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Quinalizarin Induces Apoptosis through Reactive Oxygen Species (ROS)-Mediated Mitogen-Activated Protein Kinase (MAPK) and Signal Transducer and Activator of Transcription 3 (STAT3) Signaling Pathways in Colorectal Cancer Cells

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Statistical Analysis C
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Background: Quinalizarin (1,2,5,8-tetrahydroxyanthraquinone) exhibits potentially useful anticancer effects by inducing apoptosis in several types of cancer, but its underlying mechanism of action remains unknown. The present study examined the effects of quinalizarin on the induction of cell cycle arrest, apoptosis, the generation of reactive oxygen species (ROS), other underlying mechanisms, and its role in modifying colorectal cancer cell lines.

Material/Methods: The MTT assay was used to evaluate the viability of SW480 and HCT-116 cells that had been treated with quinalizarin and 5-fluorouracil (5-FU). Cell cycle arrest and apoptosis were analyzed by flow cytometry. Western blotting was used to investigate the mitochondrial pathway; Akt, MAPK, and STAT3 signaling pathways were also investigated. The relationship between ROS generation and apoptosis was analyzed by flow cytometry and western blotting.

Results: The results indicated that quinalizarin significantly inhibits the viability of SW480 and HCT-116 cells in a dose-dependent manner. Quinalizarin induced SW480 cell cycle arrest at G_2/M by regulating cyclin B1 and CDK1/2. The apoptosis-related protein expression levels of p-p53, Bad, cleaved caspase-3, cleaved PARP and p-JNK were increased in quinalizarin-treated cells, while protein expression levels Bcl-2, p-Akt, p-ERK, and p-STAT3 were decreased. Quinalizarin induced apoptosis in colorectal cancer cells by regulating MAPK and STAT3 signaling pathways via ROS generation.

Conclusions: Quinalizarin induces apoptosis via ROS-mediated MAPK/STAT3 signaling pathways.

MeSH Keywords: Apoptosis • Colorectal Neoplasms • Mitogen-Activated Protein Kinases • Reactive Oxygen Species • STAT3 Transcription Factor

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Background

Colorectal cancer (CRC) is the third most common malignancy and the fourth most common cancer worldwide, with an estimated 1.3 million new cases diagnosed every year [1]. Predictions are that from 2017 to 2030, the incidence of CRC will increase by 60% in developing countries; it has been increasing rapidly in China [2]. CRC patients with metastasis have a poor prognosis, although chemotherapy with 5-fluorouracil (5-FU) is commonly used to treat them [3]. Such treatment has been found to prolong survival for up to 20 months, but survival remains poor for many reasons, not all of which are related to the tumor itself [4].

Quinalizarin (1,2,5,8-tetrahydroxyanthraquinone) has been regarded as a highly selective cell-permeable compound [5]. Many studies have shown that quinalizarin can regulate proliferation, migration, and apoptosis in various cancer cell lines [6,7]; however, the mode of action of quinalizarin as an anticancer drug needs further investigation and its potential signaling pathways need to be identified.

Akt has been reported to be an important regulator and mediator of various cellular activities, such as cell growth, proliferation, survival, and apoptosis, in response to extracellular stimuli [8]. Mitogen-activated protein kinase (MAPK) pathways are involved in cell survival and resistance associated with apoptosis in many cancer cells following exposure to different stresses [9]. In addition, the Akt and MAPK pathways have been shown to protect tumor cells from apoptosis and promote drug resistance in CRC [10]. Furthermore, the MAPK signaling pathway is involved in p53-independent apoptosis [11]. The signal transducer and activator of transcription 3 (STAT3) is involved in various intracellular signals, tumor initiation, apoptosis, and many other responses [12]. Importantly, STAT3 signaling is constitutively active in various human cancers, including colorectal cancer, and the activation of STAT3 signaling is significantly correlated with poor prognosis and aggressive progression in colorectal cancer patients [13–16].

Reactive oxygen species (ROS) play essential roles in maintaining biological functions such as cell proliferation and apoptosis [17]. The moderation of intracellular ROS levels can promote cell proliferation and differentiation, and the overproduction of ROS can lead to cytotoxicity in cancer cells [18,19]. There is evidence that MAPK and STAT3 are representative ROS-responsive signaling pathways that are involved in mitochondrial dysfunction and cell survival [20]. Increasing intracellular ROS levels can suppress the growth of cancer cells and induce cellular apoptosis by mediating MAPK and STAT3 signaling components [21].

In the course of this study, for the first time, we found evidence that quinalizarin induces CRC cell cycle arrest, cell apoptosis, and ROS generation. Furthermore, we also explored the underlying mechanisms in CRC to understand its anticancer effects.

Material and Methods

Chemicals and reagents

Quinalizarin (Sigma-Aldrich; St. Louis, MO, USA) and 5-FU (MedChem Express; Princeton, NJ, USA) were dissolved together (20 mM in 100% DMSO) (Sigma-Aldrich, St. Louis, MO, USA) as a stock solution and stored at –20°C. Solutions were diluted with cell culture media before use.

Cell lines and cell cultures

The CRC (SW480 and HCT-116) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, Auckland, NZ), 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified 5% CO_2 incubator at 37°C.

