospho-IRE1, XBP-1, caspase-12, CHOP, and actin, followed by a horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA). The membranes were incubated with enhanced chemiluminescence detection reagents (Amersham, Little Chalfont, Buckinghamshire, UK) and exposed to X-ray film in the dark to visualize protein bands.

### Reverse transcription–polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described (Han *et al.*, 2019). The sequences of primers used in this study are as follows: spliced *XBP-1*, forward primer (5'→3') CCTTG-TAGTTGAGAACCAGG, reverse primer (5'→3') GGGGCTTG-GTATATATGTGG; *hGAPDH*, forward primer (5'→3') TCAAGTGGGGCGATGCTGGC, reverse primer (5'→3') TGCCAGCCCCAGCGTCAAAG.

### Immunocytochemistry

Cells were seeded on a 4-well chamber glass slide at a density of  $1.5 \times 10^5$  cells/mL and incubated for 16 h. Then, the cells were directly treated with 3.3 µM shikonin, or pretreated with 1 µM of the ER stress inhibitor TUDCA for 30 min, followed by treatment with 3.3 µM shikonin. Protein expression was detected using GRP78 and CHOP antibodies, and ER-Tracker™ Blue-White DPX reagent was used for localization. Processing of the slides for immunocytochemistry was performed as previously described (Piao *et al.*, 2015).

### Transient transfection of small interfering RNA (siRNA)

The transfection of SNU-C5/5-FUR cells with control (si-Control, Santa Cruz Biotechnology) and CHOP (siCHOP, Bi-oneer, Seoul, Korea) siRNAs was performed according to a previously described method (Zhang *et al.*, 2013). The cells were subcultured and subjected to siRNA transfection. Knockdown efficiency was assessed by detecting protein expression using Western blotting. After 24 h transfection, the cells were treated with or without shikonin for 48 h. The role of shikonin in CHOP knockdown cells was examined as follows: PI staining and flow cytometry were used to identify sub-G<sub>1</sub> hypodiploid cells; DNA fragmentation was determined using a cellular DNA fragmentation ELISA kit (Roche Applied Science, Mannheim, Germany); apoptosis was confirmed using Hoechst 33342 staining reagent; cell viability was detected via MTT analysis.

