Anti-cancer effects of 2-oxoquinoline derivatives on the HCT116 and LoVo human colon cancer cell lines

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Abstract. The present study demonstrated the anti-tumor effects of the quinoline derivative [5-(3-chloro-oxo-4-phenyl -cyclobutyl)-quinoli-8-yl-oxy] acetic acid hydrazide (CQAH) against colorectal carcinoma. Substantial apoptotic effects of CQAH on HCT116 and LoVo human colon cancer cell lines were observed. Apoptosis was identified based on cell morphological characteristics, including cell shrinkage and chromatin condensation as well as Annexin V/propidium iodide double staining followed by flow cytometric analysis and detection of apoptosis-associated proteins by western blot analysis. CQAH induced caspase-3 and PARP cleavage, reduced the expression of the anti-apoptotic proteins myeloid cell leukemia-1 and B-cell lymphoma (Bcl) extra large protein and elevated the expression of the pro-apoptotic protein Bcl-2 homologous antagonist killer. In addition, pharmacological inhibition of c-Jun N-terminal kinase (JNK), but not extracellular signal-regulated kinase or p38, significantly reduced CQAH-mediated cell death as well as cleavage of caspase-3 and PARP. Co-treatment of CQAH with the commercial chemotherapeutics 5-fluorouracil and camptothecin-11 significantly improved their efficacies. Comparison of the apoptotic effects of CQAH with those of two illustrated structure-activity associations for this compound type, indicating that substitution at position-4 of the azetidine phenyl ring is pivotal for inducing apoptosis. In conclusion, the results of the present study indicated CQAH and its analogues are potent candidate drugs for the treatment of colon carcinoma.

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Introduction

Cancer is one of the greatest challenges in the clinical field worldwide and its occurrence is increasing in developed as well as in developing countries. In the United States, colorectal cancer is considered to be the second-largest cause of cancer-associated mortality (1,2). In spite of the fact that the genetics of colon cancer have been studied in depth (3), current therapies are not able to effectively treat colorectal cancer (4,5).

Although colorectal cancer can be cured at early stages, patients frequently present with metastases at the time-point of the occurrence of symptoms and diagnosis, leading to a high mortality rate (6). Hence, research efforts focus on developing novel and more potent preventive and screening methods for colon cancer (7). Numerous studies have shown that the mortality rate arising from colorectal cancer decreased to 40-50% in patients with colorectal cancer taking non-steroidal anti-inflammatory drugs and aspirin (8-13), clearly demonstrating the chemopreventive effects of these drugs. Other studies reported that sunlindac is a potent drug causing a regression of the adenoma count and size in patients with adenomatous polyposis (14-17). The primary method of colon cancer treatment is surgery. 5-fluoruracil (5-FU), camptothecin-11 (CPT-11; irinotecan) and oxaliplatin are the most common chemotherapeutic drugs used to destroy cancer cells after tumor resection (18,19). Combinatorial treatment of drug administration and surgery has enhanced therapeutic efficacy as compared with that of monotherapy (20,21). Besides from irradiation and surgical treatments, chemotherapy remains one of the primary choices for cancer treatment (22).

Quinoline moieties and their oxo-derivatives have gained substantial interest due to their occurrence in a wide range of natural products and bio-active compounds (23). 3-substituted quinoline-2-one is a key moiety present in a variety of compounds with anti-cancer properties, and quinoline-2-one-based compounds were found to be effective and promising lead structures for kinase inhibition (24).

Azetidinones are part of the structural skeleton of several antibiotics and are widely known to exhibit potent biological activities (25). Synthesis of azetidinone derivatives has provided variety of novel compounds with applications including anti-bacterial, antimicrobial, anti-convulsant, anti-inflammatory and anti-tuberculosis properties (26-30). They can

also act as enzyme inhibitors and exert effects on the central nervous system (31). Another class of heterocyclic compound, 8-hydroxy quinolines, has been demonstrated to have valuable biological activities, with derivatives being used as effective human immunodeficiency virus-1 intergrase inhibitors (32,33), as antimicrobial compounds or as herbicides (34-36). Quinoline-based synthetic azetidinone derivatives have also shown potential anti-microbial properties (37,38). Based on the abovementioned findings, the present study investigated the anti-cancer effects of compounds containing azetidine and the quinoline-2-one skeleton combined in one molecule. Madhu *et al* (39) have previously synthesized [5-(3-chloro-oxo -4-phenyl-cyclobutyl)-quinoli-8-yl-oxy] acetic acid hydrazide (CQAH), which showed efficacy against a range of bacteria and fungi. The present study explored the effects of CQAH as well as two if its derivatives, CQAH-1 and -2, bearing a methyl- or nitro-substitution, respectively, at position-4 of the azetidine phenyl ring (Fig. 1), on colorectal cancer in order to assess their potential for use as novel chemotherapeutic drugs.

Materials and methods

Compounds and reagents. CQAH and its derivatives were prepared according to the procedure of a previous study (39). The identity of the compounds was confirmed by infrared (IR) spectroscopy and nuclear magnetic resonance (NMR). The spectroscopical data, with assignments as singulet (s), multiplet (m) and duplet (d) were as follows:

2-((5-(3-chloro-2-oxo-4-phenylazetidin-1-yl) quinolin-*8-yl)oxy)acetohydrazide (CQAH)*. IR (KBr) in cm^{-1}: 674 (-Cl), 1,625 (-C=N), 1,685 (-C=O), 3,210 (-NH), 3,410 ($-NH_2$), 3,486. ^{1}HNMR [300 MHz, $(CD_3)_2SO$, tetramethylsilane (TMS)]: δ=2.09 (s, 2H, $-NH_2$), 4.7 (s, 2H, $-O-CH_2$), 5.12 (d, 1H, -CH of azetidin attached to phenyl), 5.41 (d, 1H, -CH of azetidin attached to -Cl), 6.55 (d, 1H, -CH), 6.78 (d, 1H, -CH), 7.1-7.2 (m, 5H of C_6H_5), 7.8-8.8 (m, 3H of quinoline ring), 10.08 (s, 1H, -NH). ^{13}C NMR (75 MHz, $CDCl_3$, TMS) δ=60 (C-Cl), 63 (N-CH-Ar), 68 ($O-CH_2$), 108, 115, 119, 121, 127 (Ar-C), 128, 129, 138, 134, 141, 147, 150, 161 (N-C=O), 167 (-CO-N).

