

Cepharanthine exhibits a potent anticancer activity in p53-mutated colorectal cancer cells through upregulation of p21^{Waf1/Cip1}

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Abstract. Cepharanthine (CEP), a biscochlorine alkaloid isolated from *Stephania cepharantha Hayata*, has demonstrated anticancer activity in several different types of cancer cells. Colorectal cancer (CRC) is one of the most common cancers in both men and women. Mutated p53 in CRC was reported to be associated with resistance to commonly used chemotherapeutic agents including, 5-fluorouracil, oxaliplatin and irinotecan. Many studies reported that mutation of p53 induced chemoresistance through several mechanisms, including induction of drug efflux, disruption of cell cycle regulation, evasion of apoptosis and upregulation of DNA repair. This study aimed to evaluate the anticancer activity of CEP in p53 mutant versus p53 wild-type colorectal cancer cells and determine its underlying mechanisms of action. Our results showed that CEP induced colorectal cancer cell death in a concentration-dependent manner. Remarkably, CEP was more effective in controlling the growth of the p53 mutant colorectal cancer cell lines, HT-29 and SW-620, than the p53 wild-type colorectal cancer cell lines, COLO-205 and HCT-116. Further studies on the underlying mechanisms revealed that CEP could induce cell cycle arrest and apoptosis in both HT-29 and COLO-205 cells. Treatment with CEP dramatically increased p21^{Waf1/Cip1} expression levels of the p53 mutant cell line HT-29 and to a lesser extent, the p53 wild-type cell line COLO-205. In addition, cyclin A and Bcl-2 expression levels of both cell lines were significantly downregulated following treatment with CEP. CEP also induced ROS formation in colorectal cancer cells. Taken together, we concluded that CEP effectively induced cell cycle arrest and apoptosis which may be mediated through upregulation of p21^{Waf1/Cip1},

downregulation of cyclin A and Bcl-2 and induction of ROS production in colorectal cancer cells. These findings suggested that CEP could potentially be a novel anticancer agent for p53 mutant colorectal cancer cells which are often resistant to current chemotherapeutic agents.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancer in both men and women and is the second leading cause of cancer-associated death worldwide (1). Currently, the treatment options for CRC include surgery, chemotherapy, radiotherapy and targeted therapies, which depend on stage of the disease and physical health of the patient. The chemotherapeutic agents commonly used in CRC treatments are 5-fluorouracil, oxaliplatin and irinotecan (2). Although combination therapy of these drugs has improved response rate and progression-free survival, its application is often limited due to acquired drug resistance which can occur in nearly all patients with CRC (3). Therefore, novel therapeutic compounds and strategies to overcome drug resistance are urgently needed.

Tumor suppressor p53 plays an important role in the regulation of DNA repair, cell cycle arrest, and apoptosis in the presence of cellular stress. Mutations in p53, the most common genetic change in human cancer, have been detected in ~40-50% of sporadic CRC (4). Mutant p53 has been shown to be involved in proliferation, invasion, migration, angiogenesis and cell survival. It is also responsible for drug resistance of cancer cells (5). Mutant p53 induced chemoresistance through several mechanisms, including induction of drug efflux, disruption of cell cycle regulation, evasion of apoptosis and upregulation of DNA repair (6). It has been reported that mutated p53 in CRC is associated with resistance to commonly used chemotherapeutic agents, including, 5-FU, doxorubicin, oxaliplatin and irinotecan (7-9). Therefore, finding a novel anticancer compound that remains effective without p53 chemoresistance may offer a potential strategy for the treatment of chemoresistant cancer cells.

Cepharanthine (CEP), a biscochlorine alkaloid extracted from *Stephania cepharantha Hayata*, possesses various biological activities such as anti-inflammatory, anti-malarial,

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anti-HIV-1, anti-oxidant, and anti-allergic (10). Notably, CEP has been found to exert anticancer activities against several types of cancer cells such as leukemia, oral squamous cell carcinoma, hepatocellular carcinoma, myeloma, cholangiocarcinoma, osteosarcoma, cervical adenocarcinoma, nasopharyngeal carcinoma and non-small cell lung cancer both *in vitro* and *in vivo* (11-21). It has been reported that CEP inhibited tumor growth through numerous mechanisms, including induction of host immune responses, inhibition of NF- κ B and STAT3 signaling pathways and reduction of angiogenesis by inhibiting expression of two major pro-angiogenic factors, vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) (16,18,22-24). Mechanistic studies revealed that CEP induced apoptosis via activating caspase-3 and -9, stimulating pro-apoptotic signaling pathways such as JNK, ERK and p38 MAPK pathways, inhibiting anti-apoptotic gene Bcl-x1 expression and inducing oxidative stress (12,14-16,18,24,25). Moreover, CEP was found to induce cell cycle arrest by downregulating the expression of cyclin D1, CDK-6 and c-Myc (15,18). Remarkably, CEP has been found to induce cell cycle arrest of adenocarcinoma cell carcinoma carrying p53 mutation (25).

To the best of our knowledge, there were only a few reports of antitumor effect of CEP in CRC cells (26). More importantly, the mechanisms underlying anticancer effect of CEP in CRC have never been investigated. In the present study, we therefore evaluated the anticancer activity of CEP using a p53 wild-type human colorectal cancer cell line, COLO-205, and a p53 mutant human colorectal cancer cell line, HT-29, and determined its underlying mechanisms of action.

Materials and methods

Chemical reagents. Cepharanthine was from Abcam (Cambridge, UK). Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), hydrogen peroxide (H₂O₂), N-acetylcysteine (NAC), oxaliplatin, SN-38, trypan blue, 5-fluorouracil, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich (St. Louis, MO, USA). Propidium iodide (PI) was from Santa Cruz Biotechnology (Dallas, TX, USA). Chloroform, ethanol and 2-propanol were from Merck (Kenilworth, NJ, USA). Nuclease-free water was from Qiagen (Hilden, Germany).

Cell lines and culture. Human colorectal cancer cell lines HT-29 and COLO-205 were from American Type Culture Collection (Manassas, VA, USA) whereas SW-620 and HCT-116 were kindly provided by Dr Amornpun Sereemasun (Chulalongkorn University, Thailand). HT-29 and SW-620 were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco). COLO-205 and HCT-116 were cultured in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin. The cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Cell viability assay. Cell viability was determined by using the MTT reduction assay. Briefly, cells were seeded in 96-well

plates at a density of 5x10³ cells/well. After overnight incubation, the cells were treated with 5-FU, oxaliplatin, SN-38 or CEP at 0.01, 0.1, 1, 10 and 100 μ M for 48 h. Thereafter, MTT solution was added and incubated for additional 4 h. The medium was removed and 200 μ l of DMSO was added to each well. The absorbance of the converted dye was measured at a wavelength of 570 nm using LabSystems Multiskan MS microplate reader (Thermo Scientific, Vantaa, Finland).

Cell cycle analysis. HT-29 and COLO-205 cells were seeded in 6-well plates at a density of 3x10⁵ cells/well and incubated overnight. HT-29 cells were treated with 5, 10, and 20 μ M of CEP or 0.2% DMSO (solvent control) whereas COLO-205 cells were treated with 10, 20 and 40 μ M of CEP or 0.2% DMSO. After 12, 24 or 36 h of incubation, the cells were washed with phosphate-buffered saline (PBS), harvested by trypsinization and centrifuged at 1,500 rpm for 5 min. The cell pellets were washed with cold PBS and fixed with 70% ethanol for 20 min at -20°C. The cells were then washed with cold PBS and incubated with 500 μ l of assay buffer containing 5 μ l of 4 mg/ml RNase A at room temperature. After 30-min incubation, the cells were stained with 5 μ l of 0.05 μ g/ml PI for another 30 min at room temperature in the dark. DNA content of the stained cells was analyzed using BD LSR II flow cytometer.