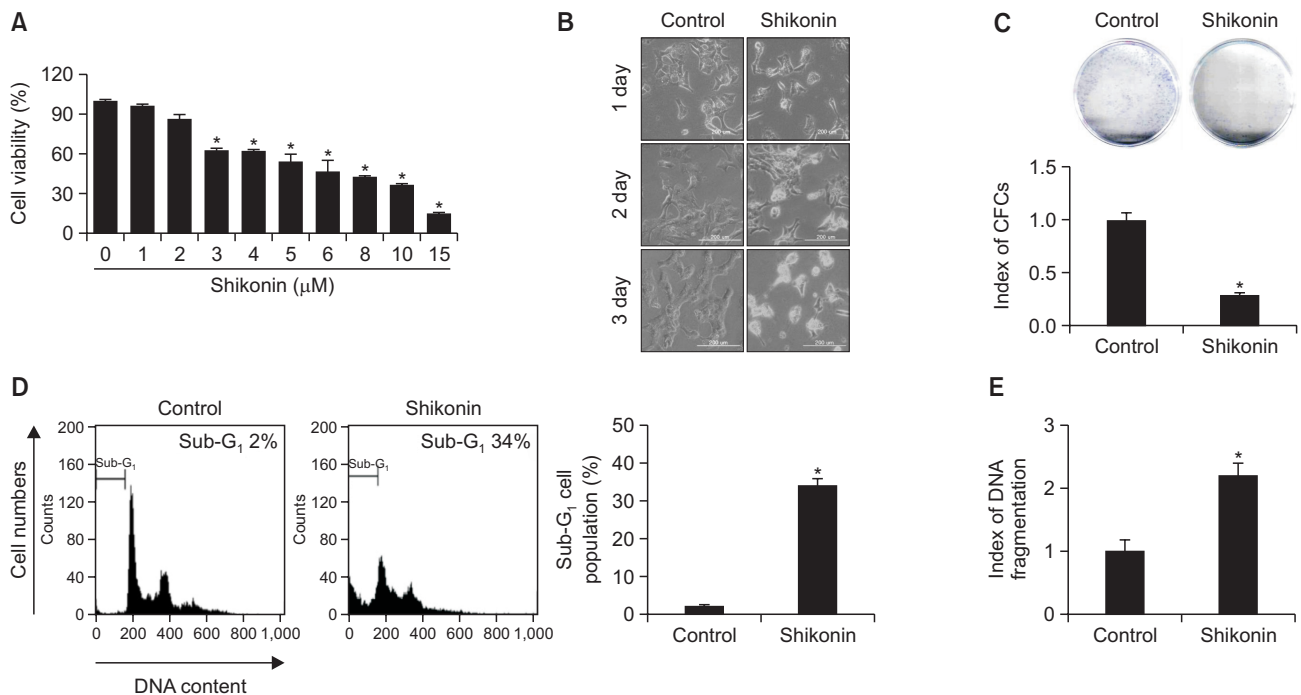
### Statistical analysis

All experiments were repeated three times, and the values presented are the mean  $\pm$  standard error of the mean. Analysis of variance (ANOVA) and Tukey's post-hoc test were performed to analyze the differences between the means. Statistical significance was set at  $p < 0.05$ .

## RESULTS

### Shikonin induces apoptosis in 5-FU-resistant colorectal cancer cells

To determine the effect of shikonin on 5-FU-resistant colorectal cancer cells, we treated SNU-C5/5-FUR cells, into a wide range of shikonin concentrations and determined the effects on cell viability. Our results indicated that shikonin exhibits concentration-dependent cytotoxicity in SNU-C5/5-FUR cells, with significant cytotoxicity evident at concentrations greater than 3 µM. The calculated IC<sub>50</sub> value was 5.7 µM (Fig. 1A). Based on past experience, it is convenient to observe cell changes when the cell survival rate is approximately 60%–70%; therefore, a concentration of 3.3 µM, at which the cell survival rate was 65%, was selected as the optimum concentration for further investigations. After 24 h treatment with 3.3 µM shikonin, morphological changes were observed in the cells, including some dead cells, although the number of dead cells was not prominent. However, after two days of incubation, a significant number of dead cells was observed, and after three days of incubation, most of the cells appeared to be dead (Fig. 1B). To determine whether shikonin inhibits cell proliferation, a colony formation assay was performed. The results showed that compared with the control group, shikonin significantly inhibited colony formation of SNU-C5/5-FUR cells (CFCs) (Fig. 1C). To confirm that the cytotoxic effect of shikonin was due to its ability to promote apoptosis, we used flow cytometry to measure the proportion of sub-G<sub>1</sub> DNA content (considered to represent apoptotic cells) after shikonin treatment. Shikonin significantly increased the rate of apoptosis relative to that in the control group (Fig. 1D). Moreover, shikonin-treated cells had a higher proportion of fragmented DNA content than the control group (Fig. 1E). These results suggested that shikonin effectively inhibits the proliferation of SNU-C5/5-FUR cells by inducing apoptosis.



**Fig. 1.** Shikonin induces apoptotic cell death of SNU-C5/5-FUR cells. (A) Shikonin was added to cells at various concentrations (1, 2, 3, 4, 5, 6, 8, 10, and 15 μM). After 48 h, cell viability was determined by MTT assay. \*Significantly different from untreated control cells ( $p < 0.05$ ). (B) Changes in cell morphology were detected by phase contrast images (magnification: 20×). (C) Long-term cytotoxic effects of shikonin were investigated using a colony formation assay. The resultant colonies were stained using a Diff-Quik kit. \*Significantly different from untreated control cells ( $p < 0.05$ ). (D) Cells treated with or without shikonin were stained with PI and analyzed via flow cytometry. (E) DNA fragmentation was assessed using ELISA. (D, E) \*Significantly different from control cells ( $p < 0.05$ ).

