



Combined effects of celecoxib and cepharanthine on human colorectal cancer cells *in vitro*

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ABSTRACT

Colorectal cancer is one of the most common cancers worldwide. We investigated the combined effects of celecoxib (CLX)–cepharanthine (CEP) on HT-29 human colorectal cancer cells. CLX at 5, 10, 20, or 40 μ M in combination with CEP at 1.25, 2.5, or 5 μ M displayed synergistic cytotoxic effects with a combination index <1 . Combinations of 20 or 40 μ M of CLX with 1.25 or 2.5 μ M of CEP increased HT-29 cell accumulation at the G1 phase of the cell cycle. The combined treatments increased the levels of p21 mRNA and decreased the levels of cyclin-A2 mRNA. Their combined effect triggered significant apoptosis of HT-29 cells when compared with the effect of each drug alone. The apoptotic effects of the drugs were correlated with increases in the levels of mRNA for BAX and decreases in the levels of mRNA for Bcl-xL. The results from this study revealed that at concentrations that were sub-IC₅₀ individually, CLX-CEP combinations had synergistic cytotoxic and apoptotic effects, suggesting that their combination is useful for reducing their nonselective toxicity and side effects when treating colorectal cancer.

INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer-related death in both men and women worldwide (Siegel *et al.*, 2017). Treatment options for CRC depend primarily on the stage of the disease. Standard treatments for CRC are surgery, radiotherapy, chemotherapy, and targeted anticancer drugs. 5-Fluorouracil combined with other chemotherapeutic drugs, such as oxaliplatin and irinotecan, has been the first line chemotherapy for CRC. However, nonselective toxicity for normal cells and acquired drug resistance remain critical challenges of chemotherapy (van der Stok *et al.*, 2017). Therefore, several strategies have been investigated to improve the use of anticancer agents for CRC treatment.

Epidemiological studies have found that nonsteroidal anti-inflammatory drugs (NSAIDs) can decrease CRC-related mortality (Hua *et al.*, 2017). NSAIDs inhibit activities of cyclooxygenases (COXs), including COX-1 and COX-2

isoforms. COX-2 is overexpressed in many types of cancers, including CRC. Overexpression of COX-2 is found in approximately 80% of colon carcinoma (Wu *et al.*, 2003). Prostaglandin E₂ (PGE₂), a major product of COX-2 catalysis, plays important roles in tumorigenesis and cancer progression by increasing cell survival, proliferation and invasion, stimulating angiogenesis, and inhibiting apoptosis (Greenhough *et al.*, 2009; Sobolewski *et al.*, 2010). Cancer cells overexpressing COX-2 are resistant to many chemotherapeutic agents such as cisplatin, 5-fluorouracil, and paclitaxel (Choi *et al.*, 2011; Ferrandina *et al.*, 2006; Saikawa *et al.*, 2004). NADPH oxidases (NOXs) are major enzymes generating superoxide anion (O₂⁻), which rapidly converts to H₂O₂. NOX1-dependent ROS is produced during the proliferation and migration of colon cancer cells (Sadok *et al.*, 2008). Celecoxib (CLX; Fig. 1), a selective COX-2 inhibitor, is approved by the U.S. Food and Drug Agency to treat patients with familial adenomatous polyposis to prevent colon cancer (Kraus *et al.*, 2013). CLX also demonstrates potent anticancer activity in various cancer cells such as lymphoma, leukemia, breast cancer, pancreatic cancer, and CRC (Grosch *et al.*, 2001; Wang *et al.*, 2017; Wun *et al.*, 2004; Zhang *et al.*, 2006; Zuo *et al.*, 2018). However, long-term use of high-dose CLX can increase the risk of cardiovascular side effects (Kraus *et al.*, 2013). Therefore,