2-(5-(3-chloro-2-oxo-4-p-tolylazetidin-1-yl) quinolin-8-yloxy)acetohydrazide (CQAH-1). IR (KBr) in cm^{-1}: 678 (-Cl), 1,620 (-C=N), 1,684 (-C=O), 3,208 (-NH), 3,410 ($-NH_2$), 3,494. ^{1}HNMR [300 MHZ, $(CD_3)_2SO$, TMS]: δ=2.10 (s, 2H, $-NH_2$), 2.30 (s, 3H, Ar-CH_3), 4.73 (s, 2H, $-O-CH_2$), 5.14 (d, 1H azetidin-CH attached to phenyl), 5.45 (d, 1H, azetidin-CH attached to Cl), 6.3 (d, 1H, -CH), 6.4 (d, 1H, -CH),7.0 (m, 4H of C_6H_4), 8-8.7 (m, 3H of quinoline ring), 10.08 (s, 1H, -NH). ^{13}C NMR (75 MHz, $CDCl_3$, TMS) δ=25 (Ar-CH_3), 62 (C-Cl), 65 (N-CH-Ar), 67 ($O-CH_2$), 114, 117, 123, 127, 128, 130, 134 (Ar-C), 135, 137, 139, 147, 150, 162 (N-C=O), 167, 168 (-CO-N).

2-((5-(3-chloro-2-(4-nitrophenyl)-4-oxoazetidin-1-yl) quinolin-8-yl)oxy) acetohydrazide (CQAH-2). IR (KBr) in cm^{-1}: 675 (-Cl), 1,614 (-C=N), 1,680 (-C=O), 3,208 (-NH), 3,412 ($-NH_2$), 3,494. ^{1}H NMR [300 MHZ, $(CD_3)_2SO$, TMS]: δ=2.10 (s, 2H, $-NH_2$), 4.80 (s, 2H, $-O-CH_2$), 5.13 (d, 1H, -CH of azetidin attached to phenyl ring), 5.46 (d, 1H, -CH of azetidin attached to -Cl), 6.54 (d, 1H, -CH), 6.74 (d, 1H, -CH), 7.0-8.0 (m, 4H of C_6H_4), 7.3-8.8 (m, 3H of quinoline ring), 10.10 (s, 1H, -NH). ^{13}C NMR (75 MHz, $CDCl_3$, TMS) δ=62 (C-Cl), 65 (N-CH-Ar), 67 ($O-CH_2$), 106, 117, 120, 122, 127, 129, 134, 138, 147 (Ar-C), 151, 153, 162 (N-C=O), 167 (-CO-N).

All chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diphenylene iodonium (DPI), MTT and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma-Aldrich. Antibodies against c-Jun N-terminal kinase (JNK; cat no. 8528; 1:5,000; rabbit polyclonal IgG, 1 h at 25˚C) and phosphorylated (p)-JNK (cat no. 4668; 1:2,000; rabbit polyclonal IgG, 1 h at 25˚C) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against B-cell lymphoma (Bcl) extra-large protein (Bcl-XL; cat no. GTX100632; 1:3,000; rabbit polyclonal IgG, 1 h at 25˚C), Bcl-2 homologous antagonist killer (Bak; cat. no. GTX100063; 1:3,000; rabbit polyclonal IgG, 1 h at 25˚C), poly(adenosine diphosphate ribose) polymerase (PARP; cat no. GTX100573; 1:3,000; rabbit polyclonal IgG, 1 h at 25˚C), caspase-3 (cat. no. GTX110543; 1:3,000; rabbit polyclonal IgG, 1 h at 25˚C), myeloid cell leukemia 1 (Mcl-1; cat. no. GTX102026; 1:1,000; rabbit polyclonal IgG, 1 h at 25˚C) and α-tubulin (cat. no. GTX112141; 1:10,000; rabbit polyclonal IgG, 1 h at 25˚C) were purchased from Gene Tex (San Antonio, TX, USA). Mitogen-activated protein kinase (MAPK) inhibitors, including extracellular signal-regulated kinase (ERK) inhibitor PD98059, p38 inhibitor SB203580 and JNK inhibitor SP600125 were obtained from Merck Millipore (Billerica, MA, USA).

Cell culture and MTT assay. The HCT116 and LoVo human colorectal cancer cell lines were purchased from the American type culture collection (Manassas, VA, USA). These cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 5% heat-activated fetal bovine serum (FBS), penicillin-streptomycin (100 U) and sodium pyruvate (1 mM) in a humidified incubator with 5% CO_2 at 37˚C.

For the MTT assay, HCT116 and LoVo cells seeded in 24-well plates at a density of $1x10^5$ per well in growth medium (Sigma-Aldrich). Subsequent to the cells being re-fed with growth medium, they were incubated with various concentrations of CQAH (0-20 μM) for 24 or 48 h. The growth medium was fully removed and the cells were incubated with MTT solution (50 μg/ml) for 2 h. The obtained formazan crystals were dissolved in isopropanol and the absorbance was recorded at a wavelength of 560 nm using an ELISA reader (SpectraMax 190; Molecular Devices Inc., Sunnyvale, CA, USA). The cell viability was determined as a percentage of the control.

In order to determine the role of the MAPK pathway in drug-induced apoptosis, cells were pretreated with inhibitors of ERK (PD98059; 10 and 20 μM); JNK (SP600125; 10 and 20 μM) or p38 (SB203580; 10 and 20 μM). CQAH (10 μM) treatment was performed for 48 h and the viability of the cells was measured using an MTT assay.