Real-time RT-PCR. Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA concentration and purity was determined using NanoDrop ND-2000 (Thermo Scientific, Rockford, IL, USA). RNA (500 ng) was reverse-transcribed into cDNA using Improm IITM Reverse Transcription system (Promega, Madison, WI, USA). Amplification of target genes was carried out on StepOnePlusTM Real-Time PCR system (Applied Biosystems, Waltham, MA, USA) using SYBR GreenERTM qPCR Supermix (Life Technologies) and the primers listed in Table I. Expression of the gene of interest was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and relative expression was calculated using the $\Delta\Delta$ CT method.

Western blotting. Cell lysates were prepared by homogenizing cells in RIPA lysis buffer (Thermo Fisher Scientific) containing protease inhibitors (Sigma). Protein concentration of supernatants was determined by a microplate assay with the Bio-Rad DC Protein assay reagents (Hercules, CA, USA) using bovine serum albumin as a standard. Twenty micrograms of protein lysate were separated on an 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a PVDF membrane. The membrane was blocked in 3% non-fat dry milk (NFD) and then incubated with antibodies against p21^{Waf1/Cip1} (dilution 1:1,000, 2947; Cell Signaling Technology, Danvers, MA, USA), cyclin A2 (dilution 1:2,000, 4656; Cell Signaling Technology), cyclin D1 (dilution 1:1,000, 2922; Cell Signaling Technology) or β -actin (dilution 1:1,000, 4970; Cell Signaling Technology) overnight at 4°C. The membrane was then washed and incubated with HRP-linked secondary antibody (Cell Signaling Technology) for 1 h at room temperature. Protein bands were detected using LuminataTM Cescendo Western HRP substrate (Millipore, Billerica, MA, USA) and analyzed using Image Studio

Table I Sequences of primers used for real-time RT-PCR analysis.

Target genes	Primer sequences
Bcl-2	F: 5'-TCA TGT GTG TGG AGA GCG TCA A-3' R: 5'-CTA CTG CTT TAG TGA ACC TTT TGC-3'
Bcl-xl	F: 5'-TTG GAC AAT GGA CTG GTT GA-3' R: 5'-GTA GAG TGG ATG GTC AGT G-3'
Bax	F: 5'-GAC GAA CTG GAC AGT AAC ATG-3' R: 5'-AGG AAG TCC AAT GTC CAG CC-3'
Bak	F: 5'-ATG GTC ACC TTA CCT CTG CAA-3' R: 5'-TCA TAG CGT CGG TTG ATG TCG-3'
Cyclin A	F: 5'-CTG CTG CTA TGC TGT TAG CC-3' R: 5'-TGT TGG AGC AGC TAA GTC AAA A-3'
Cyclin D	F: 5'-TTG TTG AAG TTG CAA AGT CCT GG-3' R: 5'-ATG GTT TCC ACT TCG CAG CA-3'
Cyclin E	F: 5'-TCC TGG ATG TTG ACT GCC TT-3' R: 5'-CAC CAC TGA TAC CCT GAA ACC T-3'
p21	F: 5'-CCT GTC ACT GTC TTG TAC CCT-3' R: 5'-GCG TTT GGA GTG GTA GAA ATC T-3'
GAPDH	F: 5'-AAG GTC GGA GTC AAC GGA TTT GGT-3' R: 5'-ATG GCA TGG ACT GTG GTC ATG AGT-3'

F, forward. R, reverse.

software (LI-COR, Lincoln, NE, USA). β -actin was used as the loading control for protein normalization.

Immunofluorescence staining. After growing HT-29 cells on 8-well Lab-Tek chamber slides (Thermo Fisher Scientific) overnight, the cells were treated with CEP at various concentrations for 24 h. The cells were then fixed in 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, the cells were permeabilized with 0.25% Triton X-100 for 10 min at room temperature. Thereafter, the cells were washed and incubated with blocking solution containing 2% normal goat serum (Abcam) and 1% bovine serum albumin for 20 min at room temperature. The cells were then washed with PBS and incubated with a p21^{Waf1/Cip1} rabbit antibody (dilution 1:800, 2947; Cell Signaling Technology) overnight at 4°C followed by incubation with Alexa Fluor 488 conjugated goat anti-rabbit secondary antibody (Invitrogen) for 30 min at room temperature in the dark. The cells were washed, mounted using fluoroshield mounting medium with DAPI (Abcam) and observed with an LSM 800 Confocal Laser Scanning Microscope (Zeiss, Oberkochen, Germany).

Measurement of ROS generation. Intracellular ROS was quantified using membrane-permeable fluorescent probe dichlorodihydrofluorescein diacetate (DCFH-DA). Cells were seeded in 96-well plates at a density of 5×10^3 cells. After overnight incubation, the culture medium was replaced with 100 μ l of Hank's buffered salt solution (HBSS) containing 10 μ M DCFH-DA and incubated for 30 min at 37°C in the dark.

Table II. IC₅₀ of anticancer agents and cepharanthine (CEP) in HT-29 and COLO-205 cells.

Drug	IC ₅₀ (μ M)	
	HT-29	COLO-205
5-Fluorouracil	12.64 \pm 2.61	2.67 \pm 1.20
Oxaliplatin	2.83 \pm 0.32	1.23 \pm 0.45
SN-38	2.40 \pm 0.37	1.20 \pm 0.14
CEP	5.18 \pm 1.23	20.74 \pm 1.47

The cells were washed with PBS and incubated with various concentrations of CEP for 1 h. The cells were then washed with cold PBS and suspended in 200 μ l of 1% Triton X-100. The fluorescence intensity was measured using a fluorescent microplate reader (Thermo Scientific) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Statistical analysis. Data are presented as mean \pm standard error of mean (SEM) from at least three independent experiments. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by a Bonferroni/Dunn post hoc comparison test. P-values <0.05 were considered statistically significant.

Results

Cepharanthine exhibits a potent anticancer activity in p53-mutated colorectal cancer cells. Initially, we evaluated the cytotoxicity of cepharanthine (CEP) compared to the most common chemotherapeutic agents for CRC, including 5-fluorouracil, oxaliplatin, and SN-38 (an active metabolite of irinotecan), in a p53 mutant HT-29 cells and a p53 wild-type COLO-205 cells. As shown in Fig. 1A, all four compounds decreased cell viability of both cell lines in a concentration-dependent manner. However, the IC₅₀ values illustrated that the COLO-205 cells were more susceptible to the three FDA-approved anticancer drugs than the HT-29 cells (Table II). Interestingly, CEP was more toxic to the HT-29 cells than the COLO-205 cells as evidenced by a ~4-fold lower IC₅₀ value.

To further confirm the anticancer activity of CEP in p53 mutant colorectal cancer cells, we determined the cell viability of 4 human colorectal cancer cell lines treated with CEP at 2.5, 5, 10, 20 and 40 μ M for 48 h. These cell lines were p53 mutant CRC cells; HT-29 and SW-620 and p53 wild-type CRC cells; HCT-116 and COLO-205. As shown in Fig. 1B, CEP decreased the viability of HT-29, SW-620, HCT-116 and COLO-205 cells in a concentration-dependent manner. However, HT-29 and SW-620 with p53 mutations were more susceptible to the cytotoxic effect of CEP than HCT-116 and COLO-205 cells with wild-type p53 (Fig. 1C). These results suggested that CEP was very effective in controlling the growth of cancer cells harboring p53 mutations.