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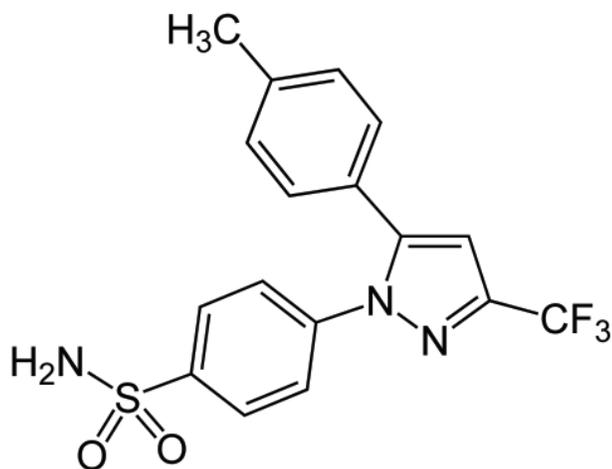


Figure 1. The chemical structure of celecoxib.

an approach of using compounds that have both anticancer and anti-inflammatory activities in combination with CLX for colon cancer treatment is highly promising because these compounds may augment cytotoxicity and decrease side effects associated with CLX.

Cepharanthine (CEP; Fig. 2), a biscoclaurine alkaloid isolated from the root of *Stephania cepharantha* Hayata, is approved by Japanese Ministry of Health for treatment of alopecia areata, leukopenia, and idiopathic thrombocytopenic purpura (Rogosnitzky *et al.*, 2011). Moreover, it demonstrates many pharmacological activities, including antimalarial, anti-HIV-1, antiallergic, anti-inflammatory, and antitumor activities. CEP exerts cytotoxicity against many types of cancer cells by inducing cell cycle arrest and apoptosis, activating ROS production, and downregulating COX-2 expression (Biswas *et al.*, 2006; Chen *et al.*, 2012; Fang *et al.*, 2013; Hua *et al.*, 2015). It may also augment the anticancer activities of several chemotherapeutic agents, such as doxorubicin and paclitaxel (Nakajima *et al.*, 2004; Zhou *et al.*, 2009). Therefore, the present study aimed to determine the combined effects of CEP and CLX and their underlying mechanism(s) in CRC cells.

MATERIALS AND METHODS

Chemicals

CEP was obtained from Abcam. CLX, resazurin, and trypan blue were obtained from Sigma-Aldrich. Other chemicals used were of analytical grade or higher.

Cell culture

Authenticated HT-29 human CRC cells were obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin at 37°C under a humidified atmosphere of 5% CO₂. For cell viability assays, HT-29 cells at low passage were seeded into 96-well plates at 5x10⁴ cells/ml. For all other assays, the cells were seeded into 6-well plates at 5x10⁴ cells/ml.

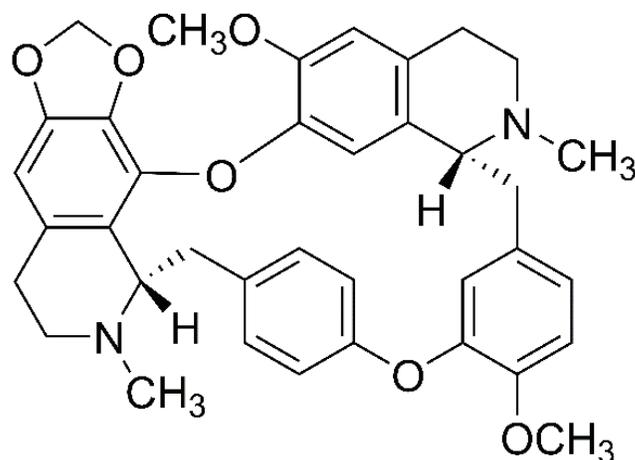


Figure 2. The chemical structure of cepharanthine.

Cell viability assay

HT-29 cells were treated with CLX at concentrations of 2.5, 5, 10, 20, and 50 μM, CEP at concentrations of 1.25, 2.5, 5, 10, and 20 μM, or 0.2% dimethyl sulfoxide (DMSO; vehicle control) for 48 hours. Five hours before the end of incubation, 15 ml of 0.05 mg/μl resazurin solution was added into each well. The absorbance of the well contents was measured at 570 and 600 nm using a microplate reading spectrophotometer. IC₅₀ values were calculated using GraphPad Prism 7 software.