### Shikonin-induced apoptosis in SNU-C5/5-FUR cells is related to mitochondrial Ca<sup>2+</sup> accumulation

In the mitochondrial matrix, although the accumulation of Ca<sup>2+</sup> can stimulate oxidative phosphorylation, high concentrations of Ca<sup>2+</sup> can also transmit and amplify apoptotic signals. We observed high levels of fluorescence in the shikonin-treated cells, compared to control cells, which was detected using flow cytometry after staining with Rhod-2 AM, a mitochondrial Ca<sup>2+</sup>-specific dye (Fig. 2A). Images obtained by confocal microscopy also confirmed this result, with shikonin-treated cells exhibiting strong red fluorescence, indicating that the accumulation of mitochondrial Ca<sup>2+</sup> was higher than that of the control cells (Fig. 2B). To confirm the effect of Ca<sup>2+</sup> on shikonin-induced cell apoptosis, we treated SNU-C5/5-FUR cells with shikonin in culture medium with or without EGTA (a Ca<sup>2+</sup> chelator) and performed a nuclear fragmentation analysis using Hoechst 33342 staining reagent to detect changes in apoptosis. Compared with the control group, shikonin treatment resulted in a significant increase in apoptosis, but this increase was significantly inhibited in the shikonin+EGTA treatment group (Fig. 2C).

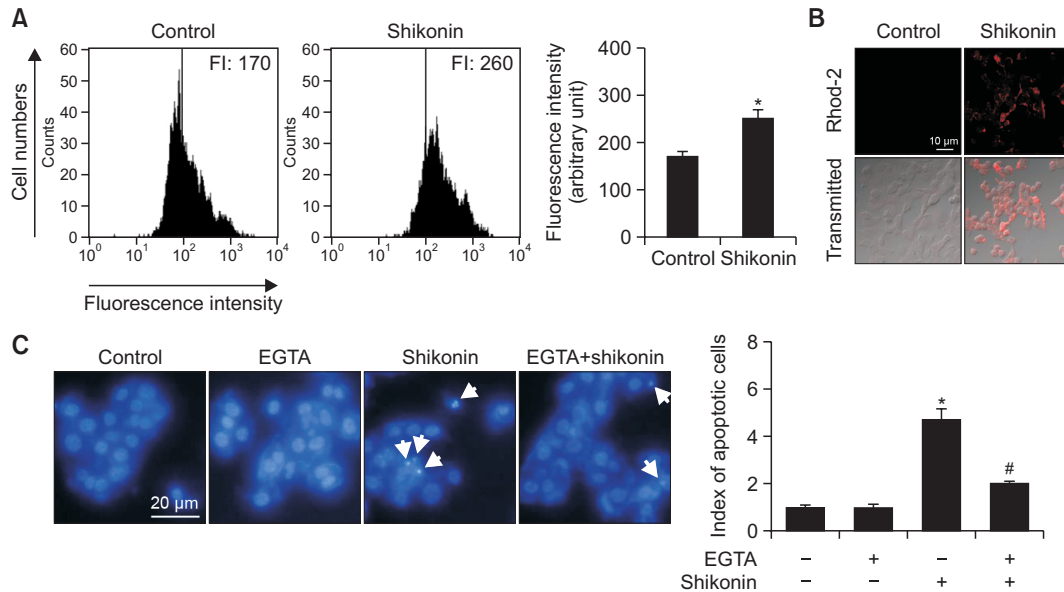
### Shikonin increases the levels of ER stress-related proteins and spliced XBP-1 mRNA