Cepharanthine induces cell cycle arrest and apoptosis in both p53-mutant and p53 wild-type colorectal cancer cells.

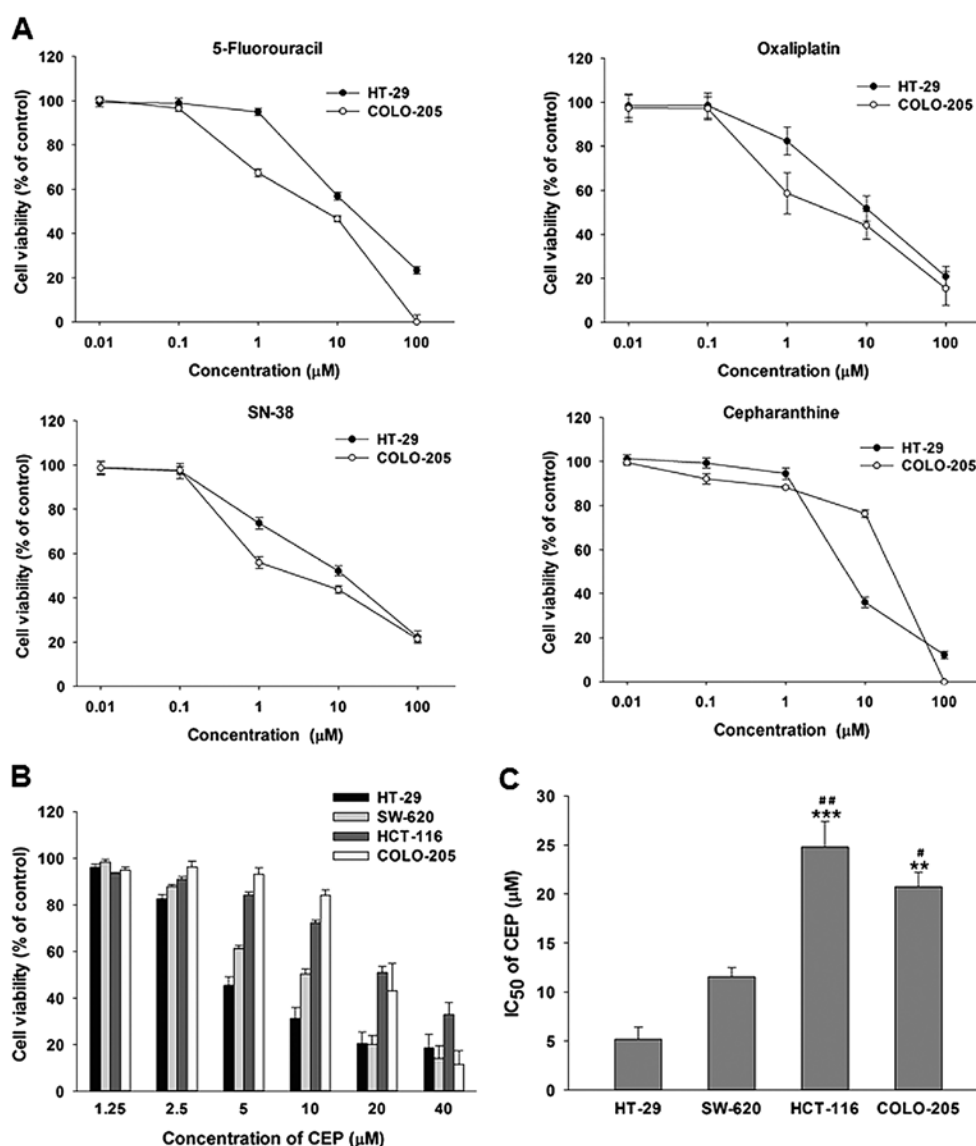


Figure 1. CEP exhibits a potent anticancer activity in p53-mutated colorectal cancer cells. (A) Cytotoxicity of 5-FU, oxaliplatin, SN-38 and CEP on HT-29 and COLO-205 cells. Cells were treated with indicated compounds for 48 h and cell viability was evaluated using MTT assay. (B) Cytotoxicity and (C) IC₅₀ of CEP on four CRC cell lines. Cells were treated with CEP for 48 h and cell viability was determined using MTT assay. **p<0.01 and ***p<0.001 vs. HT-29; #p<0.05 and ##p<0.01 vs. SW-620.

We then determined whether the growth-inhibitory effect of CEP is related to cell cycle arrest. HT-29 and COLO-205 cells were treated CEP and cell cycle was analyzed by PI staining followed by flow cytometry. As shown in Fig. 2A, the percentages of HT-29 cells in the G1 phase were significantly increased following treatment with 10 μM CEP for 12 h. Similar results were detected in HT-29 cells treated with 5 and 10 μM CEP for 24 h. However, it should be noted that 20 μM of CEP significantly increased accumulation of the sub-G1 fraction, an indicator of apoptotic cell death. At 36 h of incubation, treatment with CEP only at 5 μM caused cell accumulation at the G1 phase whereas 10 and 20 μM of CEP significantly induced a sub-G1 accumulation 10- and 24-fold above control, respectively. We also observed a significant accumulation of COLO-205 cells in the G1/S phases following treatment with CEP at 10 and 20 μM whereas CEP at 40 μM induced apoptotic cell death as early as 12 h of incubation (Fig. 2B). Interestingly, for the longer incubation time-point, CEP at 10 μM resulted

in accumulation of the COLO-205 cells in S phase while apoptosis was significantly detected in the cells treated with CEP at 20 and 40 μM. These results indicated that CEP at low concentrations effectively disturbed cell cycle progression while CEP at higher concentrations clearly induced apoptosis in both HT-29 and COLO-205 cells.

Cepharanthine induces changes in cell cycle-regulated gene expression in colorectal cancer cells. To address the mechanism responsible for the effect of CEP on cell cycle arrest, we measured the mRNA levels of cell cycle regulators: cyclins A, D and E; and p21, a CDK inhibitor using real-time reverse transcription polymerase chain reaction. As shown in Fig. 3A, CEP at 20 μM significantly decreased cyclin A mRNA level in HT-29 cells by ~65%. This compound also downregulated the expression of cyclin D in a concentration-dependent manner; however, expression of cyclin E was unaffected by CEP. Notably, the expression of p21^{Waf1/Cip1} mRNA in