Cell viability assay

SW480 and HCT-116 were harvested and seeded in 96-well culture plates at a density of 6000 cells per well. After 24 hours of incubation, the cells were treated with various concentrations (1, 3, 10, 30, and 100 µmol/L) of 5-FU or quinalizarin for 24 hours. Subsequently, the cells were incubated with 20 µL MTT (5 mg/mL) for 2 hours and the intracellular formazan dyes were solubilized with DMSO. After the cells were incubated for 20 minutes at 37°C, the absorbance of the solutions was measured at 490 nm. Percentage viabilities were calculated as the absorbance of the drug-treated sample/the absorbance of the DMSO-treated sample times 100%.

Cell cycle analysis

SW480 cells were seeded into 6-well culture plates (1×10^5 cells per well) and cultivated for 24 hours. Cells were pretreated with 10 µmol/L of quinalizarin for 3, 6, 12, and 24 hours; untreated cells were included as the control. Then, the cells were trypsinized, washed twice with chilled PBS, and fixed with 70% ethanol for 12 hours at –20°C. Cell suspensions were incubated with RNase A and propidium iodide (PI) (Beyotime Biotechnology, Shanghai, China) for 30 minutes at 37°C in the dark. The stained cells were then analyzed for DNA content by flow cytometry (Beckman Coulter, California, USA) with a 488-nm argon laser.

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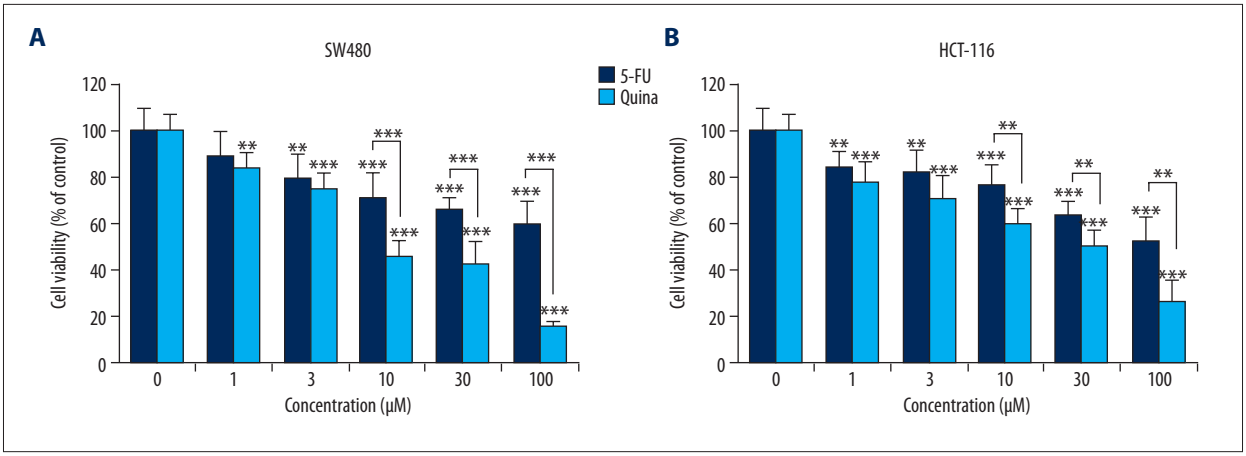

Figure 1. Cytotoxic effects of 5-FU and quinalizarin in CRC cells. (**A**) Viability of SW480 cells. (**B**) Viability of HCT-116 cells. Cells were treated with the indicated concentrations of 5-FU and quinalizarin for 24 hours, and then the cell viability was measured using the MTT assay. Cell viabilities were expressed as a percentage of viable cells compared with the 5-FU-treated groups. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ versus the 5-FU group.

Apoptosis assay

Apoptosis was performed using Annexin V-FITC/PI double staining and analyzed by flow cytometry. Cells were plated at a density of 1×10^5 cells per well in 6-well plates and incubated overnight. Then, the cells were treated with quinalizarin (10 μmol/L) for 0, 3, 6, 12, and 24 hours, collected at 5,000×g for 5 minutes at 4°C, and washed twice with PBS. Staining solutions containing Annexin V-FITC and PI (Beyotime Biotechnology) were added to cell suspensions for 20 minutes and incubated in the dark. The cells were then detected by flow cytometry.

Western blot analysis

Cells were lysed in lysis buffer at 4°C for 30 minutes and separated using centrifugation at 4°C for 30 minutes at 12,000×g. Then, the supernatant was dissolved with 5× sample loading buffer at a total of 30 μg protein. Electrophoresis was performed using 10% SDS-PAGE gels, and the proteins were subsequently transferred onto nitrocellulose membranes. Following blocking with 5% skim milk, the membranes were incubated for 12 hours at 4°C with primary antibodies (all of them were obtained from Santa Cruz Biotechnology, Dallas, TX, USA) against mouse monoclonal β-actin, α-tubulin, cyclin B1, CDK1/2, Bcl-2, Bad, cleaved caspase-3, PARP, p-p38, p-JNK, JNK, p-ERK, p-STAT3, and STAT3. Rabbit polyclonal antibodies included p-p53, p53, p-Akt1/2/3, Akt1/2/3, p38α/β, and ERK2. Peroxidase-Conjugated AffiniPure Goat Anti-Mouse IgG and Goat Anti-Rabbit IgG were used as the secondary antibodies. Protein bands were detected by an enhanced chemiluminescence (ECL) detection system and the band intensities were analyzed by ImageJ software.