Next, we examined the effect of shikonin on the expression of ER stress-related factors using Western blotting. Compared with the control group, the expression of GRP78, phospho-PERK, phospho-eIF2α, phospho-IRE1, spliced XBP-1, cleaved caspase-12, and CHOP in the shikonin-treated

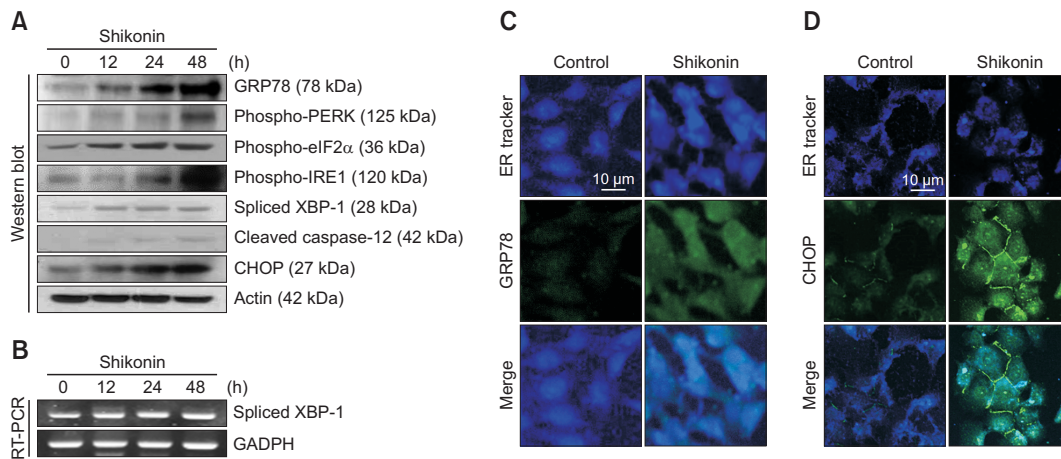
groups increased in a time-dependent manner (Fig. 3A). The activation of phospho-IRE1 leads to unconventional splicing of the mRNA encoding XBP-1. The spliced form of XBP-1 is translated into a transcription factor that promotes the expression of genes which regulate protein quality in the ER (Xu *et al.*, 2005). Compared with the control cells, the rate of conversion of XBP-1 mRNA into its spliced isoform increased in the shikonin-treated cells (Fig. 3B). To confirm this result, we examined the expression of the most representative ER stress marker proteins, GRP78 and CHOP, using immunocytochemistry. Compared with the control group, shikonin significantly upregulated the expression of GRP78 and CHOP in the treated cells (Fig. 3C, 3D).

### Downregulation of CHOP attenuates shikonin-induced apoptosis

It has been reported that overexpression of CHOP induces apoptosis through the Bcl-2 pathway, and CHOP has also been shown to regulate apoptosis during ER stress (Hu *et al.*, 2019). Therefore, we investigated whether CHOP was required for shikonin-induced apoptosis in SNU-C5/5-FUR cells. We used an siRNA against CHOP (siCHOP) to knock down the expression of CHOP in SNU-C5/5-FUR cells, with a scrambled RNA (siControl) as a negative control, and knock-down was confirmed using Western blot analysis (Fig. 4A). Our flow cytometry analyses showed that shikonin increased the rate of apoptosis in siControl-transfected cells, but this increase was attenuated in cells transfected with siCHOP (Fig. 4A). This result was confirmed by DNA fragmentation analy-



**Fig. 2.** Shikonin triggers accumulation of mitochondrial  $\text{Ca}^{2+}$  in SNU-C5/5-FUR cells. After cells were loaded with the fluorescent probe, Rhod-2 AM, mitochondrial  $\text{Ca}^{2+}$  levels were measured via (A) flow cytometry and (B) confocal microscopy. \*Significantly different from control cells ( $p < 0.05$ ). (C) Cells were inoculated in culture medium with or without EGTA (500 mM), and after 16 h, 3.3  $\mu\text{M}$  shikonin was added. After 48 h incubation, the presence of apoptotic bodies was determined using the Hoechst 33342 nuclear staining method. \*Significantly different from control cells ( $p < 0.05$ ); #significantly different from shikonin-treated cells ( $p < 0.05$ ).