The cells were treated with the antioxidant agents NAC (glutathione activator) and DPI (NAPDH inhibitor) to determine their role in drug-induced cell death. Cells were pretreated with inhibitors of glutathione (NAC; 5 and 10 μM) or NADPH (DPI; 5 and 10 μM). CQAH (10 μM) was added and cells were incubated for 48 h. The viability of the cells was assessed using an MTT assay.

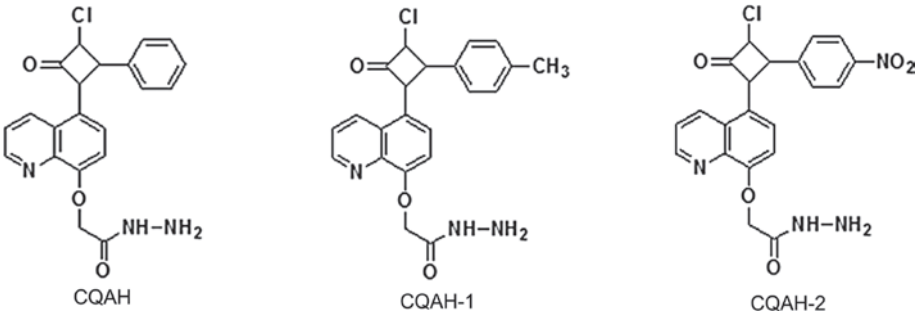

Figure 1. Chemical structures of the 2-oxoquinolines CQHA, CQAH-1 and CQAH-2.

Assessment of apoptosis. Cells at a density of $1x10^5$ per well were seeded into wells containing glass slips and treated with CQAH (10 μM) for 24 h. The slips were fixed with methanol for 10 min and treated with DAPI for 30 min. Slides were mounted with mounting medium (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The percentage of apoptotic nuclei was determined by counting condensed and bright nuclei compared with the total number of cells.

In another experiment, the cells were pretreated with inhibitors of caspase-3 (Z-DEVD-FMK; 50 and 100 μM) or caspase-9 (Z-LEHD-FMK; 50 and 100 μM) to inhibit the action of apoptosis-associated enzymes. CQAH (10 μM) was then added for 48 h. The cell viability was measured using an MTT assay.

Annexin V-propidium iodide (PI) staining. An Annexin-V-fluorescein isothiocyanate (FITC)/double staining kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to quantify the apoptosis of CQAH-treated HCT116 and LoVo cells. In brief, HCT116 and LoVo cells at a density of $1x10^5$ per well were seeded in 24-well plates and then treated with CQAH at various concentrations (0-20 μM) for 24 h. The cells were then trypsinized, treated with binding buffer and Annexin-FITC and PI were added, followed by incubation for 15 min in the dark according to the manufacturer's instructions of the apoptosis kit. The cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Western blot analysis. For western blot analysis, HCT116 and LoVo cells were washed two times using ice-cold phosphate-buffered saline (PBS) and then extracted in radioimmunoprecipitation assay buffer containing Tris-HCl (50 mM, pH 7.4), 1% nonidet P-40, NaCl (150 mM), ethylene glycol tetraacetic acid (1 mM), sodium deoxycholate (0.025%), sodium orthovanadate (1 mM), phenylmethyl sulfonylfluoride (1 mM), and NaF (1 mM). Cell extracts were examined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in order to quantify the protein concentration using bovine serum albumin (BSA) as a standard. The ELISA reader was used to measure the absorbance at a wavelength of 595 nm. Equal quantities of protein (50 μg) separated by 8% SDS-PAGE and then transferred onto immobilon polyvinylidene difluoride membranes (Merck-Millipore). 1% BSA was used to block the membranes at room temperature, and membranes were subsequently incubated with specific primary antibodies overnight. Membranes were washed three times in PBS containing Tween 20, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h. The protein expression was visualized using an enhanced chemiluminescence assay kit (cat. no. WBKLS0500; Merck-Millipore) by ImageQuant LAS4000 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

In order to determine the role of the MAPK pathway in drug-induced apoptosis, cells were pretreated with inhibitors of ERK (PD98059; 10 and 20 μM); JNK (SP600125; 10 and 20 μM) or p38 (SB203580; 10 and 20 μM). CQAH (10 μM) treatment was performed for 48 h. The protein expression of MAPK-associated proteins was assessed using western blot analysis.

In order to determine the role of antioxidants in drug-induced cell death, cells were treated with NAC (10 μM) and DPI (5 μM) in the presence of CQAH (10 μM) for 24 h, and the protein expression of PARP and caspase-3 was analyzed using western blotting.

Transient transfection. HCT116 and LoVo cells were first seeded in 6-cm dishes and transfection was performed using dominant-negative JNK (DN-JNK) or pcDNA3 with PolyJect reagent according to the manufacturer's instructions (SignaGen Laboratories, Gaithersburg, MD, USA). After 24 h of transfection, cells were trypsinized, seeded in a 24-well plate and then treated with CQAH for 48 h.

Statistical analysis. All experimental data are from three independent experiments and values are expressed as the mean ± standard error. Statistical analysis was conducted with GraphPad Prism, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences between groups were assayed using Student's t-test for each paired experiment. $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

CQAH decreases the viability of HCT116 and LoVo human colorectal cancer cells by inducing apoptosis. CQAH and its analogues (Fig. 1) have been previously shown to exhibit anti-bacterial activity against *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (39); however, their effects on cancer have not been elucidated, to the best of our knowledge. In order to assess the effects of CQAH on colon cancer, the HCT116 and LoVo cell lines were