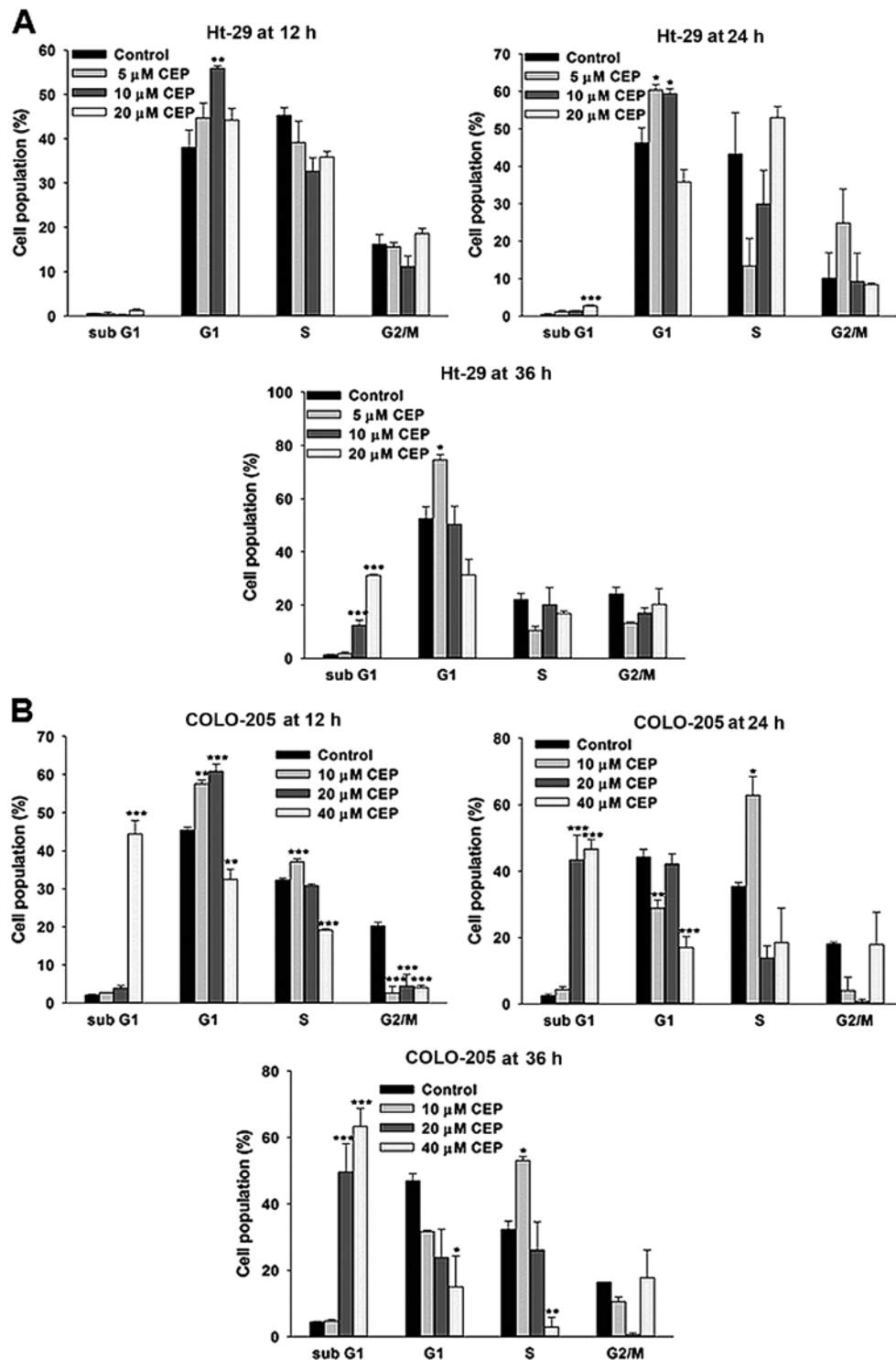


Figure 2. CEP induces cell cycle arrest and apoptosis in colorectal cancer cells. The percentage of cells with DNA content corresponding to sub-G1 fraction and G1, S and G2/M phases at 12, 24 and 36 h after CEP treatment on (A) HT-29 and (B) COLO-205 cells. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. control.

HT-29 cells was dramatically upregulated in response to CEP treatment. CEP at 5, 10 and 20 μ M increased p21^{Waf1/Cip1} mRNA levels ~5, 20 and 60 times above control, respectively. In COLO-205 cells, CEP significantly downregulated the cyclin A mRNA expression levels in a concentration-dependent manner (Fig. 3B) whereas the expression of cyclin D and E were unaltered by CEP. Similar to HT-29, CEP at 20 and 40 μ M significantly induced the expression of p21^{Waf1/Cip1} mRNA in COLO-205 cells, although to a lesser extent.

Western blot analysis also showed that CEP suppressed expression of cyclin A2 and cyclin D1 proteins in HT-29 cells (Fig. 4A). Similarly, treatment with CEP resulted in a decrease in cyclin A2 protein level in COLO-205 cells in a concentration-dependent manner (Fig. 4B). The increased expressions of p21^{Waf1/Cip1} in HT-29 cells were further confirmed by immunofluorescence staining. HT-29 cells were treated with 5, 10 and 20 μ M of CEP for 24 h then immunolabeled for the presence of p21^{Waf1/Cip1} and stained with DAPI.