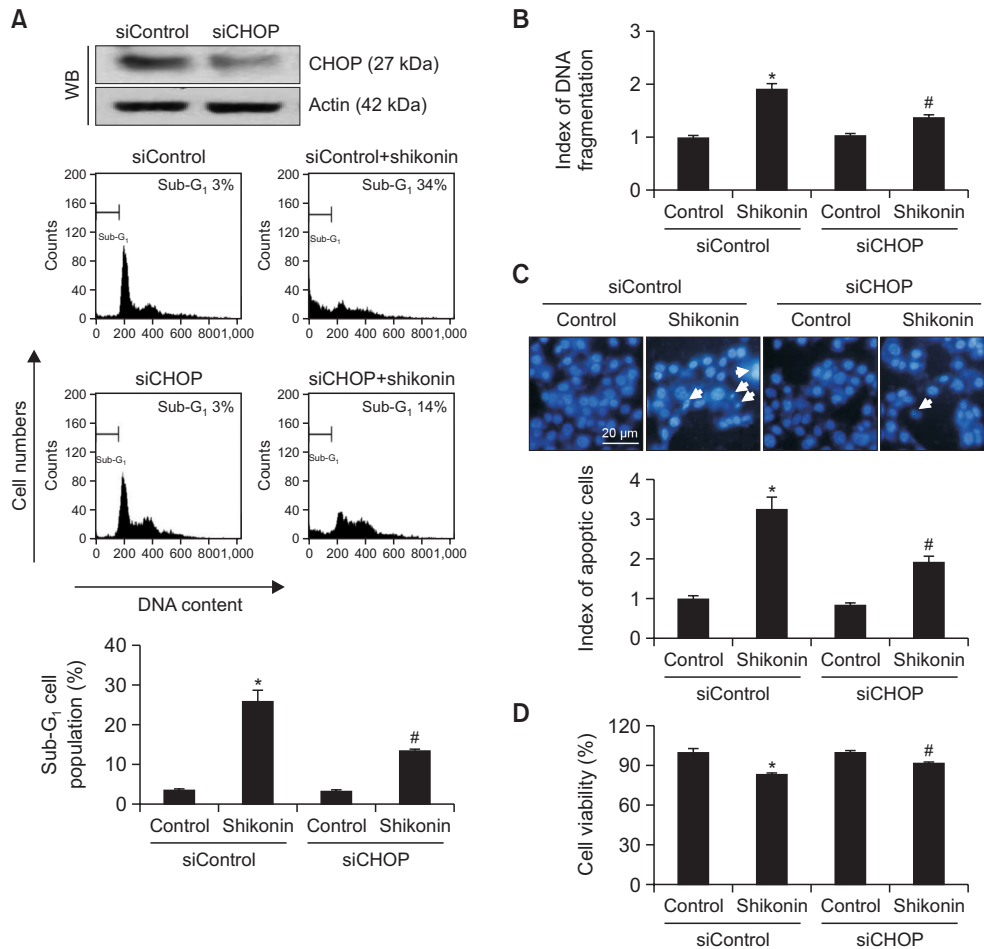


**Fig. 3.** Shikonin increases the expression of ER stress-related proteins and splicing of *XBP-1* mRNA. (A) Cell lysates were subjected to electrophoresis, and GRP78, phospho-PERK, phospho-eIF2 $\alpha$ , phospho-IRE1, spliced XBP-1, cleaved caspase-12, and CHOP were detected using Western blotting. Actin was used as a loading control. (B) The level of spliced *XBP-1* mRNA was measured using RT-PCR. GAPDH was used as a loading control. The expression of (C) GRP78 and (D) CHOP proteins were evaluated via immunocytochemistry. The ER-tracker Blue-White DPX probe was used to determine the number and morphology of ER organelles.