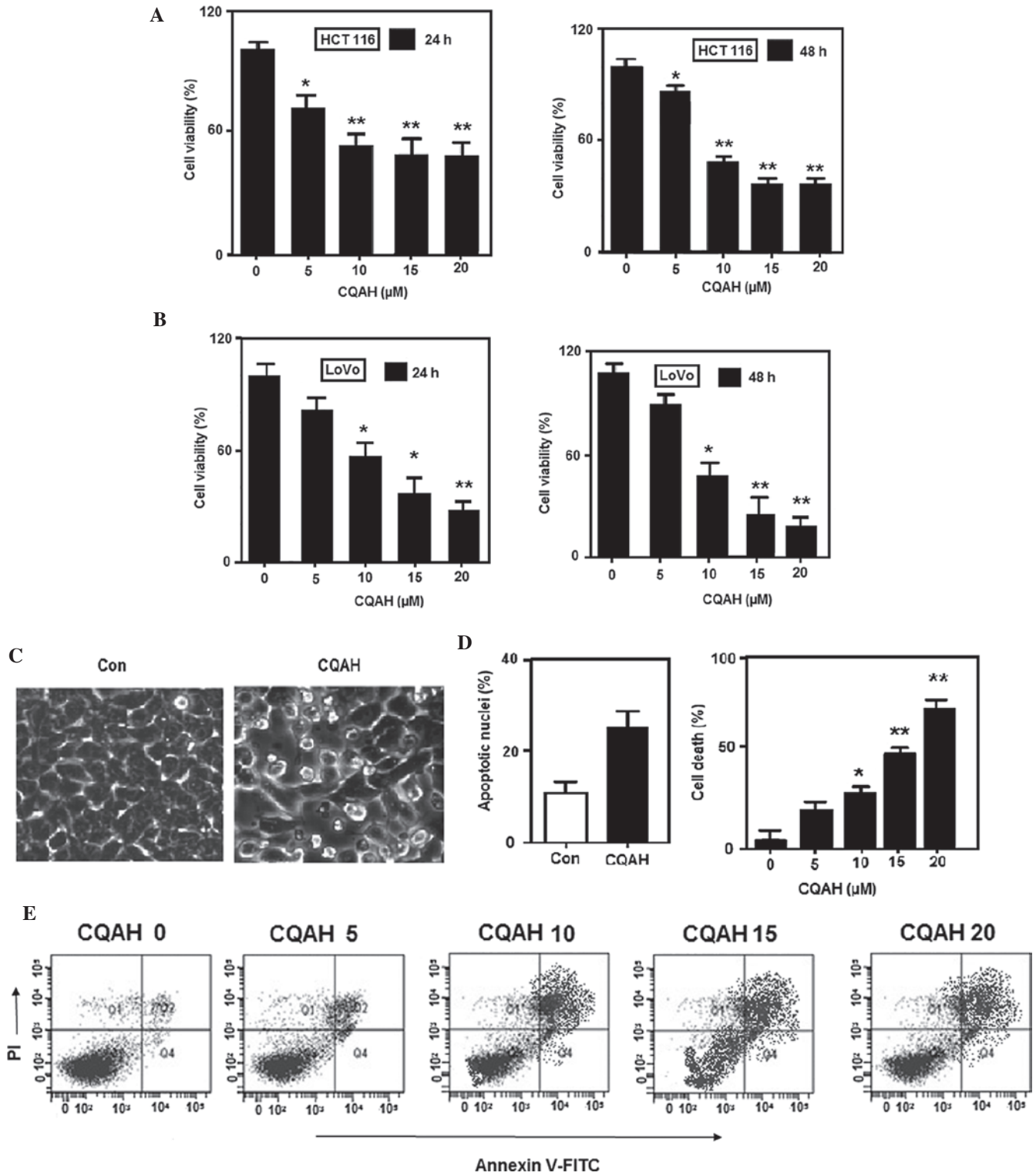

Figure 2. CQAH inhibits of cell growth and induces apoptosis in HCT116 and LoVo cells. (A) HCT116 and (B) LoVo cells were treated with CQAH (0-20 μM) for 24 and 48 h, respectively, and an MTT assay was used to assess cell viability. (C) HCT116 cells were treated with CQAH (10 μM) for 48 h and apoptotic nuclei were identified by DAPI staining (magnification, x100). (D) Number of apoptotic nuclei and percentage of apoptotic cells determined by quantification of C. (E) Annexin-FITC/PI staining followed by flow cytometric analysis was performed to assess CQAH-induced cell death. Data were obtained from at least 3 replicates of 3 independent experiments, and are expressed as the mean ± standard error. *P<0.05; **P<0.01 vs. untreated cells. FITC, fluorescein isothiocyanate; PI, propidium iodide.

treated with CQAH for 24 or 48 h and subjected to MTT assays. CQAH exerted considerable cytotoxic effects on the two cell lines in a dose-dependent manner (Fig. 2A and B) with 48-h IC_{50}-values of ~10 μM for HCT116 as well as LoVo. In order to assess whether CQAH can induce tumor-cell apoptosis, HCT116 cells were treated with CQAH and morphological changes were observed by microscopy (Leica TCS SP8 STED 3X; Leica Microsystems, Wetzlar, Germany), indicating that cell shrinkage and the formation of apoptotic bodies were induced by CQAH treatment. To further verify that apoptosis