Drug interaction analysis

We assessed the interaction of CLX and CEP using a combination index (CI). HT-29 cells were treated with 5, 10, 20, or 40 μM CLX, 1.25 or 2.5 μM CEP, or their combinations for 48 hours. Viability of the treated cells was measured using a resazurin assay. The CI was determined according to the method described by Chou–Talalay (2010) using the following formula

$$CI = \frac{(D_{\text{combination}1})_1}{(D_{\text{alone}1})} + \frac{(D_{\text{combination}2})_2}{(D_{\text{alone}2})}$$

$D_{\text{alone}1}$ and $D_{\text{alone}2}$: IC₃₀ of the first drug and second drug, respectively.

$D_{\text{combination}1}$ and $D_{\text{combination}2}$: The concentrations of the first drug and the second drug that gives 30% of cell inhibition, respectively.

CI values were interpreted as follows: CI = 1 was considered to indicate an additive effect, CI < 1 synergism, and CI > 1 antagonism.

Reactive oxygen species assay

The level of intracellular reactive oxygen species (ROS) in HT-29 cells was measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). After incubating the cells overnight, they were washed with PBS and treated with 100 μl DCFH-DA in Hank's balanced salts solution for 30 minutes. DCFH-DA was removed and cells were treated with 20 or 40 μM CLX, 1.25 or 2.5 μM CEP, or their combinations for 1 hour. The cells were then lysed with 1% Triton X-100 in 0.3 M NaOH. Subsequently,

cell lysates were transferred into 96-well black flat-bottomed polystyrene plates. The fluorescence intensity of the well contents was measured at 485 and 570 nm using a microplate reading fluorescence spectrophotometer.

ELISA for prostaglandin-E2

After incubating HT-29 cells overnight, they were treated with 20 or 40 μM CLX, 1.25 or 2.5 μM CEP, or their combinations for 24 hours. Supernatants were collected and stored at -20°C until they were assayed for prostaglandin-E2 (PGE₂). PGE₂ levels in the cells were measured using a Prostaglandin E2 Human ELISA Kit (Invitrogen–Thermo Scientific) according to the manufacturer's instructions.

Flow cytometric analysis of cell cycle stage

After incubating HT-29 cells overnight, they were treated with 20 or 40 μM CLX, 1.25 or 2.5 μM CEP, or their combinations for 48 hours. Cells were collected after trypsinization, washed with PBS, and fixed with 70% ethanol at -20°C for 20 minutes. Cells were then washed with PBS, resuspended in 500 μl PBS, and incubated with 20 μg RNase at room temperature for 1 hour. Subsequently, cells were stained with 5 μl propidium iodide (Thermo Scientific) for 15 minutes. The DNA content of 10,000 cells/sample was determined using a flow cytometer.

Cell apoptosis analysis

After incubating HT-29 cells overnight, they were treated with 20 or 40 μM CLX, 1.25 or 2.5 μM CEP, or their combinations for 24 hours. Cells were collected, washed with PBS, and resuspended in 100 μM assay buffer specified for the Annexin V-FITC Apoptosis Detection Kit (Life Technologies). Briefly, following the protocol specified by the manufacturer, cells were stained with 1 μl fluorescein isothiocyanate-labeled Annexin V for 15 minutes at room temperature followed by 1 μl propidium iodide for 5 minutes on ice. Four populations of stained cells, including viable cells (Annexin V⁻, PI⁻), early apoptotic cells (Annexin V⁺, PI⁻), late apoptotic cells (Annexin V⁺, PI⁺), and necrotic cells (Annexin V⁻, PI⁺) were counted using a flow cytometer.

qPCR analysis

After incubating HT-29 cells overnight, they were treated with 20 or 40 μM CLX, 1.25 or 2.5 μM of CEP, or their combinations for 24 hours. Total RNA was isolated using TRIzol reagent according to manufacturer's instructions (Life Technologies). The RNA was transcribed to cDNA using an ImProm-II Reverse Transcription System (Promega). The genes of interest were amplified using SYBR Green I with specific primers listed in Table 1. Real-time qPCR reactions were conducted using a StepOnePlus Real-Time PCR System (Applied Biosystems) using the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Gene expression was calculated using the $2^{-\Delta\Delta\text{CT}}$ method.