sis (Fig. 4B). Moreover, Hoechst 33342 staining for apoptotic bodies also yielded results that were consistent with these findings (Fig. 4C). The results from MTT cell viability assays showed that shikonin inhibited the survival of siControl-transfected cells, but the shikonin-induced decrease in cell numbers was reduced in cells transfected with siCHOP (Fig. 4D). These data demonstrated that CHOP plays an essential role in apoptosis during ER stress induced by shikonin.

### Shikonin promotes cell death in SNU-C5/5-FUR cells by activating ER stress

To further confirm that ER stress is involved in the apoptosis of SNU-C5/5-FUR cells induced by shikonin, we examined the effect of the ER stress inhibitor, TUDCA, in our experiments. As shown in Fig. 5A, TUDCA effectively restored the shikonin-induced decrease in cell viability. Moreover, pretreatment with TUDCA significantly inhibited the emergence of apoptotic bodies (visualized by Hoechst 33342 staining) in the shikonin-treated group (Fig. 5B). To confirm this result, we also ana-



**Fig. 4.** Knockdown of CHOP attenuates shikonin-induced apoptosis in SNU-C5/5-FUR cells. After confirming the successful transfection of siControl and siCHOP cells via Western blot analysis, the inoculated cells were treated with or without shikonin for 48 h. (A) Cells with apoptotic sub-G<sub>1</sub> DNA content were detected using flow cytometry following PI staining, and (B) DNA fragmentation was quantitated using ELISA. After 48 h incubation, (C) the apoptotic bodies were identified via Hoechst 33342 nuclear stain and (D) cell viability was evaluated via MTT analysis. (A-D) \*Significantly different from siControl-transfected cells ( $p < 0.05$ ); #significantly different from shikonin-treated, siControl-transfected cells ( $p < 0.05$ ).

lyzed the expression of the ER stress marker proteins, GRP78 and CHOP, via immunocytochemistry after TUDCA treatment. TUDCA significantly inhibited the expression of GRP78 and CHOP that were induced by shikonin (Fig. 5C, 5D).