Detection of intracellular ROS

Flow cytometry was performed to measure the intracellular ROS generation of quinalizarin-treated SW480 and HCT-116 cells. Cells were seeded in 6-well culture plates (1×10^5 cells per well) and treated with quinalizarin (10 μmol/L) for 24 hours. A fluorescent probe comprising 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Merck, Shanghai, China) was used to evaluate intracellular ROS. DCFH-DA (10 mmol/L) was dissolved in PBS at a concentration of 20 μL/mL for 30 minutes at 37°C. The substrate solution was then removed and the cells were washed with PBS. The levels of ROS were analyzed by flow cytometry.

Statistical analysis

Data were presented as the mean ±SD and all of the experiments were replicated 3 times. Statistical analyses were performed using Excel. $P<0.05$ indicated statistically significant differences, which are noted in the figures as * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

Results

Quinalizarin inhibits cells proliferation in colorectal cancer

To determine the effect of quinalizarin on cell survival, SW480 and HCT-116 cells were treated with 5-FU and quinalizarin for 24 hours and cell viabilities were detected by MTT assay. As shown in Figure 1A and 1B, quinalizarin had significant inhibitory effects on the proliferation of SW480 and HCT-116 cells in a dose-dependent manner. The cell numbers of the quinalizarin groups were significantly less than those of the 5-FU groups. The proliferation inhibition rate of quinalizarin on SW480 cells

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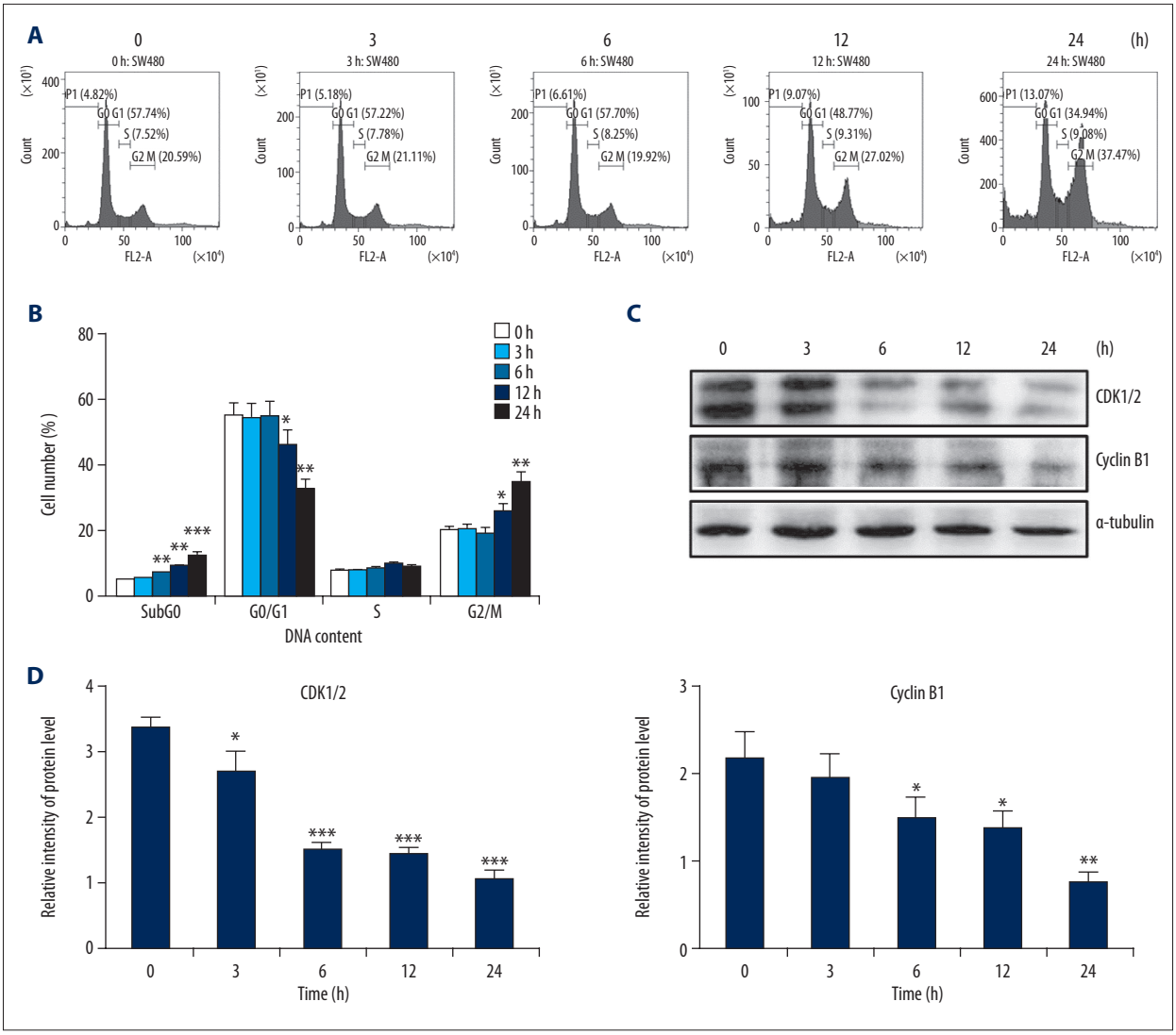

Figure 2. Effect of quinalizarin on the cell cycle distribution and cell cycle checkpoint related proteins in SW480 cells. (**A**) Cells were stained with PI; DNA content was then analyzed for cell cycle phase distribution by flow cytometry. The percentages of apoptotic cells were evaluated by SubG0 DNA content (hypodiploid DNA). (**B**) The quantified data of cell cycle distribution from A. (**C**) Cells were treated with quinalizarin for various lengths of time (0, 3, 6, 12, and 24 hours) and subjected to western blotting with antibodies against cyclin D1 and CDK1/2. (**D**) The intensities of the bands were quantified by the Image J program. * P<0.05, ** P<0.01, and *** P<0.001 versus 0 hours.