Statistical analysis

The data are presented as mean \pm standard error of the mean (SEM) of three independent experiments. Groups were compared using a one-way analysis of variance (ANOVA)

Table 1. The sequencing primers used for qPCR.

Gene		Sequences
GAPDH	Forward	5'-ATGGCATGGACTGTGGTCATGAGT-3'
	Reverse	5'-AAGGTCGGAGTCAACGGATTGGT-3'
CDKN1A (p21)	Forward	5'-CCTGTCACTGTCTTGTACCCT-3'
	Reverse	5'-GCGTTTGAGTGGTAGAAATCT-3'
CCND1 (Cyclin D1)	Forward	5'-TTGTTGAAGTTGCAAAGTCCCTGG-3'
	Reverse	5'-ATGGTTTCCACTTCGCAGCA-3'
CCNE1 (Cyclin E1)	Forward	5'-TCCTGGATGTTGACTGCCTT-3'
	Reverse	5'-CACCAGTATACCCTGAAACCT-3'
CCNA2 (Cyclin A2)	Forward	5'-CTGCTGCTATGCTGTAGCC-3'
	Reverse	5'-TGTTGGAGCAGCTAAGTCAAAA-3'
PTGS2 (COX-2)	Forward	5'-CCCTGAGCATCTACGGTTTG-3'
	Reverse	5'-TCGCATACTCTGTTGTGTCC-3'
NOX1	Forward	5'-GGTTTACCCTCCAGCAGAA-3'
	Reverse	5'-GGATGCCATTCCAGGAGAGAG-3'
BCL2	Forward	5'-TCATGTGTGTGGAGAGCGTCAA-3'
	Reverse	5'-CTACTGCTTTAGTGAACCTTTTGC-3'
BCL2L1 (Bcl-xL)	Forward	5'-TTGGACAATGGACTGGTTGA-3'
	Reverse	5'-GTAGAGTGGATGGTCAAGT-3'
BAX	Forward	5'-GACGAACTGGACAGTAACATG-3'
	Reverse	5'-AGGAAGTCCAATGTCCAGCC-3'
BAK	Forward	5'-ATGGTCACCTTACCTCTGCAA-3'
	Reverse	5'-TCATAGCGTCGGTTGATGTGC-3'

followed by a Bonferroni post hoc test. Differences with $p < 0.05$ were considered significant.

RESULTS

Celecoxib with cepharanthine displayed synergistic cytotoxicity for HT-29 cells

Initially, we assessed the individual effects of CLX or CEP on the viability of HT-29 CRC cells using a resazurin assay. Treatment with CLX at 40 or 50 μM significantly decreased the viability of HT-29 cells with an $\text{IC}_{50} > 50 \mu\text{M}$ (Fig. 3A). In contrast to CLX, CEP decreased the viability of HT-29 in a concentration-dependent manner with an IC_{50} of $5.22 \pm 0.28 \mu\text{M}$ (Fig. 3B). We then determined the effect of CLX at 5, 10, 20, or 40 μM combined with CEP at 1.25 or 2.5 μM , concentrations less than their IC_{50} s, respectively, on HT-29 cell viability. As shown in Fig 1C, only 40 μM CLX induced cell death significantly compared with the vehicle control. However, treatment with 5, 10, 20, or 40 μM CLX combined with 1.25 μM CEP resulted in significant cell death compared with the vehicle control and each drug alone. In contrast to 1.25 μM CEP, we found that 2.5 μM CEP was cytotoxic for HT-29 cells, but cytotoxicity that enhanced significantly more than that for each drug alone was found after treatment of HT-29 cells with 20 or 40 μM CLX combined with 2.5 μM CEP.