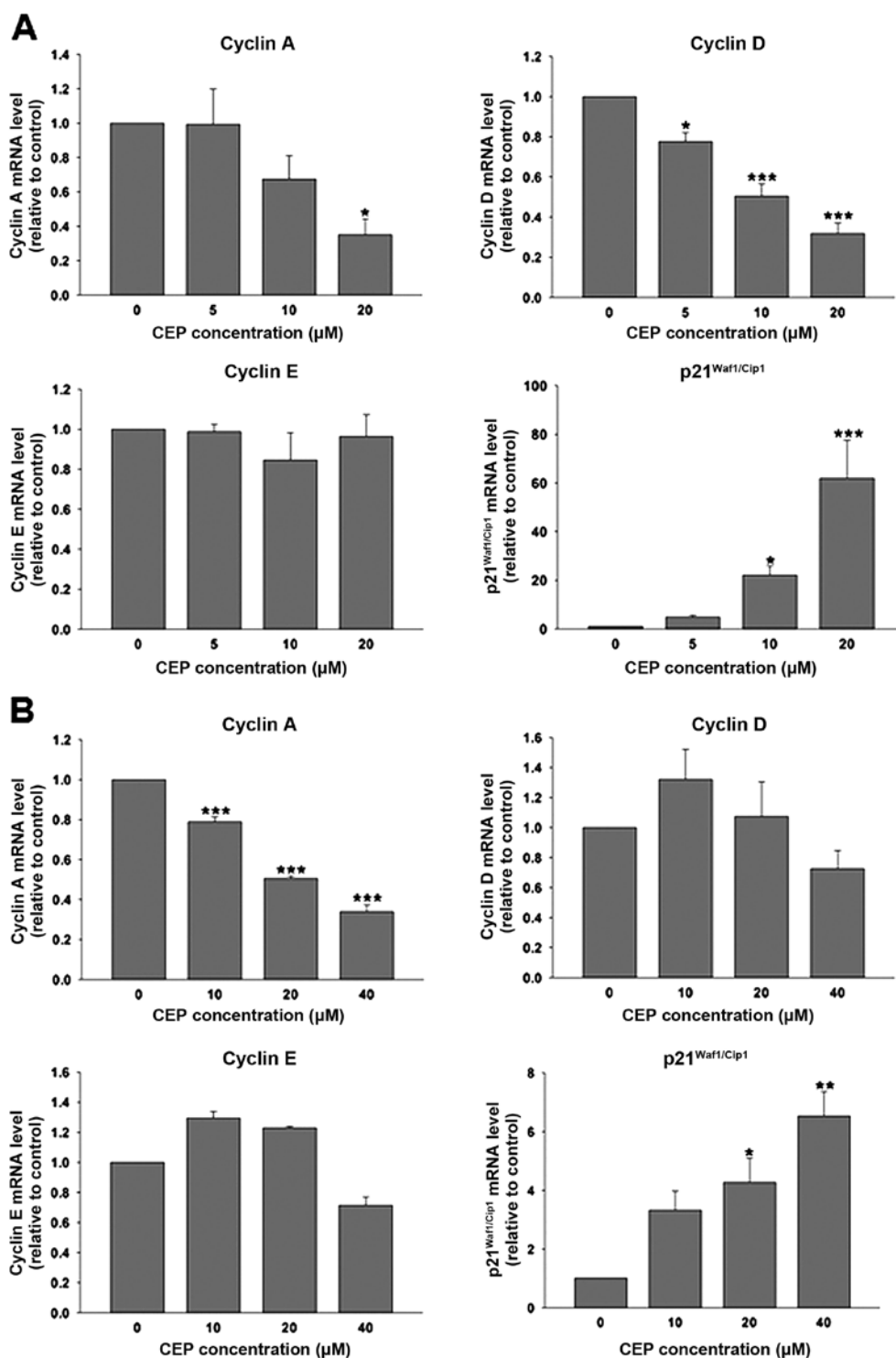


Figure 3. CEP downregulates the mRNA expression of cyclins and upregulates the mRNA expression of p21^{Waf1/Cip1} in colorectal cancer cells. Cells were treated with CEP and the mRNA levels of cyclins A, D and E and p21 were determined in (A) HT-29 and (B) COLO-205 cells using real-time RT-PCR. *p<0.05, **p<0.01 and ***p<0.001 vs. control.

In agreement with mRNA and protein expression results, CEP increased p21^{Waf1/Cip1} fluorescence intensity in the nucleus of the HT-29 cells in a concentration-dependent manner (Fig. 5).

Cepharanthine decreases Bcl-2 expression in colorectal cancer cells. The intrinsic pathway of apoptosis is mainly involved in anticancer activity of several chemotherapeutic agents. This pathway is regulated by members of the Bcl-2

family proteins (27). Therefore the mRNA levels of Bcl-2 family, such as anti-apoptotic proteins, including Bcl-2 and Bcl-xl; and pro-apoptotic proteins, including Bax and Bak were evaluated after CEP treatment. Real-time RT-PCR analysis revealed that CEP at 20 μM significantly downregulated the mRNA expression of anti-apoptotic proteins, Bcl-2 and Bcl-xl in HT-29 cells (Fig. 6A). In contrast, CEP did not alter mRNA expression of pro-apoptotic proteins, Bak and Bax.

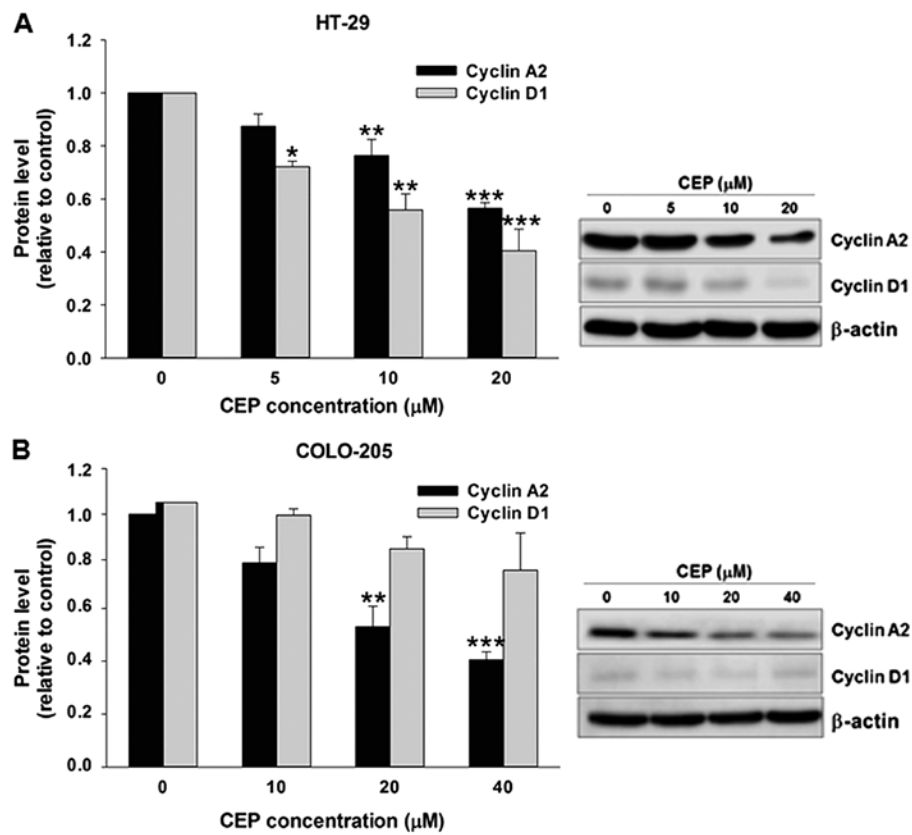


Figure 4. CEP downregulates the protein expression of cyclins in colorectal cancer cells. Cells were treated with CEP and the protein levels of cyclins A2 and D1 were determined in (A) HT-29 and (B) COLO-205 cells using western blot analysis. *p<0.05, **p<0.01 and ***p<0.001 vs. control.

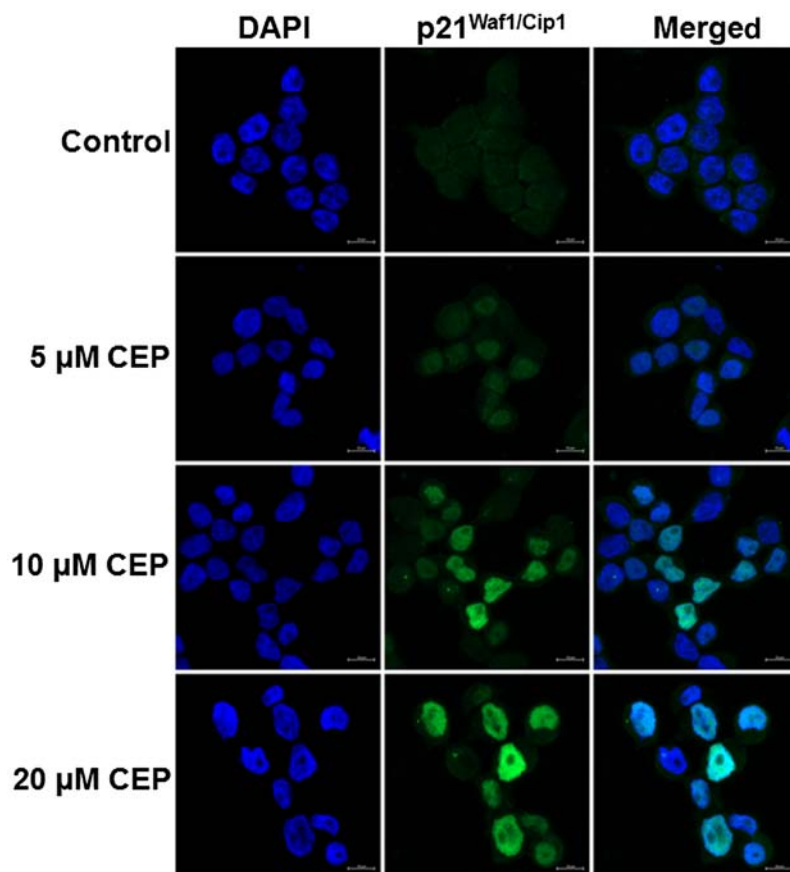


Figure 5. CEP induces p21^{Waf1/Cip1} intensity, especially in the nucleus of HT-29 cells. The immunofluorescence staining of HT-29 cells treated with CEP for 24 h. The HT-29 cells were then immunolabeled for the presence of p21^{Waf1/Cip1} and stained with DAPI.