(IC_{50}, 10.13 μmol/L) was higher than that on HCT-116 cells (IC_{50}, 13.65 μmol/L). These results indicate that quinalizarin has excellent cytotoxic effects on CRC cells. Since SW480 cells were more sensitive to quinalizarin, we chose SW480 cells as the subjects of the subsequent experiments.

Quinalizarin induces cell cycle arrest in SW480 cells

To investigate the possible mechanisms of quinalizarin-induced growth inhibition, cell cycle distribution was analyzed by flow cytometry. As shown in Figure 2A and 2B, quinalizarin induced cell cycle arrest at the G_2/M phase. Compared with the control group, the percentage of cells in the G_2/M phase of the cell cycle was significantly increased, accompanied by a decrease in the number of cells in the G_0/G_1 phase. To further investigate the mechanism of quinalizarin-induced cell cycle arrest at the G_2/M phase, the expression levels of two key regulatory proteins of the cell cycle were examined. As shown in Figure 2C and 2D, SW480 cellular protein expression levels of cyclin B1 and CDK1/2 were repressed in a time-dependent manner. These results indicate that quinalizarin can induce cell cycle arrest at the G_2/M phase by decreasing the expression levels of cyclin B1 and CDK1/2.

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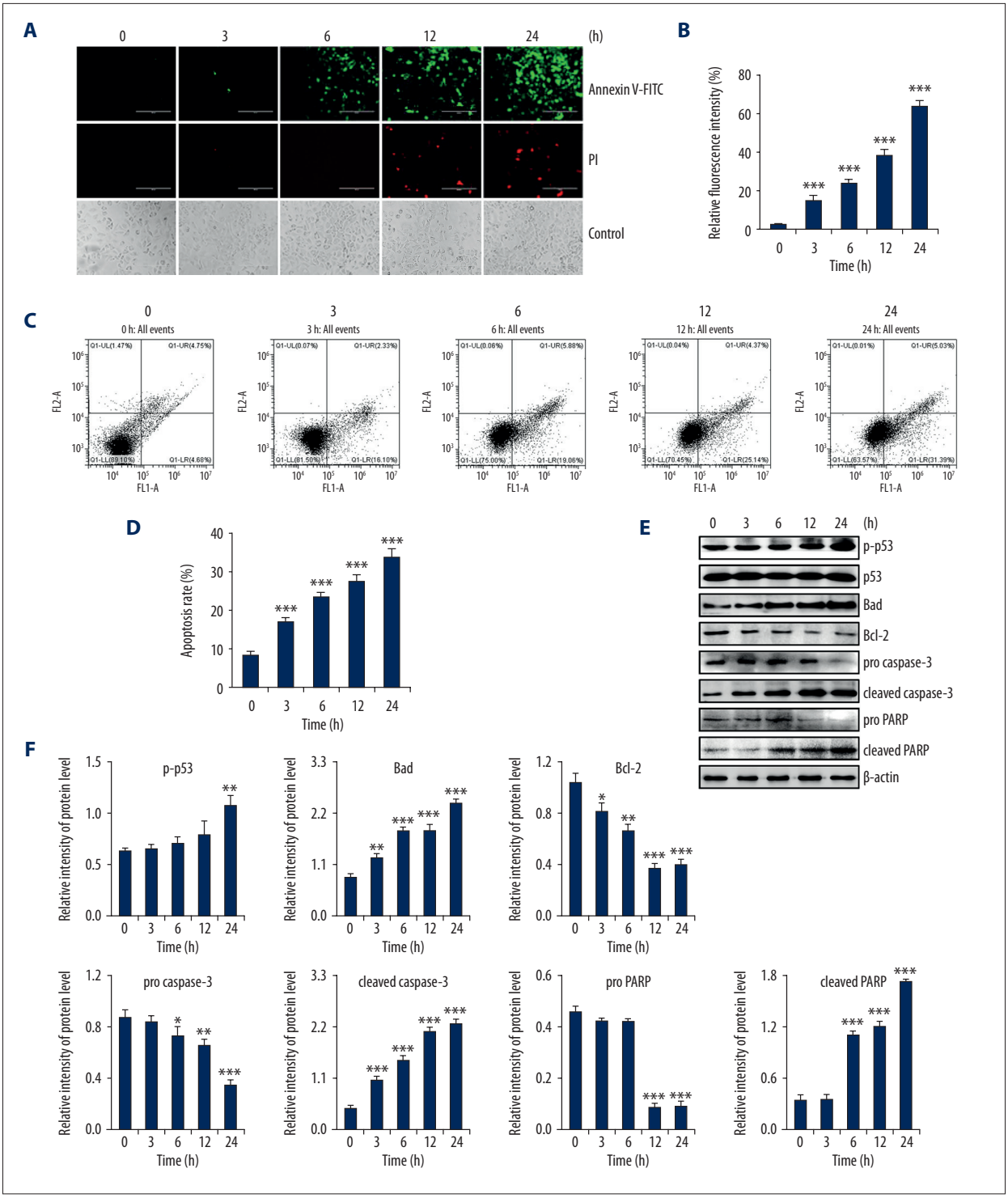