The effect of the CLX–CEP combination on the viability of HT-29 cells was also interpreted as the CI. The IC_{50} values of CLX ($48.18 \pm 1.53 \mu\text{M}$) and CEP ($3.08 \pm 0.22 \mu\text{M}$) were used to calculate the CI for each combination. The results shown in Table 2 indicate the CIs of the combinations were < 1 . Thus, the combinations had a nearly additive effect (CI = 0.964) or from

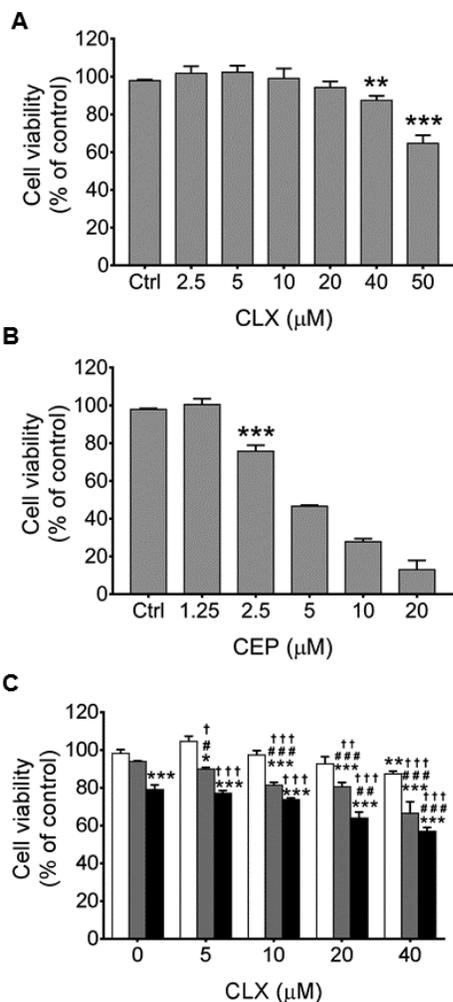


Figure 3. Effects of celecoxib, or cepharanthine, or their combination on HT-29 cell viability. Cells were treated with (A) celecoxib (CLX) at 2.5, 5, 10, 20, 40, or 50 μM , (B) cepharanthine (CEP) at 1.25, 2.5, 5, 10, or 20 μM , or (C) combinations of 5, 10, 20, or 40 μM of CLX alone (unfilled bars) with 1.25 (gray bars) or 2.5 μM (black bars) CEP for 48 h. The viability of the treated cells was assayed using resazurin. The data are presented as mean \pm SEM from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. 0.2% DMSO vehicle control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. CEP alone; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ vs. CLX alone.

moderate to strong synergistic effects (CI range: 0.424–0.777) on the viability of HT-29 cells. Combinations of 20 or 40 μM CLX with 1.25 or 2.5 μM CEP, which had a CI range of 0.424–0.694, were chosen for further investigation.

Effects of celecoxib and cepharanthine combined on COX-2 mRNA levels and PGE₂ production by HT-29 cells

CLX (40 μM) significantly decreased COX-2 mRNA levels in HT-29 cells by approximately 45% compared with the vehicle control (Fig. 4A). In contrast, neither 1.25 μM nor 2.5 μM of CEP affected the level of COX-2 mRNA in HT-29 cells. It is noteworthy that COX-2 mRNA levels in HT-29 cells treated with the drug combinations were no different from those in cells treated with the vehicle control or either drug alone. CLX (20 or

Table 2. Combination index values for CLX–CEP.