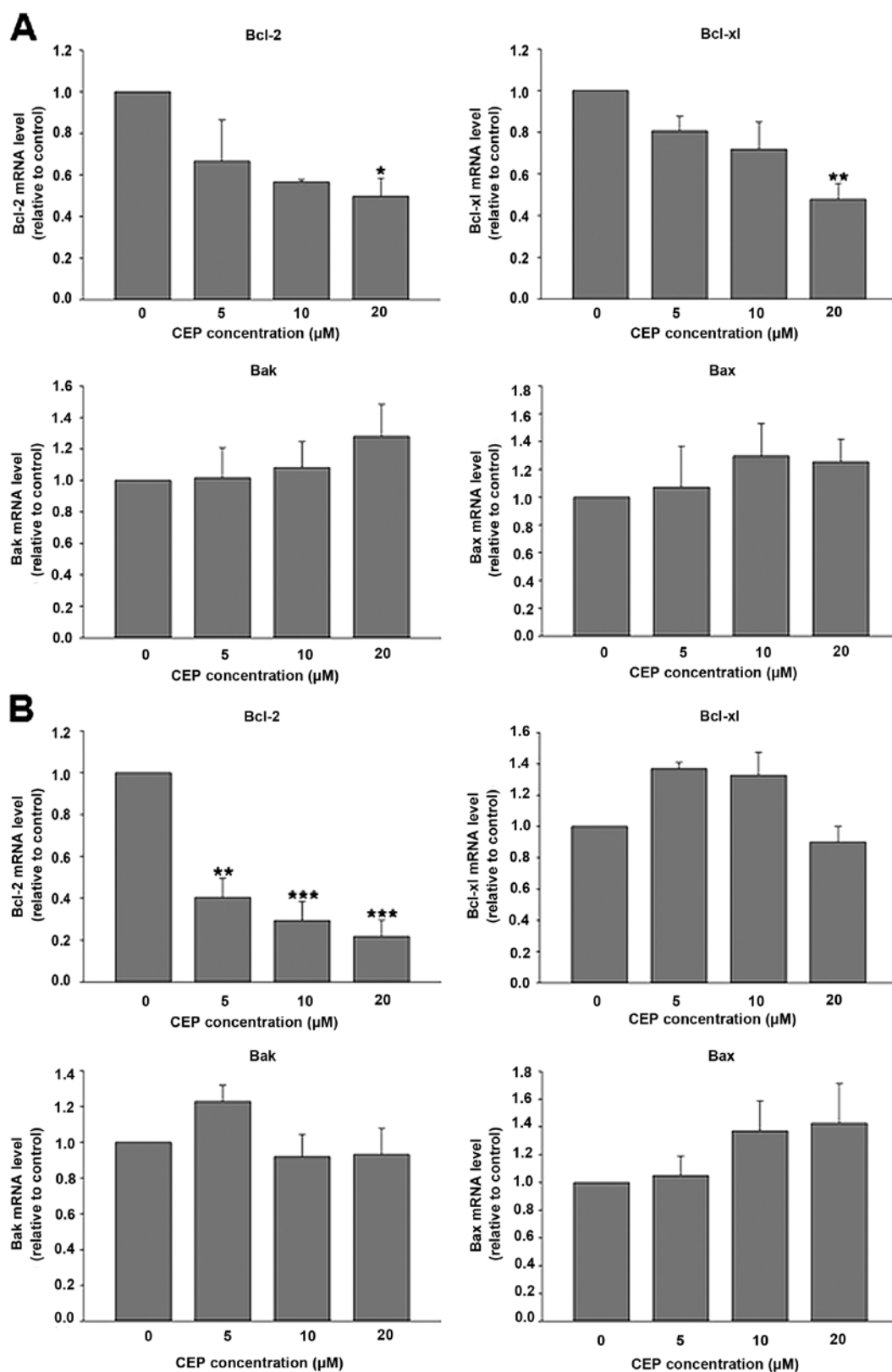


Figure 6. CEP downregulates the mRNA expression of Bcl-2 and Bcl-xl in colorectal cancer cells. Cells were treated with CEP and the mRNA levels of Bcl-2, Bcl-xl, Bak and Bax were determined in (A) HT-29 and (B) COLO-205 cells using real-time RT-PCR. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. control.

As illustrated in Fig. 6B, CEP significantly decreased Bcl-2 mRNA expression in COLO-205 cells in a concentration-dependent manner. However, mRNA levels of Bcl-xl, Bak and Bax were unaffected following CEP treatment. These results indicated that downregulation of Bcl-2 mRNA expression was

likely responsible for CEP-induced apoptosis in colorectal cancer cells.

Cepharanthine induces ROS production in colorectal cancer cells. Overproduction of reactive oxygen species (ROS) can

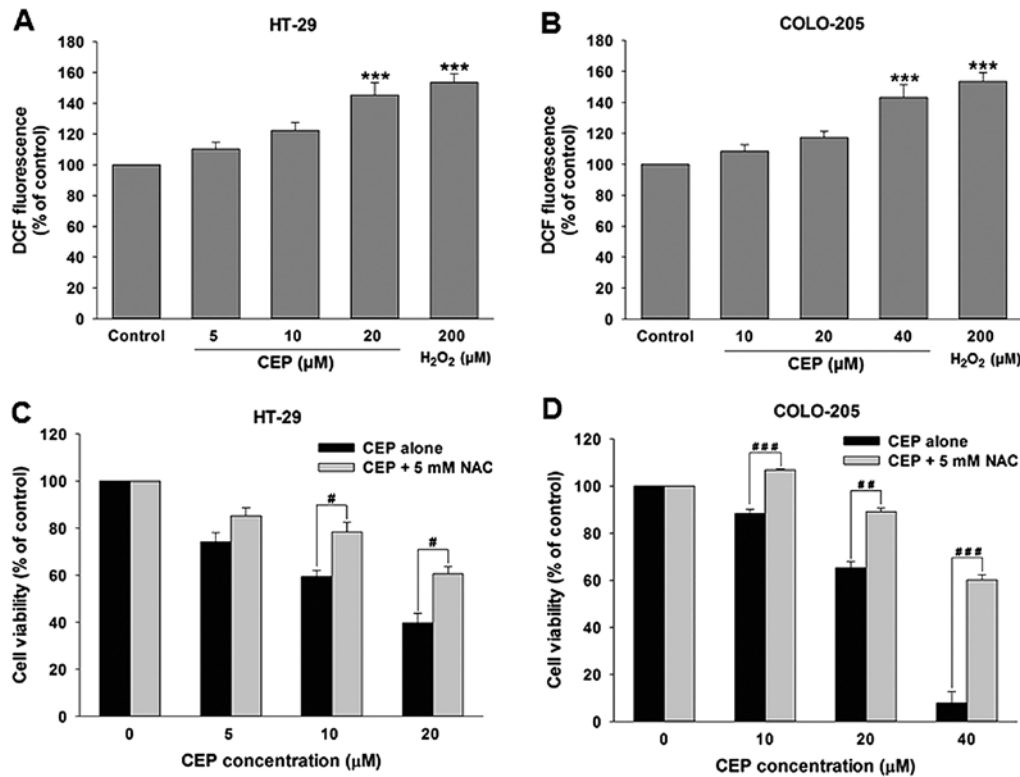


Figure 7. CEP induces ROS generation in colorectal cancer cells. The ROS levels (DCF fluorescence intensity) in (A) HT-29 and (B) COLO-205 cells were measured by dichlorodihydrofluorescein diacetate (DCFH-DA) assay following CEP treatment. The viability of (C) HT-29 and (D) COLO-205 cells treated without NAC vs. with NAC, prior to CEP treatment. *** p <0.001 vs. control. # p <0.05, ## p <0.01 and ### p <0.001 vs. CEP alone.