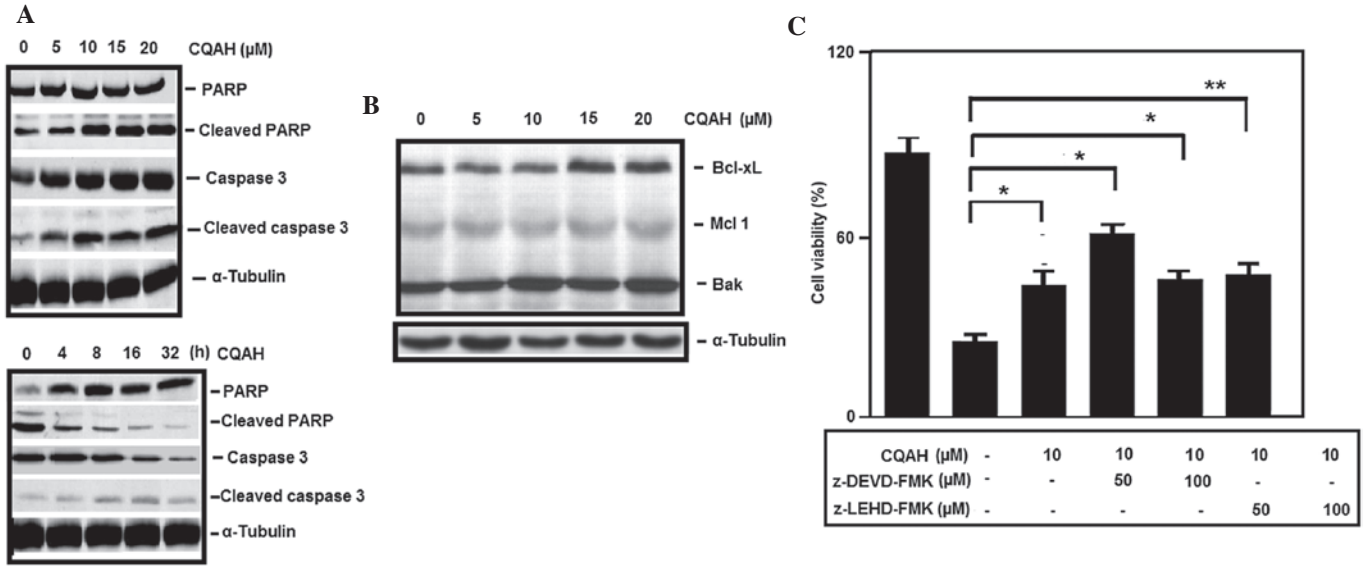

Figure 3. CQAH induces the expression of apoptotic proteins. (A) Concentration- and time-dependent induction of PARP and caspase-3 cleavage in the presence of CQAH. HCT116 cells were treated with 0-20 μM CQAH for 24 h or with 10 μM CQAH for 0, 4, 8, 16 and 32 h, and caspase-3 and PARP expressions were assessed using western blot analysis. α-Tubulin was used as the loading control. (B) HCT116 cells were treated with CQAH (0-20 μM) for 24 h and the expression of Bcl-2 family members Mcl-1, Bcl-XL and Bak was assessed using western blot analysis. (C) Cells were pre-treated with inhibitors of caspase-3 (z-DEVD-FMK; 50 and 100 μM) or caspase-9 (z-LEHD-FMK; 50 and 100 μM). CQAH (10 μM) was then added for 48 h. The cell viability was measured using an MTT assay. Data were obtained from at least three replicates of three independent experiments, and are expressed as the mean ± standard error. *P<0.05; **P<0.01 as indicated. PARP, poly(adenosine diphosphate ribose) polymerase; Bcl-2, B-cell lymphoma 2; Bcl-XL, Bcl extra large; Bak, Bcl-2 homologous antagonist killer; Mcl, myeloid cell leukemia.