CLX	1.25 μM CEP		2.5 μM CEP	
	CI	CI effect	CI	CI effect
5	0.964	Nearly additive	0.777	Moderate synergistic
10	0.720	Moderate synergistic	0.534	Synergistic
20	0.694	Synergistic	0.502	Synergistic
40	0.610	Synergistic	0.424	Synergistic

CI = combination index.

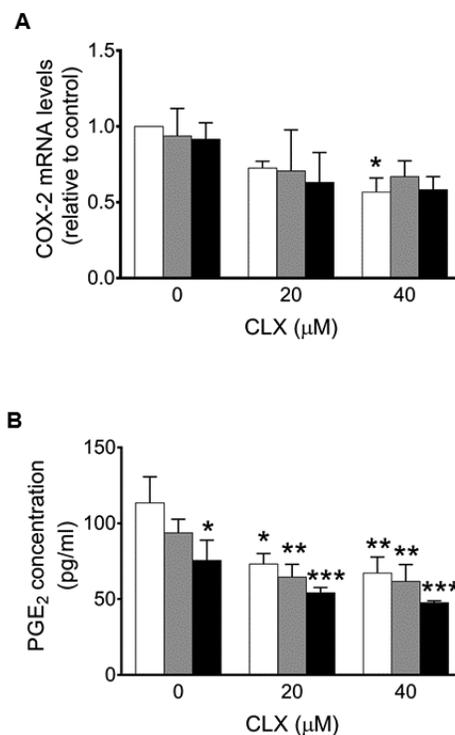


Figure 4. Effects of treatment of HT-29 cells with celecoxib, or cepharanthine, or their combination on COX-2 mRNA levels and PGE₂ production. Cells were treated with 20 or 40 μM of celecoxib (CLX) alone (unfilled bars), or 1.25 μM (gray bars) or 2.5 μM (black bars) of cepharanthine (CEP), or their combination for 24 h. Levels of (A) COX-2 mRNA were determined by qPCR and (B) PGE₂ production by competitive ELISA. The data are presented as mean \pm SEM from three independent experiments. * $P < 0.01$, ** $P < 0.01$, *** $P < 0.001$ vs. 0.2% DMSO vehicle control; ## $P < 0.01$, ### $P < 0.001$ vs. CEP alone.

40 μM) significantly decreased PGE₂ levels in HT-29 cells to 73% and 60% of the level produced after treatment with vehicle control, respectively (Fig. 4B). Similarly, we found that CEP at 2.5 μM significantly decreased PGE₂ production by HT-29 cells compared with the level produced after treatment with vehicle control (Fig. 4B). Although 1.25 μM CEP had no detectable effect on PGE₂ production by HT-29 cells, the combination of 40 μM CLX and 1.25 μM CEP significantly decreased the level of PGE₂ production by the cells compared with the level produced after treatment with CLX alone. In contrast to the low concentration of CEP, 20 or 40 μM CLX combined with 2.5 μM CEP had no detectable effect on PGE₂ production by HT-29 cells compared with the effect of treatment of HT-29 cells with either agent individually.