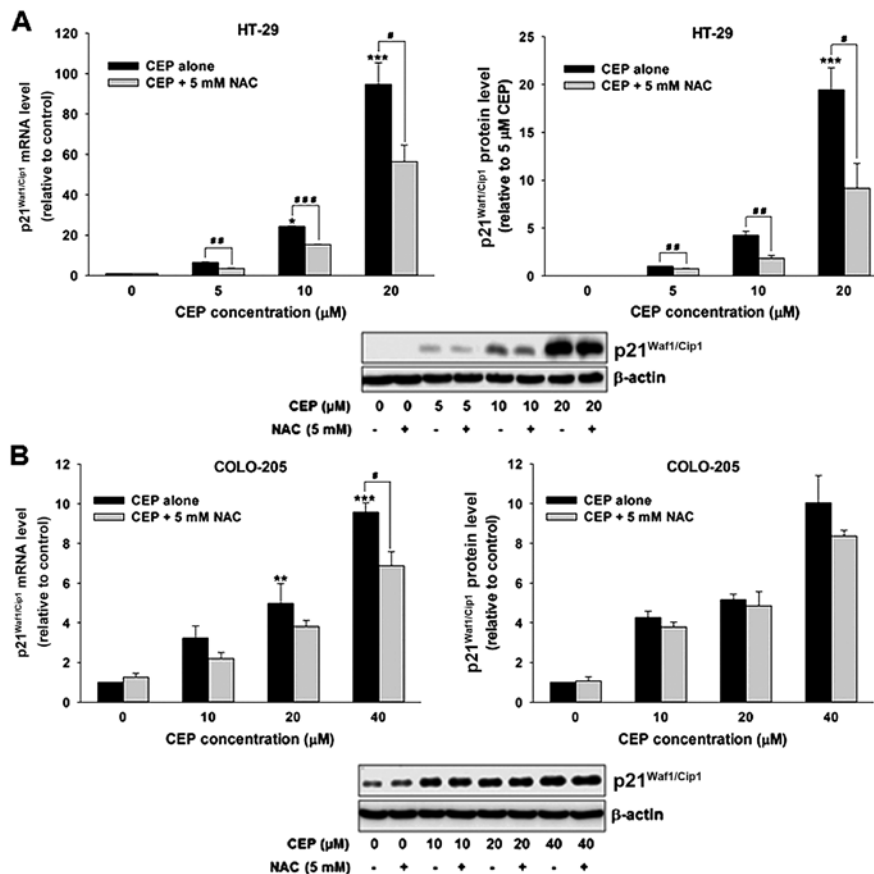


Figure 8. CEP induces p21^{Waf1/Cip1} expression via ROS production in p53 mutant colorectal cancer cells. Cells were treated with CEP in the absence or presence of NAC for 24 h. The mRNA and protein levels of p21^{Waf1/Cip1} were measured in (A) HT-29 and (B) COLO-205 cells. * p <0.05, ** p <0.01, and *** p <0.001 vs. control. # p <0.05, ## p <0.01 and ### p <0.001 vs. CEP alone.

lead to oxidative stress causing cell death. It has been shown that various anticancer drugs such as cisplatin, vinblastin, doxorubicin and camptothecin induce cell death through generation of ROS in several cancer cells (28). We therefore determined whether CEP is capable of triggering ROS production in colorectal cancer cells. HT-29 cells were treated with CEP at concentrations of 5, 10 and 20 μ M and COLO-205 cells were treated with CEP at concentrations of 10, 20 and 40 μ M. One hour after treatment ROS level was determined by measuring the dichlorodihydrofluorescein (DCF) fluorescence intensity with dichlorodihydrofluorescein diacetate (DCFH-DA) assay. As illustrated in Fig. 7A, treatment of HT-29 cells with 10 and 20 μ M of CEP significantly induced ROS production. Similarly, ROS level was significantly elevated in COLO-205 cells after treatment with 40 μ M of CEP (Fig. 7B).

To confirm whether ROS production is involved in CEP-mediated cytotoxicity, both HT-29 and COLO-205 cells were pre-incubated with 5 mM of N-acetyl cysteine (NAC), a ROS inhibitor, for 30 min before treatment with CEP. As shown in Fig. 7C and D, the cytotoxicity of CEP to both HT-29 and COLO-205 cells was significantly reduced by pretreatment with NAC, suggesting that ROS production is partly responsible for CEP-induced colorectal cancer cell death.

Many studies have reported links between ROS and p21^{Waf1/Cip1}, we therefore determined whether ROS is involved in the induction of p21^{Waf1/Cip1} following CEP treatment in colorectal cancer cells. As shown in Fig. 8A, CEP increased p21^{Waf1/Cip1} expression on the mRNA and protein levels in HT-29 cells. Remarkably, co-incubation with NAC significantly attenuated the p21^{Waf1/Cip1} induction in CEP-treated cells. Although treatment with CEP resulted in upregulation of p21^{Waf1/Cip1} mRNA and protein levels in COLO-205, the significant difference in p21^{Waf1/Cip1} mRNA level between cells treated with CEP alone and in combination with NAC was only detected after treatment with 40 μ M CEP (Fig. 8B). Taken together, these results suggest that ROS is critically required for p21^{Waf1/Cip1} induction in response to CEP treatment in p53 mutant colorectal cancer cells.

Discussion

Many lines of evidence have demonstrated that p53 mutations, the most common genetic change identified in human cancer, are associated with resistance to several types of chemotherapeutic agents, including alkylating agents, topoisomerase I/II inhibitors, and antimetabolites (29-31). This highlights the need for a novel anticancer agent that remains effective in cancer cells harboring mutant p53. Cepharanthine (CEP), a biscoclaurine alkaloid isolated from *Stephania cepharantha* Hayata, was shown to possess anticancer activities in many types of cancer cells (11-21). Interestingly, it was reported that CEP could inhibit the growth of human adenosquamous cell carcinoma containing p53 mutation (25). In the present study, we investigated the anticancer activity of CEP against colorectal cancer cells using a p53 wild-type human colorectal cancer cell line, COLO-205, and a p53 mutant human colorectal cancer cell line, HT-29. We have found that CEP induced colorectal cancer cell death *in vitro* in a time- and concentration-dependent manner. Interestingly, CEP was more cytotoxic

to the p53 mutant HT-29 cells than the p53 wild-type COLO-205 cells. The IC₅₀ value of the HT-29 cells treated with CEP was ~4 times lower than that in the COLO-205 cells. In contrast, the commonly used chemotherapeutic agents, 5-FU, oxaliplatin and SN-38, an active metabolite of irinotecan were more cytotoxic to the COLO-205 cells than the HT-29 cells. In addition to approved drugs, promising chemotherapeutic agents such as 3'-hydroxypterostilbene, 3,5,4'-trimethoxystilbene, and myricetin (32-34) were shown to be more toxic to the COLO-205 cells than the HT-29 cells.

