

Naphthazarin suppresses cell proliferation and induces apoptosis in human colorectal cancer cells via the B-cell lymphoma 2/B-cell associated X protein signaling pathway

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Received May 1, 2015; Accepted September 9, 2016

DOI: 10.3892/ol.2016.5319

Abstract. Colorectal cancer is the most common gastrointestinal cancer in the USA. Naphthazarin, one of the naturally available 1,4-naphthoquinone derivatives, is a natural bioactive molecule that exhibits an antitumor effect. To the best of our knowledge, this is the first study to investigate the anticancer effect of naphthazarin on cell proliferation and apoptosis in human SW480 colorectal cancer cells. In the present study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase assays were performed to assess the effect of naphthazarin on cell proliferation and cytotoxicity of SW480 cells, respectively. In addition, an Annexin V-fluorescein isothiocyanate/propidium iodide apoptosis assay and 4',6'-diamidino-2-phenylindole staining were used to analyze cell and nuclei apoptosis of SW480 cells, respectively, following treatment with naphthazarin. Poly (ADP-ribose) polymerase (PARP), B-cell lymphoma 2 (Bcl-2) and B-cell associated X protein (Bax) protein expression was analyzed by western blot. Furthermore, caspase-3 activation was analyzed using a commercial kit. The results revealed that naphthazarin exhibited cell growth inhibition, an increase in cytotoxicity and apoptosis induction in SW480 cells, which was associated with activation of the Bax/Bcl-2 signaling pathway and cleaved caspase-3 activation. However, no significant differences in PARP expression were identified following treatment with naphthazarin in SW480 cells. Taken together, these results suggest that naphthazarin decreased cell viability and induced apoptosis of SW480 cells, indicating that naphthazarin may present a potential therapeutic agent for human colorectal cancer treatment.

Introduction

Colorectal cancer is the third and second most common malignant tumor in males and females worldwide, respectively (1). According to the International Agency for Research on Cancer, 1.2 million novel cases of colorectal cancer were diagnosed worldwide in 2008, accounting for 8% of all cancer-related mortalities (2,3). The incidence of colorectal cancer is highest in developed countries and regions: Due to economic development and rapid urbanization in recent years in China, which has resulted in dietary and lifestyle changes within the population, the colorectal cancer morbidity and mortality rates in China are now higher than the average rates, worldwide (4,5).

In 2013, the incidence of colorectal cancer was 45.1 per 100,000 individuals, with >5,000 deaths per year and an average of 13.1 years of life lost (1). Colorectal cancer operation is an important method for the treatment of colorectal cancer (5). The clinical manifestations of colorectal cancer may appear in the following ways: Altered defecation habits, stomach ache, abdominal masses, gastrointestinal hemorrhage, jaundice and a change in character of the stool. All patients have postoperative complications, with have a great impact on the patient's quality of life, and even endanger the patient's life (6). Therefore, it is important that physicians investigate multiple potential treatments for colorectal cancer, with the aim of preventing these treatment complications (7).

Naphthazarin is a natural bioactive substance present in numerous plants that has been demonstrated to exhibit antitumor effects (8). Naphthazarin is an important active ingredient, which exhibits extensive pharmacological activities, including antitumor activity, and due to its low toxicity, it has gained considerable attention (9,10). However, to date the anticancer effects of naphthazarin on human colorectal cancer cells have not been reported. In the present study, the anticancer effects of naphthazarin, as well as its effect on the B-cell lymphoma 2 (Bcl-2)/B-cell associated X protein (Bax) signaling pathway were investigated in human SW480 colorectal cancer cells.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), naphthazarin (Fig. 1) and

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Key words: naphthazarin, human colorectal cancer, B-cell lymphoma 2; B-cell associated X protein

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution and a lactate dehydrogenase (LDH) assay were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis and caspase-3 activation kits were purchased from KeyGen Biotech Co., Ltd., (Nanjing, China).

Cell culture and cell viability assay. The human colorectal cancer SW480 cell line was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM (Sigma-Aldrich; Merck Millipore) supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ at 37°C. A total of 1x10⁴ cells/well were seeded in a 96-well plate and incubated at 37°C in a 5% CO₂ incubator for 24 h. After incubation, SW480 cells were treated with 0, 0.5, 1, 5, 10 and 20 µM naphthazarin for 24 h. Subsequently, 20 µl MTT solution (Sigma-Aldrich; Merck Millipore) was added to each well and incubated at 37°C in a 5% CO₂ incubator for 4 h. Following incubation, the culture medium was replaced and 200 µl DMSO was added to each well and agitated for 20 min at room temperature. Cell viability was analyzed at a wavelength of 490 nm using an ELISA reader (Multiskan EX; Thermo Labsystems, Helsinki, Finland).