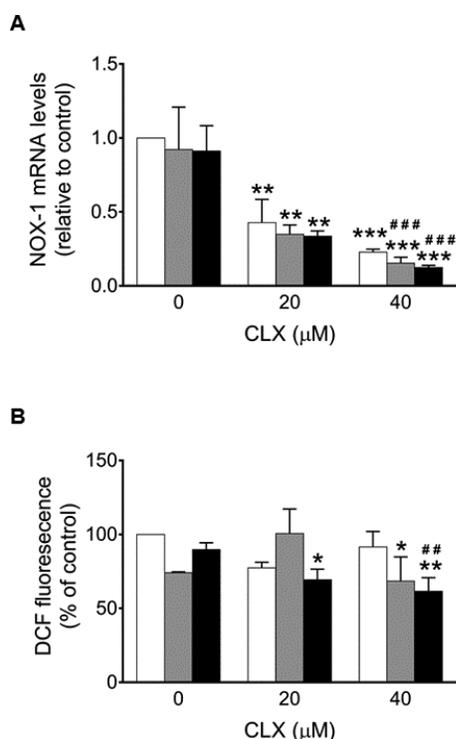


Figure 5. Effects of celecoxib, or cepharanthine, or their combination on NOX-1 mRNA levels and ROS production in HT-29 cells. (A) Cells were treated with 20 or 40 μM celecoxib (CLX) alone (unfilled bars), or 1.25 μM (gray bars) or 2.5 μM (black bars) of cepharanthine (CEP), or their combinations for 24 h. Levels of NOX-1 mRNA were determined by qPCR. (B) Cells were pretreated with 50 μM DCFH-DA for 30 min and treated with 20 or 40 μM CLX, 1.25 μM or 2.5 μM CEP, or their combinations for 1 h. The fluorescence of DCF product was determined using a microplate reading fluorescence spectrophotometer. The data are presented as mean \pm SEM from three independent experiments. * $P < 0.01$, ** $P < 0.01$, *** $P < 0.001$ vs. 0.2% DMSO vehicle control; ### $P < 0.01$, #### $P < 0.001$ vs. CEP alone.

Effects of celecoxib and cepharanthine combined on NOX1 mRNA levels and ROS production by HT-29 cells

CLX dramatically downregulated *NOX1* transcription in a concentration-dependent manner (Fig. 5A). After treatment of HT-29 cells with 20 or 40 μM CLX, cellular NOX1 mRNA levels were decreased by approximately 60% or 80%, respectively, compared with those after treatment with the vehicle control. In contrast, we found that neither 1.25 nor 2.5 μM CEP altered NOX1 mRNA levels (Fig. 5A). Moreover, CEP alone had no effect on CLX-induced *NOX1* downregulation (Fig. 5A). NOX1 mRNA levels in HT-29 cells treated with CLX combined with CEP were not different from those in cells treated with CLX alone. Neither CLX nor CEP altered ROS production by HT-29 cells compared with the vehicle control (Fig. 5B). However, we found that 40 μM CLX combined with 1.25 μM CEP and 20 μM CLX combined with 2.5 μM CEP decreased ROS levels significantly compared with the levels after treatment with the vehicle control. Furthermore, 40 μM CLX combined with 2.5 μM CEP decreased ROS levels significantly compared with the levels after treatment with either the vehicle control or CEP alone (Fig. 5B).

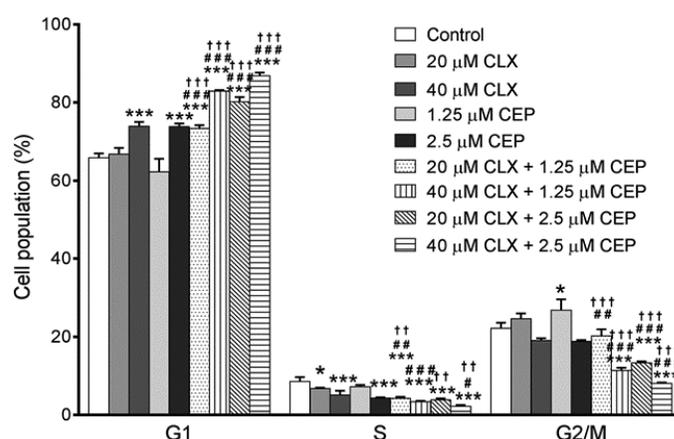


Figure 6. Effects of celecoxib, or cepharanthine, or their combination on cell cycle progression in HT-29 cells. Cells were treated with 20 or 40 μM celecoxib (CLX), 1.25 μM or 2.5 μM cepharanthine (CEP), or their combinations for 48 h. The treated cells were fixed and stained with PI and analyzed using a flow cytometer to determine the percentage of the treated cells in each phase of the cell cycle. The data are presented as mean \pm SEM from three independent experiments. * $P < 0.01$, *** $P < 0.001$ vs. 0.2% DMSO vehicle control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. CEP alone; †† $P < 0.01$, ††† $P < 0.001$ vs. CLX alone.