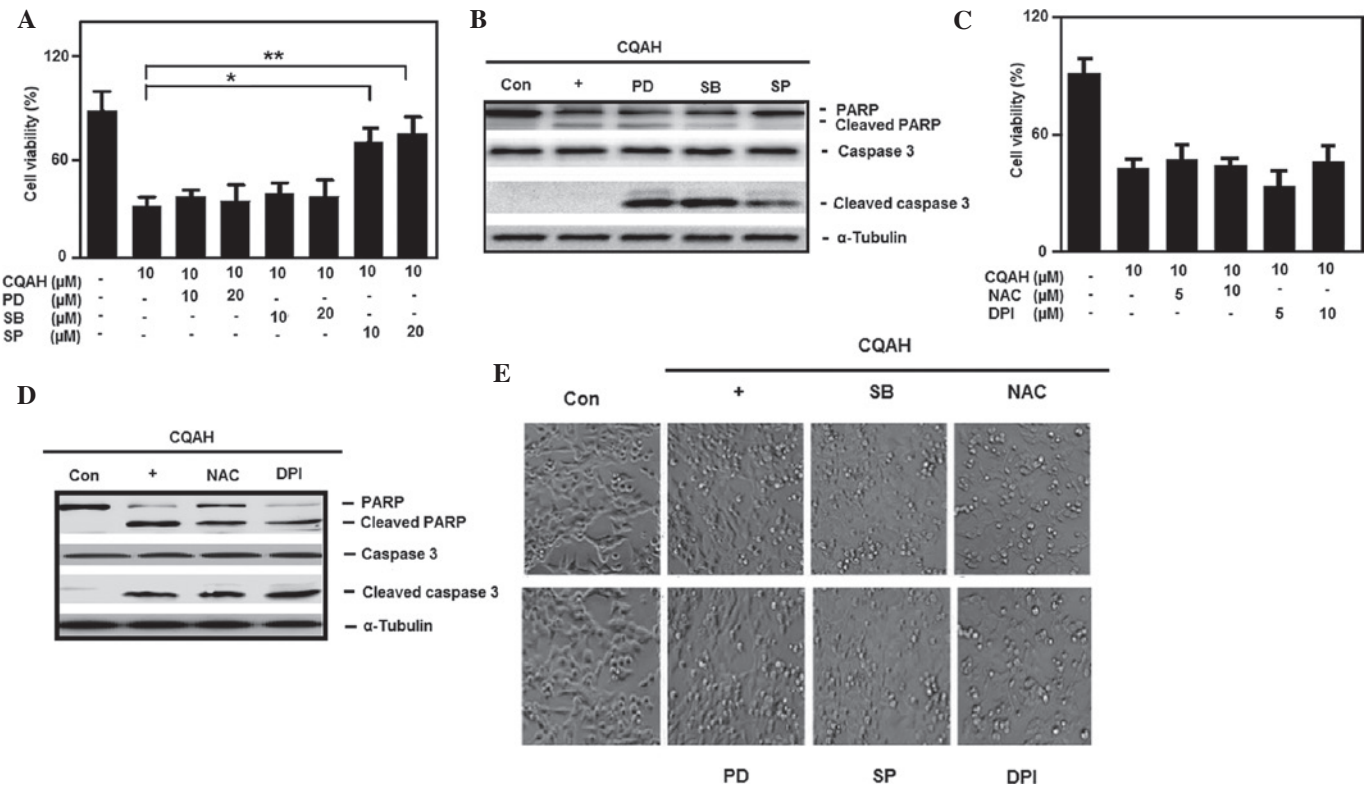

Figure 4. JNK activation-mediated CQAH induction of apoptosis, independent of reactive oxygen species involvement. (A) Cells were pre-treated with inhibitors of extracellular signal-regulated kinase (PD; 10 and 20 μM), JNK (SP; 10 and 20 μM) or p38 (SB; 10 and 20 μM). CQAH (10 μM) treatment was performed for 48 h and the viability of the cells was measured using an MTT assay. (B) Cells were pre-treated with 10 μM PD, SP or SB. CQAH (10 μM) treatment was performed for 24 h, and protein expression of PARP and caspase-3 was assessed using western blot analysis. (C) Cells were pre-treated with inhibitors of glutathione (NAC; 5 and 10 mM) or NADPH (DPI; 5 and 10 μM), CQAH (10 μM) was added, and cells were incubated for 48 h. The viability of the cells was assessed using an MTT assay. (D) Cells were treated with NAC (10 mM) and DPI (5 μM) in the presence of CQAH (10 μM) for 24 h, and protein expression of PARP and caspase-3 was analyzed using western blotting. (E) Bright-field images of cellular morphology were captured in the presence of the indicated inhibitors (20 μM PD, 20 μM SP, 20 μM SB, 10 mM NAC or 5 μM DPI) and CQAH (10 μM) for 48 h (magnification, x100). Values are expressed as the mean ± standard error. *P<0.05; **P<0.01 as indicated. JNK, c-Jun N-terminal kinase; PD, PD98059; SP, SP600125; SB, SB203580; NAC, *N*-acetyl-L-cysteine; DPI, diphenylene iodonium; Con, control; PARP, poly(adenosine diphosphate ribose) polymerase.

was induced by CQAH, chromatin condensation was observed by staining the cells with DAPI (Fig. 2C), revealing that CQAH treatment produced a marked increase in chromatin condensation and considerably increased the amount of apoptotic nuclei from 10 to 30% (Fig. 2D). Furthermore, Annexin V-PI staining and flow cytometric analysis demonstrated an increase in the apoptotic rate from 20 to 85% upon CQAH treatment (Fig. 2E).

CQAH induces apoptosis-associated signaling in colorectal cancer cells. To examine the apoptotic pathways activated by CQAH in colorectal cancer cells, the present study assessed the effects of CQAH on two key apoptotic proteins, caspase-3 and PARP, which participate in a proteolytic signaling cascade that is activated during the apoptotic process. Western blot analysis indicated that treatment of HCT116 cells with CQAH at 0-20 μM induced apoptosis in a concentration-dependent manner by enhancing cleaved caspase-3 and PARP (Fig. 3A). Time-dependent analysis revealed that pro-caspase-3 was reduced within 16 h of incubation with CQAH, whereas increased levels of cleaved caspase-3 and PARP where identified following CQAH treatment for 16-32 h (Fig. 3A). Furthermore, upon CQAH treatment, an increase in the levels of pro-apoptotic protein Bak and a decrease of anti-apoptotic proteins Bcl-XL and Mcl-1 were observed (Fig. 3B). In addition, pre-treatment with caspase-3-specific inhibitor z-DEVD-FMK and caspase-9-specific inhibitor z-LEDH-FMK significantly decreased CQAH-induced cell death (Fig. 3C).

CQAH-induced apoptosis is mediated via JNK activation. It is well known that MAPKs are broadly involved in physiological regulatory processes, which are responsible for the transduction of intracellular signaling (40). ERK, p38 and JNK have key roles in regulating cell growth and death upon MAPK activation. The present study investigated whether MAPK was involved in CQAH-induced apoptosis. For this, HCT116 cells were pre-treated with the ERK, JNK and p38 inhibitors PD98059, SP600125 and SB203580, respectively, followed by CQAH treatment. As shown in Fig. 4A, only JNK inhibition, but not p38 or ERK inhibition, significantly reduced CQAH-induced cell death. Furthermore, western blot analysis indicated that treatment with JNK inhibitor SP600125 abrogated CQAH-mediated PARP and caspase-3 cleavage (Fig. 4B), while p38 and ERK inhibitors had no marked effects. Morphological changes also confirmed that only SP600125 efficiently prevented CQAH-induced cell shrinkage and apoptotic body formation, as shown in Fig. 4E. Furthermore, intracellular oxidative stress triggered the involvement of chemopreventive product-induced cell death, which has a critical role in the activation of MAPK and hence, the present study examined the participation of reactive oxygen species (ROS) formation. As indicated in Fig. 4C, the anti-oxidant agents NAC (glutathione activator) and DPI (NAPDH inhibitor) failed to prevent CQAH-induced cell death. Similarly, the anti-oxidants failed to block the cleavage of PARP and caspase-3 (Fig. 4D) or apoptotic body formation (Fig. 4E) following treatment with CQAH. Since JNK inhibition significantly decreased CQAH-induced apoptosis, the present study further investigated the involvement of JNK by assessing JNK phosphorylation following treatment with CQAH for 4 h (Fig. 5A). Pre-treatment with SP600125 reduced CQAH-mediated phosphorylation of JNK (Fig. 5B) in parallel with an elevated expression of Bcl-XL protein (Fig. 5C). In addition, the cytotoxic effects of CQAH were restored following transfection with DN-JNK (Fig. 5D), further demonstrating the involvement of the JNK pathway in CQAH-induced cytotoxicity.

CQAH enhances the therapeutic efficacy of 5-FU and CPT-11. The present study further demonstrated the synergistic effects of CQAH upon combined treatment with the pyrimidine analogue anti-colon cancer drugs 5-FU and CPT-11, which act via blocking thymidylate synthase and inhibiting topoisomerase I (41). The results clearly indicated that the cell viability was considerably reduced upon combination with 5-FU or CPT11 (Fig. 6A). Accordingly, western blot analysis showed that combined treatment of CQAH with 5-FU or CPT-11 enhanced caspase-3 and PARP cleavage (Fig. 6B). Furthermore, the present study examined the structure-activity association of two derivatives of CQAH, CQAH-1 and CQAH-2. CQAH-1 carries a methyl group at position-4 of the azetidine phenyl ring, whereas CQAH-2 carries a nitro group in the same position. Morphological observation, MTT assay and western blot analysis indicated that CQAH-1 treatment at a concentration of 20 μM had a higher cytotoxic effect compared to that of CQAH, whereas CQAH-2 displayed reduced cytotoxicity (Fig. 6C-E).