LDH assay. SW480 cells (1x10⁴ cells/well) were seeded in a 96-well plate and incubated at 37°C in a 5% CO₂ incubator for 24 h. After incubation, SW480 cells were treated with 0, 0.5, 1, 5, 10 and 20 µM naphthazarin for 24 h. Subsequently, 100 µl LDH solution was added to each well and cultured for 30 min. The absorbance was read at a wavelength of 490 nm using an ELISA reader (Multiskan EX; Thermo Labsystems).

Annexin V-FITC/PI apoptosis assay. SW480 cells (1x10⁶ cells/well) were seeded in a 6-well plate and incubated at 37°C in a 5% CO₂ incubator for 24 h. After incubation, SW480 cells were treated with 0, 0.5, 1 and 5 µM naphthazarin for 24 h. SW480 cells were trypsinized (Sangon Biotech Co., Ltd., Shanghai, China), washed with phosphate-buffered saline (PBS) and fixed in precooled 75% ethanol at 4°C overnight. Next, Annexin-V FITC and PI were added and incubated for 10 min at room temperature in the dark. Flow cytometry analysis was performed on a FACScan flow cytometer (BD Biosciences, San Diego, CA, USA) using emission filters of 525 and 575 nm. Data were analyzed using CellQuest Pro software version 5.1 (BD Biosciences).

4',6-diamidino-2-phenylindole (DAPI) staining assay. SW480 cells (1x10⁶ cells/well) were seeded in a 6-well plate and incubated at 37°C in a 5% CO₂ incubator for 24 h. Following incubation, SW480 cells were treated with 0, 0.5, 1 and 5 µM naphthazarin for 24 h. SW480 cells were then incubated with 0.1% sodium citrate containing 0.1% Triton X-100 (Beyotime Institute of Biotechnology, Haimen, China) for 5 min at 4°C. Cell nuclei were observed using a fluorescent microscope (Zeiss Axio Observer A1; Carl Zeiss, Inc., Oberkochen, Germany).

Western blot analysis. SW480 cells (1x10⁶ cells/well) were seeded in a 6-well plate and incubated at 37°C in a 5% CO₂

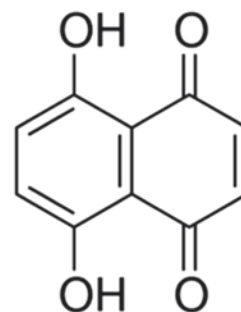


Figure 1. Chemical structure of naphthazarin.

incubator for 24 h. After incubation, SW480 cells were treated with 0, 0.5, 1 and 5 µM naphthazarin for 24 h. SW480 cells were harvested with PBS and extracted in cold radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology). Protein concentrations were determined using the by Pierce BCA protein assay kit (BD Biosciences). Equal amounts of protein were resolved on 6-15% SDS-PAGE gel and transferred to polyvinylidene fluoride membranes (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Membranes were then incubated with antibodies against poly (ADP-ribose) polymerase (PARP; 1:1,000; D161071; Santa Cruz Biotechnology, Inc.), Bax (1:500; AF0057; Beyotime Institute of Biotechnology), Bcl-2 (1:500; AF0060; Beyotime Institute of Biotechnology) and β-actin (1:500; AA128; Beyotime Institute of Biotechnology) overnight at 4°C. Membranes were next washed with TBS containing Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:2,000; Sangon Biotech, Co., Ltd.) at 37°C for 2 h. The protein band was detected using ImageLab 3.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Caspase-3 activation assay. SW480 cells (1x10⁶ cells/well) were seeded in a 6-well plate and incubated at 37°C in a 5% CO₂ incubator for 24 h. After incubation, SW480 cells were treated with 0, 0.5, 1 and 5 µM naphthazarin for 24 h. SW480 cells were harvested with PBS and extracted in cold RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein concentrations were determined using the Pierce BCA protein assay kit (BD Biosciences). Equal amounts of the total protein were mixed with Ac-DEVD-pNA (Beyotime Institute of Biotechnology) for caspase-3 expression and incubated at 37°C for 2 h in the dark. Caspase-3 activation was analyzed at a wavelength of 405 nm using an ELISA reader (Multiskan EX; Thermo Labsystems).