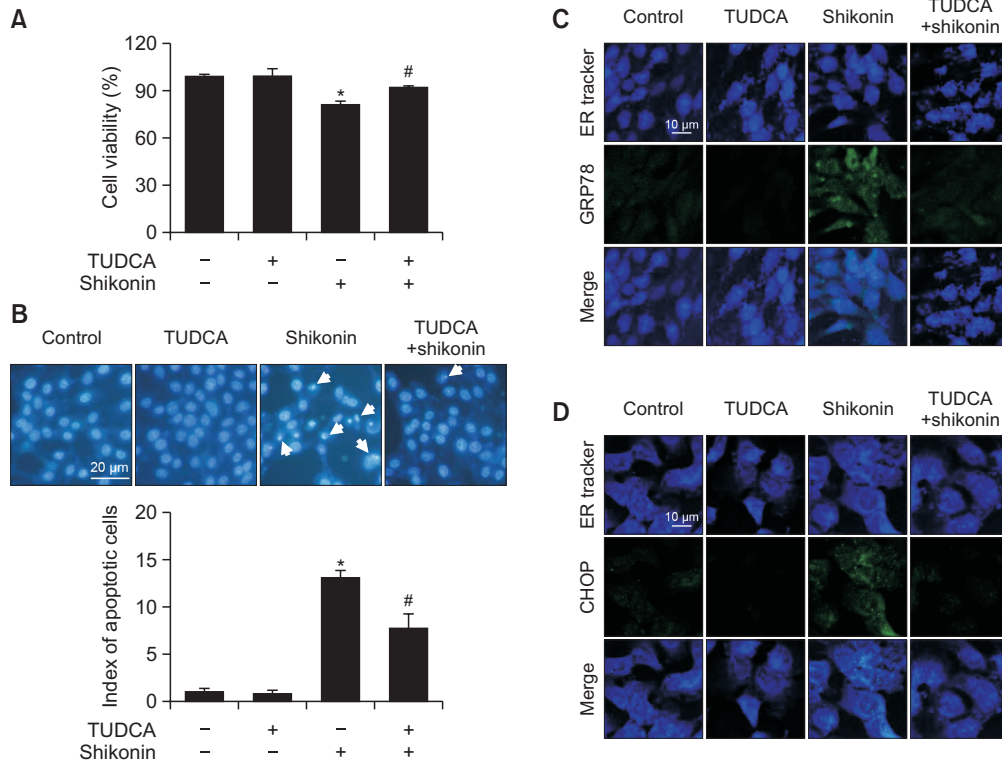
## DISCUSSION

Shikonin has a variety of biological activities, making it an attractive compound. We have previously reported that shikonin induces apoptosis in SNU-407 colon cancer cells by triggering mitochondrial dysfunction and activating the caspase cascade (Han *et al.*, 2019). In that study, we used shikonin at the IC<sub>50</sub> concentration of 3.3 μM in our experiments. However, in SNU-C5/5-FUR cells, the IC<sub>50</sub> value is approximately 5.7 μM, which is nearly twice the IC<sub>50</sub> value of 3.3 μM in the SNU-407 cells. Comparing these results, it is apparent that while the sensitivity of shikonin is slightly lower in 5-FU-resistant cells, it still has obvious apoptotic activity in these cells. In this study, we observed that shikonin significantly increased the

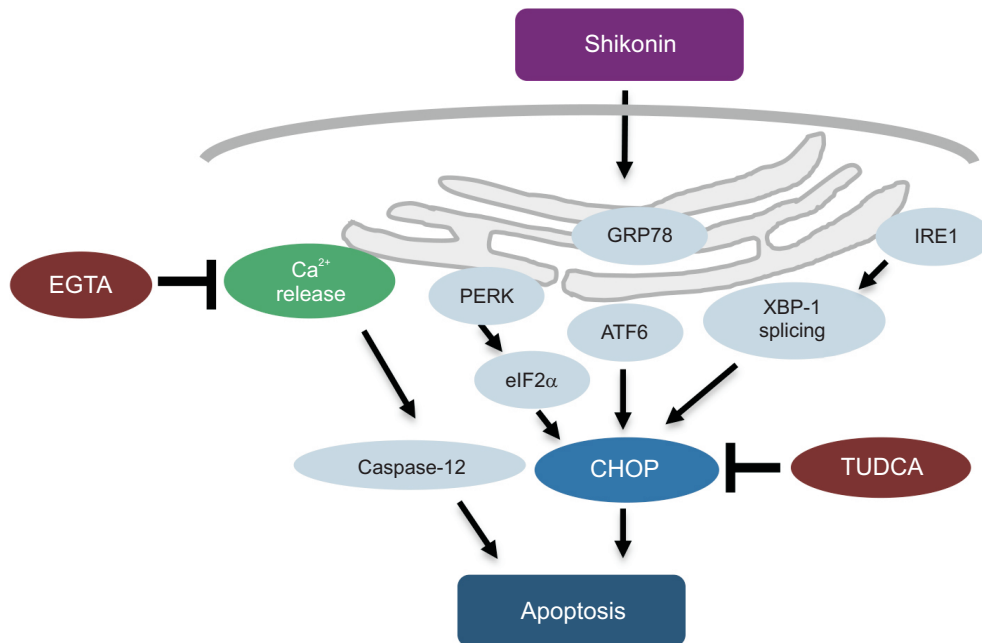
proportion of SNU-C5/5-FUR cells with apoptotic sub-G<sub>1</sub> content and triggered DNA fragmentation.