To further confirm the anticancer activity of CEP against p53 mutant colorectal cancer cells, SW-620, another p53 mutant colorectal cancer cell line and HCT-116, a p53 wild-type colorectal cancer cell were treated with CEP. The MTT assays revealed that CEP was more cytotoxic to the two p53 mutant colorectal cancer cell lines, HT-29 and SW-620, than the two p53 wild-type colorectal cancer cell lines, HCT-116 and COLO-205, suggesting that CEP has potential to be used as a novel chemotherapeutic agent for colorectal cancer cells harboring p53 mutation.

Many chemotherapeutic agents exert their anticancer activity via induction of cell cycle arrest and apoptosis. Previous studies demonstrated that CEP could induce cell cycle arrest at G1 and S phase in various types of cancer (13,15,18). Similar to previous studies, we have found that CEP caused accumulation of HT-29 and COLO-205 cells at the G1 and the G1 and S phase of the cell cycle, respectively. Remarkably, cell cycle arrest effect was detected following CEP treatment at the lowest concentration while its apoptotic-induction effect was noted at the highest concentration in both HT-29 and COLO-205 cells. The time course studies revealed that CEP at the intermediate concentration (10 μ M) significantly caused accumulation of the HT-29 cells at the G1 phase after 24-h treatment and induced apoptosis at 36 h whereas treatment of COLO-205 cells with CEP at the intermediate concentration (20 μ M) significantly disrupted progression of cell cycle as early as 12 h while significant apoptotic cell death was observed at 24 h after treatment. These results suggested that CEP was able to induce both cell cycle arrest and apoptosis and its anticancer activity likely depended on concentration and duration of the treatment. Accumulating evidence has demonstrated that mutations of p53 are capable of not only disrupting the antiproliferative properties but also promoting various oncogenic responses to cell growth, metastasis, invasion, chemoresistance, and apoptosis resistance (5). Moreover, it was reported that mutant p53 could delay cell growth arrest in fibroblasts (35). Thus, it is likely that mutation of p53 is responsible for the delayed cell cycle arrest in HT-29 cells relative to COLO-205 cells.

Bcl-2 family proteins, including anti-apoptotic proteins such as Bcl-2 and Bcl-xl and pro-apoptotic proteins such as Bax and Bak are regulators of mitochondria or intrinsic apoptotic pathway (36). It has been reported that CEP induced apoptosis through upregulation of Bax and downregulation of Bcl-2 and Bcl-xl in various cancer cells (18,19,21). In agreement with these observations, this study revealed that CEP decreased level of Bcl-2 mRNA in COLO-205 cells in a concentration-dependent manner. Notably, significant reductions of mRNA levels of the two key anti-apoptotic proteins, Bcl-2 and Bcl-xl were observed in HT-29 cells following CEP treatment at high

concentration only. Taken together, these results suggested that apoptotic-inducing effect of CEP was mediated through downregulation of Bcl-2 and Bcl-xl in colorectal cancer cells. Previously, several studies demonstrated that CEP reduced expression of Bcl-2 and Bcl-xl through inhibition of STAT3 signaling pathway in cervical adenocarcinoma and osteosarcoma (18,19). Therefore, apoptosis-inducing effects of CEP on human colorectal cancer cells may be mediated through inhibition of STAT3 resulting in suppression of downstream gene products, including Bcl-2 and Bcl-xl. However, the effects of CEP on the STAT3 signaling pathway in colorectal cancer cells require further elucidation.

The cell cycle is regulated at the checkpoints by cyclin/cyclin-dependent kinase (CDK) complexes, which are inactivated by CDK inhibitors (CKIs) (37). In the present study, we have documented that CEP downregulated the expression of cyclin A and both cyclin A and D in COLO-205 and HT-29 cells, respectively. Numerous previous studies demonstrated that CEP downregulated the expression of cyclins D1 and E in several cancer cells including osteosarcoma, oral squamous cell carcinoma and myeloma (13,15,18). However, one of the most surprising finding of this study was that CEP was able to decrease the expression of cyclin A in both COLO-205 and HT-29 cells, suggesting that cyclin A may be a therapeutic target of CEP in human colorectal cancer cells.

Oxidative stress is an overproduction of reactive oxygen species (ROS) beyond the cellular antioxidant capacity. It was reported that oxidative stress can trigger p53-dependent and p53-independent apoptotic cell death pathway in cancer cells (38). Numerous natural agents with potent anticancer activity such as epigallocatechin-3-gallate (EGCG), resveratrol, and curcumin were shown to induce apoptosis in cancer cells via a ROS-dependent pathway (39). Previous studies demonstrated that CEP triggered apoptosis in human hepatocellular carcinoma cells and non-small cell lung cancer cells through ROS (14,21). Similarly, the results of this study showed that CEP significantly increased ROS level in HT-29 and COLO-205 cells. Moreover, the cytotoxicity of CEP was significantly abolished by N-acetylcysteine (NAC), a specific ROS inhibitor, indicating that cytotoxic effect of CEP is partly mediated by ROS.

Although p53 is a known transcriptional regulator of p21^{Waf1/Cip1}, several different compounds such as phenoxodiol, ascofuranone, and MLN4924, an inhibitor of NEDD8 activating enzyme, have been demonstrated to induce p21^{Waf1/Cip1} independently of p53 status (40-42). Similarly, CEP was shown to induce cell cycle arrest at G1 phase by activating p21^{Waf1/Cip1} in a human adenosquamous cell carcinoma cell line harboring mutant p53 (25). Our results also showed that CEP dramatically increased mRNA and protein levels of p21^{Waf1/Cip1} in HT-29 cells. Remarkably, NAC was able to attenuate CEP-induced p21^{Waf1/Cip1} expression in HT-29 cells. Together, these findings suggest that upregulation of p21^{Waf1/Cip1} by ROS mainly contribute to anticancer activity of CEP in p53 mutant colorectal cancer cells. Although several studies demonstrated that upregulation of p21^{Waf1/Cip1} led to increased production of ROS in various cancer cells such as prostate, bladder and head and neck (43,44), our results raised the possibility that CEP induced production of ROS, leading to transcription of p21^{Waf1/Cip1}, independently of p53. The high

levels of p21^{Waf1/Cip1}, in turn, activated ROS production. A recent study demonstrated that cells undergoing p21^{Waf1/Cip1}-dependent cell death had higher sensitivity to oxidants (43), thus it is likely that upregulation of p21^{Waf1/Cip1} makes HT-29 cells susceptible to cytotoxicity of CEP. In the present study, we also uncovered that CEP increased p21^{Waf1/Cip1} in COLO-205 cells but less extensive than in HT-29 cells and NAC had no significant effect on CEP-induced p21^{Waf1/Cip1} expression. Since p53 regulates growth arrest and apoptosis mainly through the activation or suppression of various target genes, therefore expression of p21^{Waf1/Cip1} might not play an important role in anticancer activity of CEP in p53 wild-type colorectal cancer cells. In fact, we observed that CEP dramatically downregulated Bcl-2 expression in COLO-205 cells.

In conclusion, these results clearly illustrated that CEP has a remarkable anticancer activity against both p53 wild-type and p53 mutant colorectal cancer cells. Mechanistic studies revealed that the anticancer effect of CEP in colorectal cancer cells involves induction of cell cycle arrest and apoptosis which may be associated with upregulation of p21^{Waf1/Cip1}, downregulation of cyclin A and Bcl-2 and induction of ROS. It should be noted, however, that this study was only performed on two cell lines. Further elucidation and verification of these observations both *in vitro* and *in vivo* are warranted. In addition, toxicity of CEP should be thoroughly tested for clinical application.

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