Discussion

The MAPK signaling pathway regulates a variety of cellular responses with the aid of numerous intracellular and extracellular stimuli (42). The status of MAPK signaling controls cell fate, including proliferation and apoptosis. The present study demonstrated that treatment with CQAH triggered phosphorylation of JNK, whereas inhibition of JNK blocked CQAH-mediated phosphorylation of JNK as well as cleavage of caspase-3 and PARP; however, the mechanism of CQAH-mediated phosphorylation of JNK remains elusive. In spite of oxidative stress being a common trigger of cell death upstream of MAPK, participation of ROS generation was not identified following CQAH treatment, since pre-treatment with anti-oxidant agents did not affect CQAH-mediated cell death. Certain quinoline derivatives are capable of inhibiting MAPK, which activates the pro-apoptotic JNK and p38 signaling pathways (43). In a previous study, screening for potential kinase inhibitors identified a series of dihydropyrrolo pyrazole quinolines as effective potential inhibitors of the catalytic activity of MLK7 *in vitro* (44). A reduction in the activation of JNK and p38 with no evident effect on the activation of ERK indicated that this compound type may be suitable for blocking MLK7-mediated activation of the MAPK signaling pathway *in vivo*, while the underlying mechanism has remained elusive. JNK activation usually induces p53 expression, which has a major role in cell cycle regulation and activation of pro-apoptotic proteins; however, in the present study, it was observed that p53 protein levels were not altered upon CQAH treatment (data not shown), implying that CQAH-mediated apoptosis p53-independent.

Experimental results demonstrated that microtubules inhibition by paclitaxel and vincristine end up in elevation of

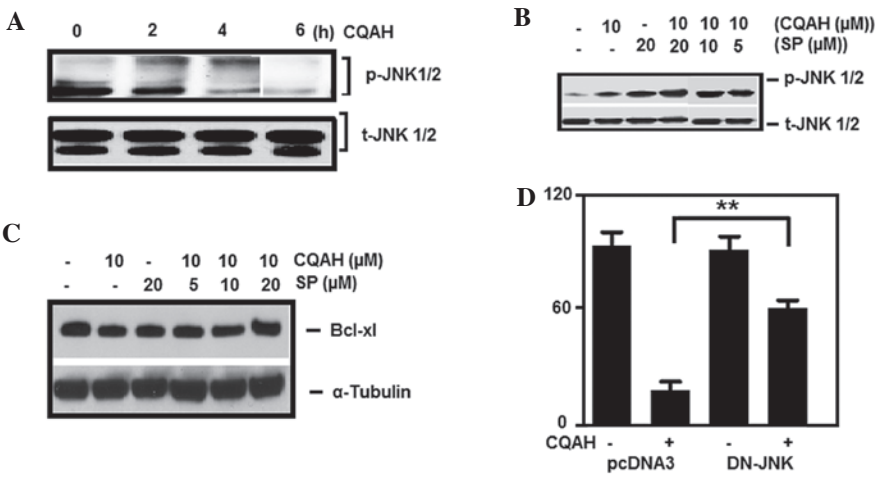

Figure 5. Activation of JNK is crucial for CQAH-induced apoptosis. (A) HCT116 cells were treated with CQAH (10 μM) for 1-6 h, and phosphorylation of JNK was analyzed using western blotting. Cells were pre-treated with SP600125 (5, 10 and 20 μM) and then incubated with CQAH (10 μM) for 2 h to detect (B) p-JNK and for 24 h to detect (C) Mcl-1 and Bcl-XL. (D) Cells were transiently transfected with DN-JNK for 18 h and then treated with CQAH for 48 h. Cell viability was measured using an MTT assay. Data were obtained from at least 3 replicates of 3 independent experiments, and are expressed as the mean ± standard error. **P<0.01. p/t-JNK, phosphorylated/total c-Jun N-terminal kinase; Bcl-2, B-cell lymphoma 2; Bcl-XL, Bcl extra large; Bak, Bcl-2 homologous antagonist killer; Mcl, myeloid cell leukemia; SP, SP600125; DN, dominant-negative.