Figure 3. Apoptotic effect of quinalizarin on SW480 cells. (**A**) Cells were treated with quinalizarin for various lengths of time (0, 3, 6, 12, and 24 hours) and stained with Annexin V-FITC/PI. Data shown represent fluorescence microscopic images (original magnifications, 200×). (**B**) Quantification of fluorescence intensities from A. (**C**) For the quantitative analysis of apoptosis, cells were incubated with Annexin V-FITC/PI and analyzed by flow cytometry. (**D**) Quantification of flow cytometry from C. (**E**) Cells were treated with quinalizarin for various lengths of time (0, 3, 6, 12, and 24 hours) and subjected to western blotting with antibodies against p-p53, Bad, Bcl-2, cleaved caspase-3, and cleaved PARP. (**F**) The intensities of the bands were quantified by the ImageJ program. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ versus 0 hours.

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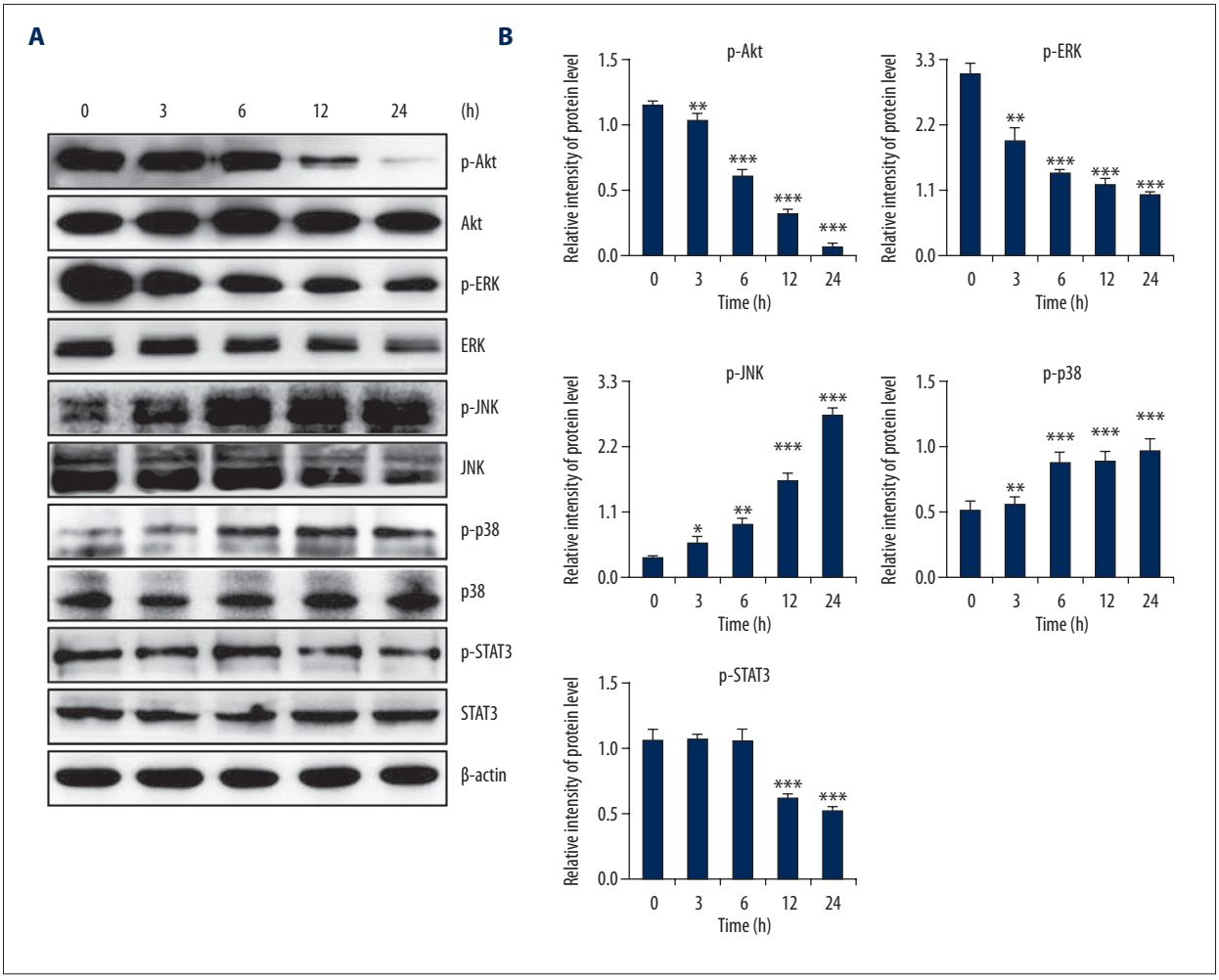

Figure 4. Quinalizarin induces the phosphorylation of Akt, ERK, JNK, p38, and STAT3 in SW480 cells. (**A**) Cells were treated with quinalizarin for various lengths of time (0, 3, 6, 12, and 24 hours). The expression levels of p-Akt, p-ERK, p-JNK, p-p38, and p-STAT3 were analyzed by western blotting. (**B**) Quantitative results for p-Akt, p-ERK, p-JNK, p-p38, and p-STAT3 protein levels, which were adjusted with their total protein levels. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ versus 0 hours.