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments. Differences between groups were analyzed using the Student's t-test and the SPSS 17.0 program (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Naphthazarin decreases SW480 cell viability. The effect of naphthazarin on cell viability was investigated by MTT assay in SW480 cells. Following treatment with 5, 10 and 20 µM naphthazarin significantly decreased cell viability of SW480

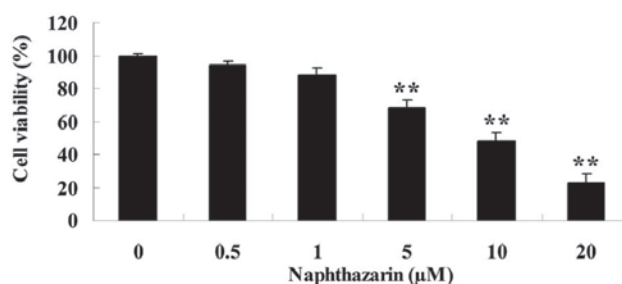


Figure 2. Effect of naphthazarin on cell viability of SW480 cells was analyzed by MTT assay. Treatment with 5, 10 and 20 μ M naphthazarin for 24 h significantly decreased cell viability of SW480 cells. ** $P < 0.01$ vs. 0 μ M naphthazarin treatment group. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

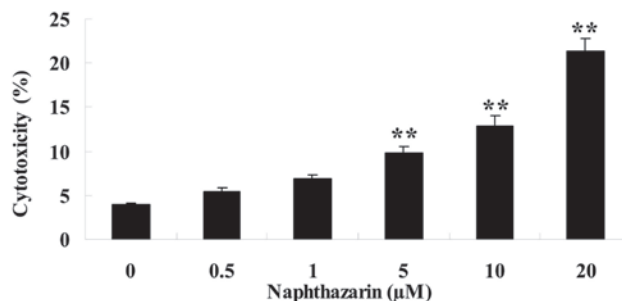


Figure 3. Effect of naphthazarin on SW480 cell cytotoxicity was analyzed by lactate dehydrogenase assay. Treatment with 5, 10 and 20 μ M naphthazarin for 24 h significantly increased cell cytotoxicity in SW480 cells. ** $P < 0.01$ vs. 0 μ M naphthazarin treatment group.

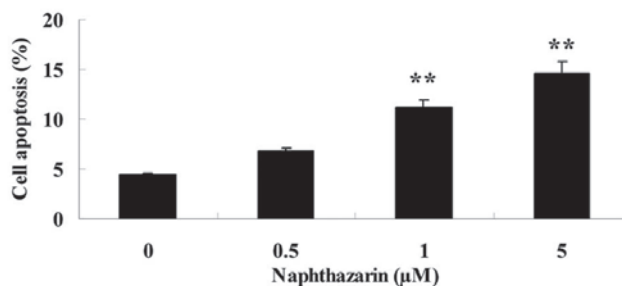


Figure 4. Effect of naphthazarin on cell apoptosis of SW480 cells was analyzed by flow cytometry. Treatment with 1 and 5 μ M naphthazarin for 24 h significantly increased the apoptotic rate of SW480 cells. ** $P < 0.01$ vs. 0 μ M naphthazarin treatment group.

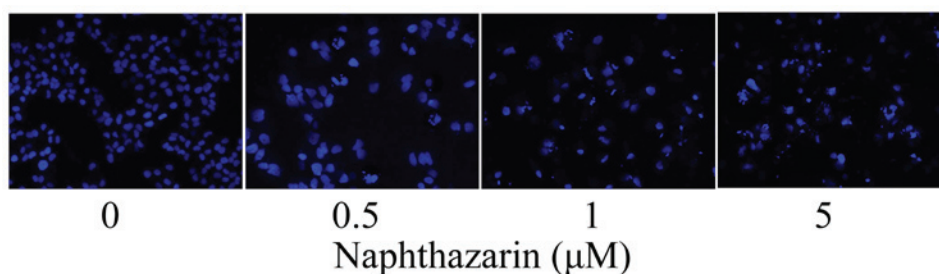


Figure 5. Effect of naphthazarin on nuclear apoptosis of SW480 cells was analyzed by DAPI staining. Treatment with 0.5, 1 and 5 μ M naphthazarin for 24 h effectively promoted nuclear apoptosis in SW480 cells compared with the 0- μ M naphthazarin treatment group. Magnification, $\times 10$.

cells in a dose-dependent manner ($P < 0.01$; Fig. 2). These results indicate that naphthazarin exhibits an anticancer effect on human colorectal cancer cells.