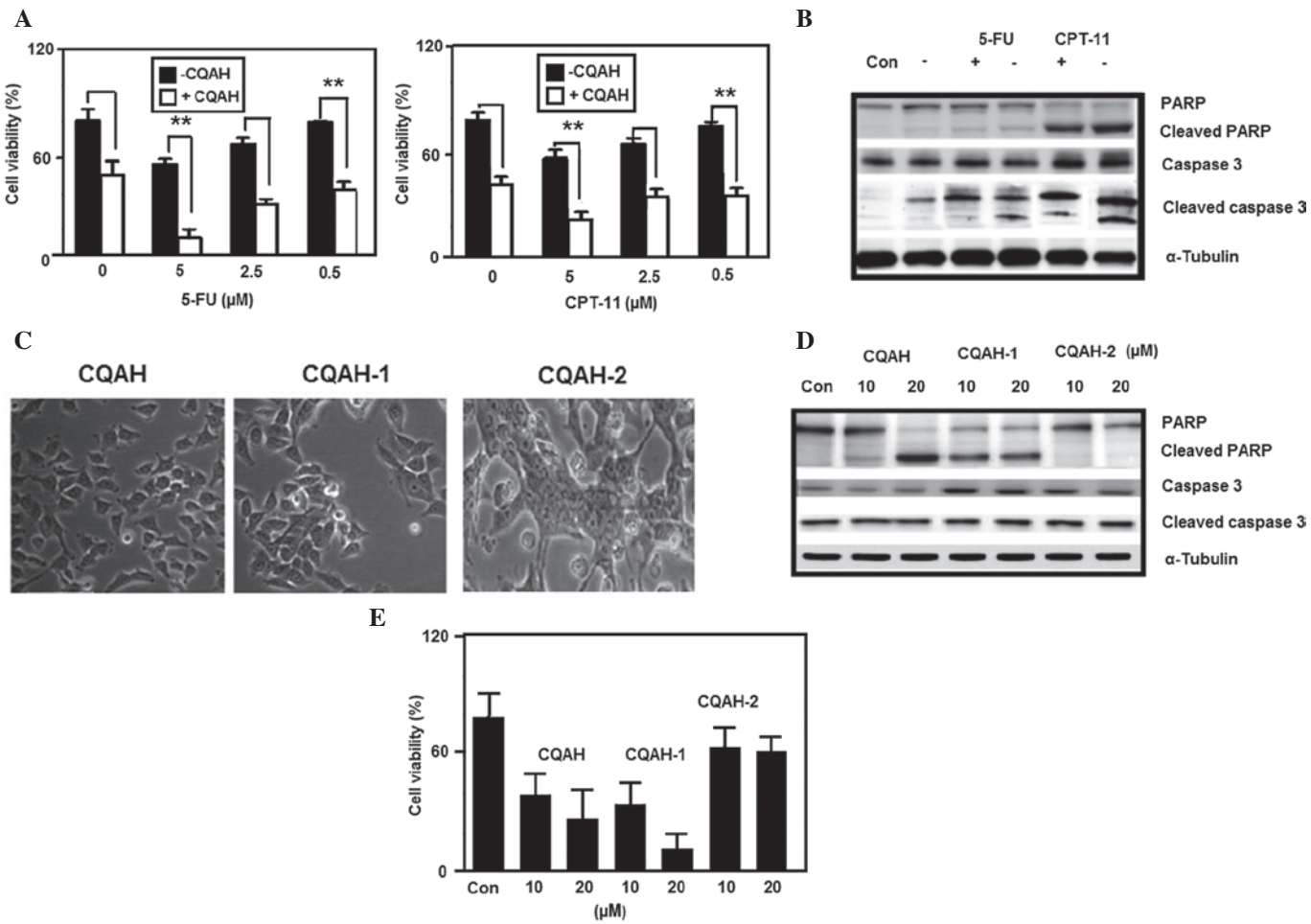

Figure 6. CQAH potentiates the therapeutic efficacy in combination with 5-FU and CPT-11, and its analogues CQAH-1 and CQAH-2 exert apoptotic effects on HCT116 cells. (A) Cells were treated with CPT-11 or 5-FU (0, 5, 2.5 or 0.5 μM) in the presence or absence of CQAH (10 μM) for 24 h, and cell viability was assessed using an MTT assay. (B) Cells were treated with CPT-11 or 5-FU (5 μM) in the presence or absence of CQAH (10 μM) for 24 h, and caspase-3 and PARP protein expressions were analyzed using western blotting. (C) Cells were treated with CQAH, CQAH-1 or CQAH -2 (10 μM) for 48 h and the cell morphology was observed using a light microscope (magnification, x100). (D) Cells were treated with CQAH, CQAH-1 or CQAH-2 (10 or 20 μM) for 24 h and the expression of PARP and cleaved caspase-3 was analyzed using western blotting. (E) Cell viability was assessed using an MTT assay after 24 h of incubation. Data were obtained from at least three replicates of three independent experiments, and are expressed as the mean ± standard error. **P<0.01. 5-FU, 5-fluorouracil; PARP, poly(adenosine diphosphate ribose) polymerase; CPT-11, camptothecin-11; Con, control.

phosphorylation of JNK and eventually Bcl-2 phosphorylation which is also unassociated with p53 expression (45). In contrast, oxidative stress dependent apoptosis mediated through arsenite is p53 dependent and JNK independent (46). Further, JNK activation mediated apoptosis by direct phosphorylation of Bcl which includes Bcl-XL, Bcl-2, Bad and Bim (47-49).

ROS are involved with the upstream signaling of all members of the MAPK family. Han *et al* (50) reported that ROS-mediated JNK and p38 activation are necessary for sanguinarine-induced HCT116 cell death. Furthermore, Zhao *et al* (51) identified that JNK phosphorylation and not p38 was involved downstream of ROS signaling as part of the mechanism of olaquindox-induced apoptosis of HepG2 cells. In the present study, ROS were not involved in CQAH-mediated apoptosis; however, JNK inhibition by SP600125 significantly reduced CQAH-induced downregulation of Bcl-XL and cell death. This finding proved that JNK-dependent Bcl signaling participates in CQAH-induced apoptosis. ROS are involved in various cellular functions, including cell proliferation, differentiation, necrosis and apoptosis. In addition, ROS can modify the mitochondrial permeability, which induces the loss of the mitochondrial membrane potential. As various anti-cancer drugs induce ROS-dependent mitochondrial malfunction (52,53), the present study used two effective anti-oxidants, NAC and DPI, to identify whether ROS is involved in CQAH-induced cell death. However, the results showed that pre-treatment with NAC and DPI did not inhibit CQAH-mediated cell death or PARP and caspase-3 cleavage, which clearly demonstrated that neither hydrogen peroxide generation nor superoxide generation is associated with CQAH induced apoptosis and that ROS were not involved in JNK activation.

In conclusion, the results of the present study showed that substitution at position-4 of the phenyl ring attached to azetidine affected the cytotoxicity of CQAH. As substitution at other positions or moieties may lead to further optimization of the activity of the compounds, the present study may serve as a basis for structure-based drug design of quinoline derivatives for the treatment of colon cancer. The present study identified that CQAH-induced apoptosis was mediated via JNK activation and caspases, while it was ROS-independent. Combined treatment with chemotherapeutic drugs showed that CQAH enhances their therapeutic efficacy against colon cancer. Therefore, CQAH and its derivatives are a promising class of compounds which may be developed into novel drugs for the treatment of colon cancer.

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