Quinalizarin induces apoptosis in SW480 cells

SW480 cells were treated with quinalizarin for various lengths of time (3, 6, 12, and 24 hours) to investigate whether quinalizarin promotes apoptosis in CRC cells. As shown in Figure 3A and 3B, the fluorescence intensities of Annexin V-FITC and PI were increased in a time-dependent manner. As shown in Figure 3C and 3D, the number of apoptotic cells increased from 8.55% to 34.51% after treatment with quinalizarin for 24 hours. These results suggest that quinalizarin is a potent inducer of apoptosis in colorectal cells. To investigate the mechanism of quinalizarin-induced apoptosis in SW480 cells, the expression levels of apoptosis-related proteins were determined by Western blot. As shown in Figure 3E and 3F, p53 was activated after treatment with quinalizarin for 12 hours, and total p53 showed no significant change. The expression level of Bcl-2 was decreased and Bad, cleaved caspase-3, and cleaved Poly (ADP-ribose) polymerase (PARP) were significantly increased after treatment with quinalizarin. These results indicate that the induction of apoptosis is associated with the mitochondria-dependent signaling pathway in SW480 cells.

Quinalizarin regulates Akt, MAPK and STAT3 signaling pathways

The expression levels of Akt, MAPK, and STAT3 were detected by Western blot to investigate possible signaling pathways in quinalizarin-induced apoptosis. As shown in Figure 4A and 4B, the phosphorylation level of Akt was significantly decreased, and there was no apparent change in total Akt under the same conditions. We next investigated the effects of quinalizarin on the activation of MAPK, including ERK, JNK, and p38, in SW480 cells. Quinalizarin significantly decreased the phosphorylation level of ERK and increased the phosphorylation

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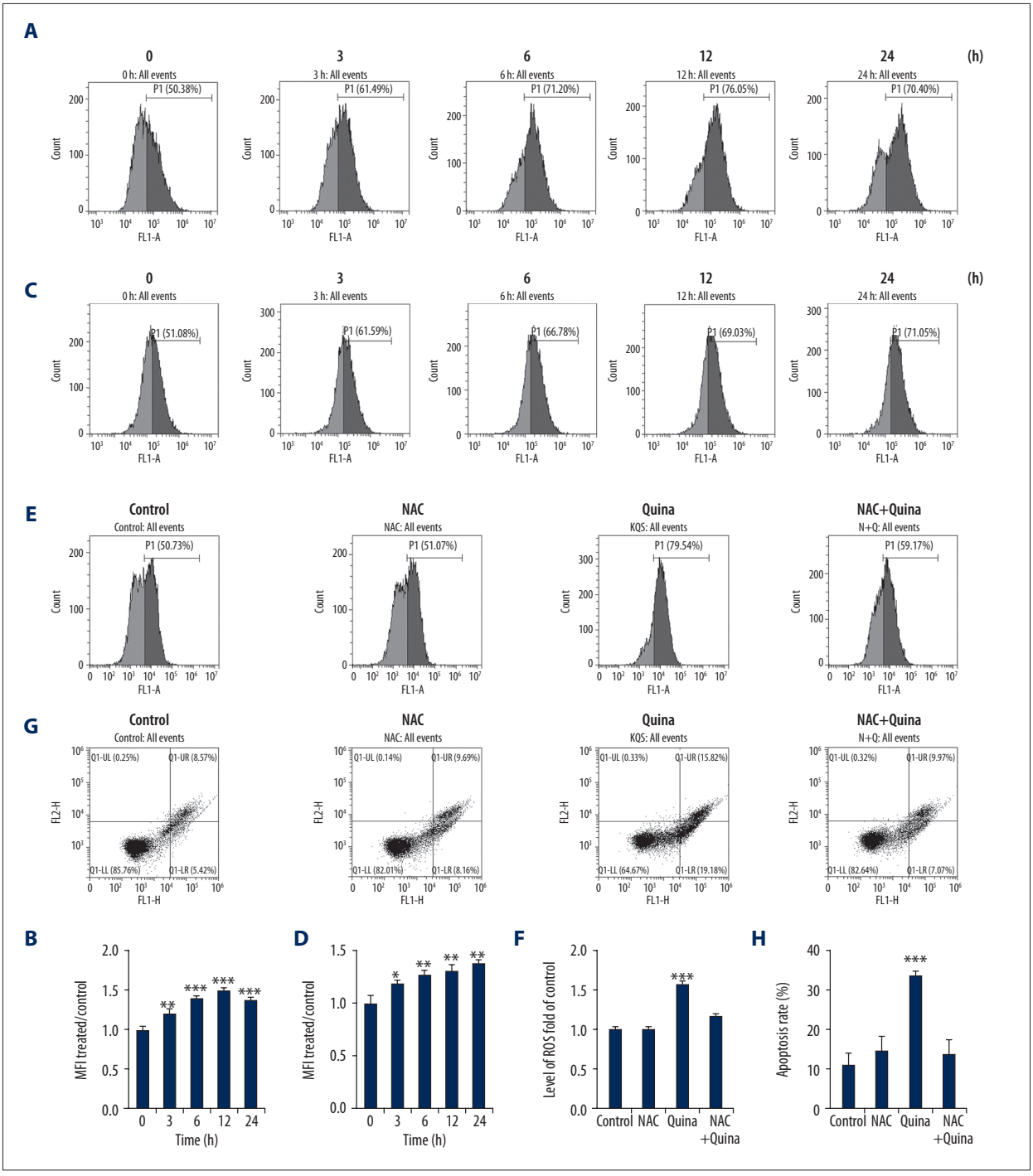