Naphthazarin increases cytotoxicity of SW480 cells. A LDH assay was performed to investigate the effect of naphthazarin

on SW480 cell cytotoxicity. Naphthazarin significantly increased cytotoxicity of SW480 cells in dose-dependent manner following treatment with 5, 10 and 20 μ M naphthazarin for 24 h (Fig. 3).

Naphthazarin induces apoptosis of SW480 cells. The effect of naphthazarin on SW480 cell apoptosis was investigated. Upon

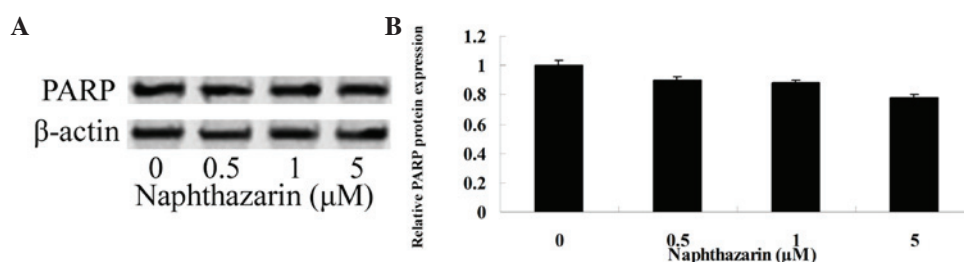


Figure 6. Effect of naphthazarin on PARP of SW480 cells was analyzed by western blotting. (A) Western blot analysis and (B) quantification revealed no evident significant changes in PARP expression following 24 h treatment with 0.5, 1 and 5 μ M naphthazarin. PARP, poly (ADP ribose) polymerase.

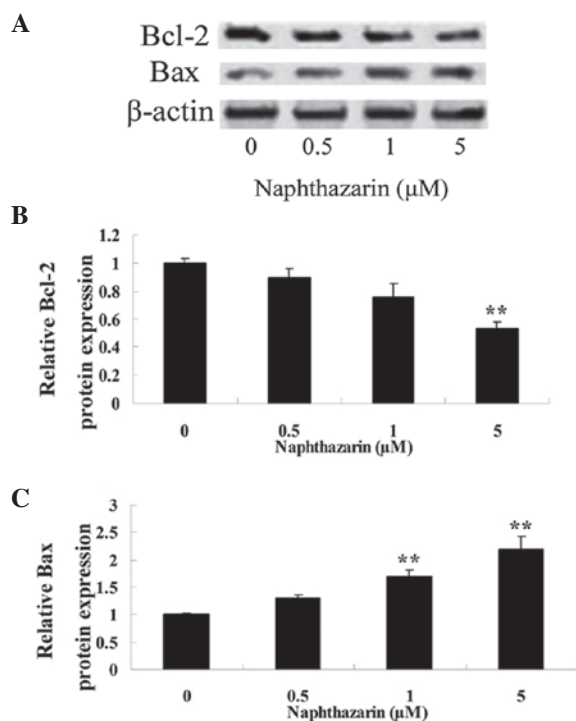


Figure 7. Effect of naphthazarin on Bcl-2 and Bax protein expression in SW480 cells was analyzed by (A) western blot analysis. Quantification of western blotting revealed that (B) Bcl-2 expression was significantly decreased following 24 h treatment with 5 μ M naphthazarin while (C) Bax expressions was significantly increased following 24 h treatment with 1 and 5 μ M naphthazarin in SW480 cells ** P <0.01 vs. 0 μ M naphthazarin treatment group. Bcl-2, B-cell lymphoma 2; Bax, B-cell associated X protein.

treatment with 1 and 5 μ M naphthazarin for 24 h, the rate of cell apoptosis was significantly increased in SW480 cells in a dose-dependent manner (Fig. 4).

Naphthazarin induces nuclear apoptosis in SW480 cells. SW480 cells were stained with DAPI and observed under a fluorescent microscope to investigate the effect of naphthazarin on nuclear apoptosis. Nuclear apoptosis was observed in SW480 cells following treatment with 0.5, 1 and 5 μ M naphthazarin for 24 h (Fig. 5).