Generally, the ER stress response is a short-term, homeostasis-linked event that is critical for cell survival, although long-term and severe ER stress can trigger apoptosis through ER stress-specific cell death signals, such as CHOP and caspase-12 (Coker-Gürkan *et al.*, 2015). The ER is the main Ca<sup>2+</sup> storage site in cells, and it is worth noting that excessive Ca<sup>2+</sup> storage in the ER can result in leaks into the cytoplasm, allowing Ca<sup>2+</sup> participation in ER stress-mediated apoptosis (Ryoo, 2015). Here, we used the calcium chelator, EGTA, to verify that treatment with shikonin significantly increased the concentration of Ca<sup>2+</sup> in the mitochondrial matrix.

We also studied specific markers of ER stress. Under stress-free conditions, the luminal domain of the ER stress sensor binds to the ER chaperone, binding immunoglobulin protein (BiP), keeping it inactive. When unfolded or misfolded proteins accumulate, BiP preferentially binds to these abnormal proteins and releases the inhibition of PERK, ATF6, and IRE1 (Mei *et al.*, 2013). The cytoplasmic domain of IRE1 possesses



**Fig. 5.** ER stress inhibitor, TUDCA, attenuates shikonin-induced cell death. Cells were incubated in the presence of 1  $\mu$ M TUDCA for 30 min and then treated with 3.3  $\mu$ M shikonin. After 48 h incubation, (A) cell viability was evaluated by MTT analysis, and (B) the presence of apoptotic bodies was determined by Hoechst 33342 nuclear staining. \*Significantly different from control cells ( $p < 0.05$ ); #significantly different from shikonin-treated cells ( $p < 0.05$ ). The expression of (C) GRP78 and (D) CHOP proteins was evaluated using immunocytochemistry. The ER-tracker Blue-White DPX probe was used to determine the number and morphology of ER organelles.



**Fig. 6.** Diagram of shikonin-induced ER stress signaling resulting in apoptosis. Shikonin induces the expression of ER stress-related proteins, such as GRP78 and CHOP, and releases  $Ca^{2+}$ , thereby causing 5-FU-resistant SNU-C5 colorectal cancer cells to undergo apoptosis. The calcium chelator, EGTA, and the ER stress inhibitor, TUDCA, protected cells against ER stressed-apoptotic cell death.

serine/threonine kinase activity, but the only known substrate of this kinase is IRE1. ER stress-induced homodimerization and trans-autophosphorylation activate IRE1 endoribonuclease activity, allowing it to excise a 26-nucleotide intron from the *XBP-1* mRNA to generate the spliced isoform, *XBP-1s* (Li *et al.*, 2010). Activated PERK phosphorylates eIF2 $\alpha$ , thereby reducing protein synthesis and reducing ER protein overload (Yan *et al.*, 2017). Here, we observed that shikonin induced the phosphorylation of PERK, eIF2 $\alpha$ , and IRE1, as well as XBP-1 splicing, caspase-12 cleavage, and GRP78 and CHOP overexpression, in a time-dependent manner.

Although CHOP is expressed at very low levels under physiological conditions, it is strongly induced at the transcriptional level under conditions of ER stress (Gotoh *et al.*, 2011). The overexpression of CHOP promotes growth arrest, and eventually, cell death. Therefore, CHOP plays a very important role in ER stress-induced apoptosis. We observed that siRNA-mediated knockdown of CHOP attenuated shikonin-induced apoptosis and cell death. In addition, treatment with the ER stress inhibitor, TUDCA, significantly restored cell viability, and greatly suppressed the emergence of apoptotic bodies due to shikonin exposure. This further supports a role for ER stress in the cytotoxic activity of shikonin in SNU-C5/5-FUR cells (Fig. 6). We believe that shikonin is a valuable candidate resource that is worthy of further research as a possible colorectal cancer treatment.

## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

## ACKNOWLEDGMENTS

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