Figure 5. Effects of quinalizarin on induced ROS generation and apoptosis in SW480 cells. (**A**) SW480 cells were treated with quinalizarin for various lengths of time, and then stained with DCFH-DA (10 μM); ROS generation was then determined by flow cytometry. (**B**) Quantification of the SW480 intracellular ROS levels from A. (**C**) HCT-116 cells were treated with quinalizarin for various lengths of time, and ROS generation was then determined by flow cytometry. (**D**) Quantification of the HCT-116 intracellular ROS levels from C. (**E**) Cells were treated with NAC for 30 minutes before being treated with quinalizarin and stained with DCFH-DA (10 μM). Intracellular ROS levels were analyzed by flow cytometry. (**F**) Quantification of the intracellular ROS levels from E. (**G**) Cells were treated with NAC for 30 minutes and then incubated with quinalizarin for 24 hours. Early and late apoptosis cell distributions were determined using flow cytometry (cells were stained with Annexin V-FITC and PI). (**H**) Quantification of early and late apoptosis cell distributions from G. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ versus 0 hours. Quina – quinalizarin; ROS – reactive oxygen species; NAC – N-acetyl-L-cysteine; PI – propidium iodide.

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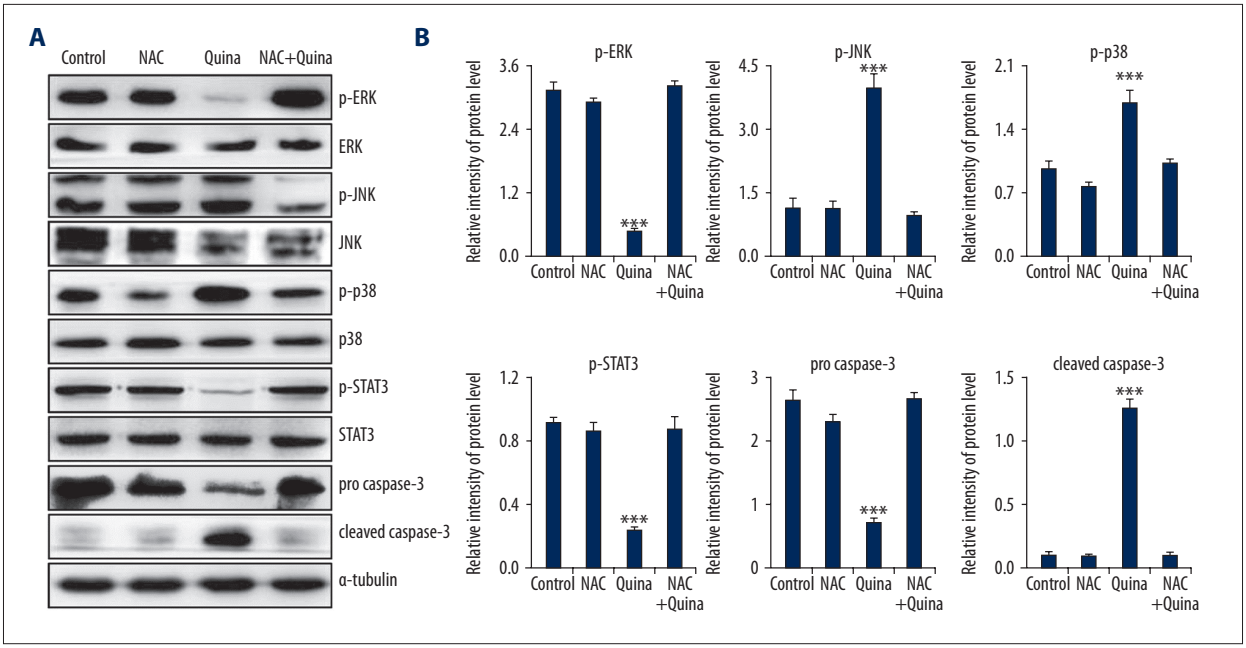

Figure 6. Quinalizarin induces ROS-mediated apoptosis via MAPKs and STAT3 signaling pathways. (**A**) Detection of the protein expression levels of p-ERK, p-JNK, p-p38, p-STAT3, and cleaved caspase-3 by western blotting. (**B**) Quantitative results of p-ERK, p-JNK, p-p38, p-STAT3, and cleaved caspase-3 protein levels, which were adjusted with their total protein levels from A. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$. Quina – quinalizarin; ROS – reactive oxygen species.

levels of JNK and p38. Of note, the protein expression level of total ERK was slightly decreased after treatment with quinalizarin. Furthermore, quinalizarin suppressed the phosphorylation level of STAT3 in a time-dependent manner. Taken together, these results suggest that quinalizarin induced apoptosis by regulating the Akt, MAPK, and STAT3 signaling pathways in the SW480 cells.