Effect of naphthazarin on PARP of SW480 cells. To determine whether PARP is associated with the effects of naphthazarin on cell viability and apoptosis in SW480 cells, PARP protein expression was evaluated by western blotting. As shown in Fig. 6, treatment with 0.5, 1 and 5 μ M naphthazarin for 24 h

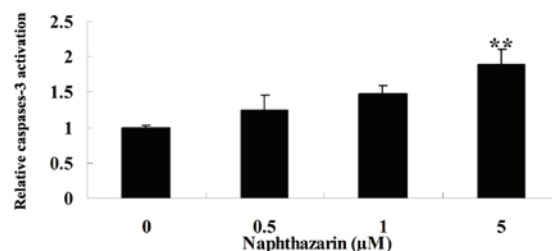


Figure 8. Effect of naphthazarin on caspase-3 activation in SW480 cells was analyzed by caspase-3 activation assay. Treatment with 5 μ M naphthazarin for 24 h significantly increased caspase-3 activation in SW480 cells. ** P <0.01 vs. 0 μ M naphthazarin treatment group.

decreased PARP protein expression, however, no significant differences were observed when compared with the control group.

Naphthazarin decreases Bcl-2 and increases Bax expression in SW480 cells. To further investigate the anticancer effect of naphthazarin, the protein expression of Bcl-2 and Bax in human colorectal SW480 cancer cells was analyzed by western blotting following naphthazarin treatment (Fig. 7A). The results revealed that treatment with 5 μ M naphthazarin for 24 h significantly decreased Bcl-2 expression in cells compared with the control (P <0.01; Fig. 7B). Furthermore, treatment with 1 and 5 μ M naphthazarin for 24 h significantly increased Bax protein expression in a dose-dependent manner compared with the control (Fig. 7C).

Effect of naphthazarin on caspase-3 of SW480 cells. To confirm that potential mechanism of naphthazarin on cell apoptosis of human colorectal cancer cell, we also examined the activation of caspase-3 after naphthazarin treatment with for 24 h. Treatment with 5 μ M naphthazarin resulted in significantly increased levels of caspase-3 activation in SW480 cells compared with the control (Fig. 8).

Discussion

Globally, colorectal cancer is the third most common type of malignant tumor, after lung and breast cancer (11). The incidence of colorectal cancer exhibits differences in regional distribution: Incidence is highest in developed countries and regions, such as Australia, New Zealand, Europe and North America, and lower in Asia and Africa (12-14). The highest mortality rates occur in Central and Eastern European countries, and the lowest

mortality rates are observed in central African regions (15). In China in 2009, colorectal cancer incidence and mortality rates were higher than the world averages: Rates were than lower than that observed in Japan, Singapore and South Korea, but higher than that of countries such as Iran, Laos and India (16,17). In the present study, it was demonstrated that naphthazarin significantly decreased cell viability, increased cytotoxicity and induced cellular and nuclear apoptosis of SW480 cells in a dose-dependent manner. Recent studies have demonstrated that naphthazarin induces apoptosis of human breast cancer (18) and gastric cancer cells (19).

PARP is involved in DNA damage recognition and signal transduction: PARP inhibitors can selectively prevent defects in the DNA of tumor cells (20). A previous clinical trial revealed that PARP inhibitors used for the treatment of ovarian cancer patients harboring mutations in breast cancer susceptibility genes achieved a good response rate, with few side effects (21). However, in the present study, treatment with naphthazarin did not affect PARP protein expression levels in SW480 cells. These results indicate that the PARP signaling pathway may not be involved with the anticancer effects of naphthazarin on human colorectal cancer cells.

Colorectal cancer is one of the most common malignant tumors worldwide (22). It has been demonstrated that the occurrence of tumor development depends on the dynamic balance between cell proliferation and apoptosis (23). Caspase-3 is a important apoptotic protein for various cells, and caspase-3 activation can induce apoptosis in cancer cells (24). The Bcl-2 family of apoptosis-related proteins includes important regulatory factors: Bcl-2 inhibits apoptosis, whereas Bax and Bak promote apoptosis. Therefore, changes in expression of these proteins affects the apoptosis of both normal cells and tumor cells (25,26). The results of the present study revealed that naphthazarin promoted Bax expression and inhibited Bcl-2 protein expression, and increased caspase-3 activation in SW480 cells. These results are in accordance with those of Acharya *et al* (9) who reported that naphthazarin increases the Bax/Bcl-2 protein ratio in A549 lung cancer cells.

In conclusion, the present study demonstrated that naphthazarin suppressed cell proliferation and induced apoptosis in human colorectal cancer cells via the Bcl-2/Bax signaling pathway. Thus, we hypothesize that naphthazarin may present a potential chemotherapeutic agent for colorectal cancer. However, further studies are required to investigate the mechanisms underlying the anticancer effects of naphthazarin on human colorectal cancers.

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