Quinalizarin induces ROS-mediated apoptosis in SW480 cells

Because ROS can initiate various types of stimulus-induced apoptosis, we investigated whether ROS were involved in quinalizarin-induced apoptosis in SW480 and HCT-116 cells. As shown in Figure 5A–5D, ROS generation was observed in SW480 and HCT-116 cells in a time-dependent manner. As shown in Figure 5E and 5F, quinalizarin-induced intracellular ROS generation was significantly decreased after treatment with ROS scavenger N-acetyl-L-cysteine (NAC) in SW480 cells. As shown in Figure 5G and 5H, quinalizarin-induced apoptotic cells were significantly decreased after scavenging by intracellular ROS. These results clearly demonstrate the important role of ROS in the induction of apoptosis. As shown in Figure 6A and 6B, the decreased phosphorylation levels of ERK and STAT3, and increased phosphorylation levels of p38, JNK, and cleaved caspase-3 induced by quinalizarin were suppressed by scavenging intracellular ROS. These results show that ROS regulate MAPK and STAT3 signaling pathways to induce apoptosis of SW480 cells.

Discussion

This study is the first to demonstrate the antiproliferation- and apoptosis-inducing effects of quinalizarin on CRC cell lines. The cell cycle checkpoints involved DNA repair when cells were damaged, and they undermined cell cycle arrest signaling pathways. That is, apoptotic pathways were activated, leading to irreparable cell death [22]. This study indicates that quinalizarin exerts anticancer effects, including the suppression of cell growth and induction of cell cycle arrest. Furthermore, quinalizarin has been shown to induce prostate cell cycle arrest at the G_2/M phase [23]. The G_2/M transition usually requires functional cyclin B/CDK protein complexes, and activation of the cyclin B1/CDK1 complex protein initiates the G_2 phase into the M phase [24]. Flow cytometric analyses indicated that quinalizarin induces SW480 cell cycle arrest at the G_2/M phase. Western blot results show that quinalizarin causes cell cycle arrest at G_2/M by suppressing the expression levels of CDK1/2 and the cyclin B1 protein. Therefore, our findings indicate that quinalizarin can induce the arrest of SW480 cells at the G_2/M phase by reducing the cyclin B1/CDK1 complex. However, the association between quinalizarin and apoptosis in SW480 cells remains unclear.

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The induction of apoptosis in cancer cells is one of the most important and direct ways of controlling the development of tumor cells [25]. Cellular apoptosis is initiated by key molecular mechanisms of the caspase cascade and mitochondrial dysfunction [22,23]. The mitochondria-dependent pathway is the intrinsic pathway that could mediate the expression levels of the key apoptosis proteins, including the Bcl-2 family, caspase-3, and PARP [25]. The Bcl-2 family plays an important role in the apoptosis of cancer cells; it has proapoptotic protein Bad and anti-apoptotic protein Bcl-2 [26]. Caspase-3 is one of the most important proteases in the cascade of reactions, and it has been recognized as a target in the design of cancer therapeutics [27]. PARP is a substrate for caspase-3 and regulates various cellular processes, including DNA repair and apoptosis [28]. P53 is one of the most important proteins involved in the mitochondria pathway; it can regulate Bad and Bcl-2, ultimately inducing cell cycle arrest and apoptosis [29]. In this study, we investigated whether quinalizarin-induced apoptosis is mediated by the activation of p53 and the mitochondria-dependent pathway. Our study showed that treatment with quinalizarin results in reduced the protein expression levels of Bcl-2, thus increasing the protein expression levels of p53, Bad, cleaved caspase-3, and cleaved PARP in a time-dependent manner. These results suggest that quinalizarin can induce caspase-3-dependent apoptosis via the mitochondrial pathway and by activating p53, upregulating Bad, and downregulating Bcl-2. Therefore, our study shows that quinalizarin induces the apoptosis of SW480 cells by regulating p53 and mitochondrial pathways.

We also investigated the involvement of the Akt, MAPK, and STAT3 signaling pathways in quinalizarin-induced apoptosis. Akt is an intracellular signaling pathway that plays important roles in regulating cell survival, growth, migration, and apoptosis [30]. Akt activation is known to regulate tumor development and progression in CRC by inducing cell cycle arrest and apoptosis [31]. The MAPK signaling pathway is known as a stress-activated kinase, and it participates in the regulation of cellular responses [32]. MAPK has been shown to promote drug resistance and the regulation of p53, and Bcl-2 may activate MAPK to induce apoptosis [33]. STAT3 is correlated with poor prognosis in cancer patients and has been regarded as a specific target of cancer treatment [34]. In our study, quinalizarin time-dependently inhibited the phosphorylation of Akt, ERK, and STAT3, thus promoting the phosphorylation of p38 and JNK. These results suggest that quinalizarin exhibits antitumor activity in CRC by regulating the Akt, MAPK, and STAT3 signaling pathways.

ROS are mainly produced by mitochondria and act as second messengers to control various cellular processes such as apoptosis and cell proliferation [35]. The overproduction of intracellular ROS generally causes mitochondrial dysfunction and leads to cellular oxidative stress-induced apoptosis [36,37]. Our study investigated the role of ROS production and the induction of apoptosis in quinalizarin-treated SW480 cells, showing that the generation of ROS influenced quinalizarin-induced cell apoptosis, MAPK, and STAT3 phosphorylation. These results show that quinalizarin induces SW480 cell apoptosis by increasing the generation of intracellular ROS and regulating the MAPK and STAT3 signaling pathways.

Conclusions

The results of this study indicate that quinalizarin can inhibit cell proliferation, induce cell cycle arrest at the G_2/M phase, and promote apoptosis in SW480 cells. That is, quinalizarin plays a pivotal role in inducing apoptosis by regulating MAPK and STAT3 signaling pathways with the accumulation of ROS.

Conflicts of interest

None.

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