Celecoxib combined with cepharanthine induced HT-29 cell cycle arrest synergistically

Flow cytometry indicated that treatment of HT-29 cells with 1.25 μM CEP or 40 μM CLX individually caused significant cell accumulation at the G2/M or G1 phase, respectively (Fig. 6). Remarkably, treatment of HT-29 cells with 20 μM or 40 μM CLX combined with 1.25 μM CEP caused significant cell accumulation at the G1 phase compared with that after treatment with either drug alone. In contrast to 1.25 μM CEP, 2.5 μM CEP blocked cell cycle progression at the G1 phase significantly. Accumulation of cells in the G1 phase was also detected after treatment of HT-29 cells with 20 μM CLX combined with 2.5 μM CEP or 40 μM CLX combined with 2.5 μM CEP. It is noteworthy that a significant increase of cells in the G1 phase was associated with a significant reduction of cells in the S and G2/M phases.

Treatment of HT-29 cells with either CLX or CEP alone downregulated cyclin A2 mRNA levels significantly in a concentration-dependent manner (Fig. 7A). Remarkably, treatment of HT-29 cells with 40 μM CLX combined with 1.25 or 2.5 μM CEP downregulated cyclin A2 mRNA levels significantly compared with the levels seen after treatment with either drug alone. In contrast, treatment with the same concentrations of either CLX or CEP alone or their combination did not apparently affect cyclin D1 mRNA levels (Fig. 7B). Although we observed that treatment of HT-29 cells with 20 or 40 μM CLX alone decreased cyclin E mRNA levels significantly compared with levels found after treatment with the vehicle control, there was no synergistic effect of CLX and CEP on cyclin E1 mRNA levels (Fig. 7C). Cyclin E1 mRNA levels in cells treated with CLX alone were not different from those in cells treated with CLX and CEP combined. In contrast to

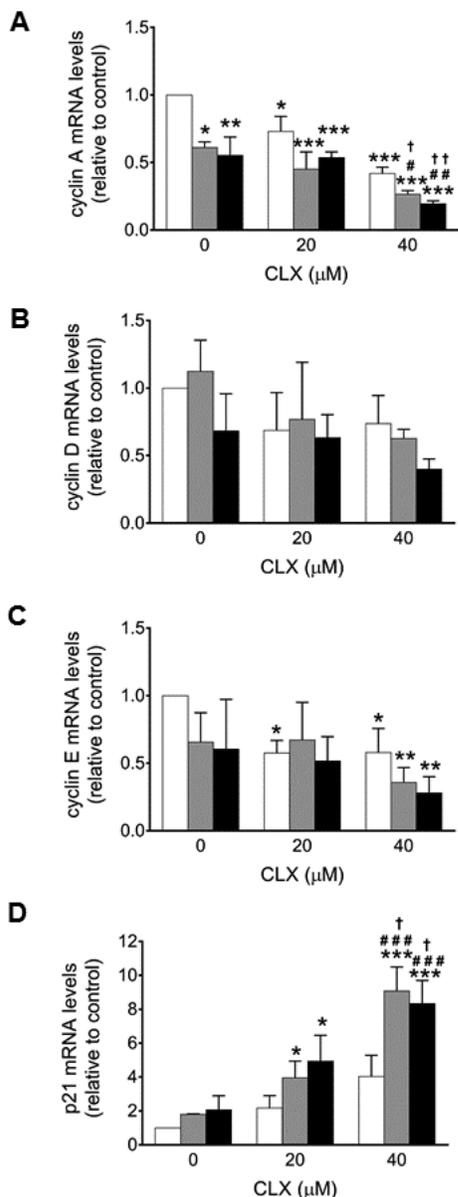


Figure 7. Effects of celecoxib, or cepharanthine, or their combination on mRNA levels of cell cycle regulators in HT-29 cells. Cells were treated with 20 or 40 μM celecoxib (CLX) alone (unfilled bars), or 1.25 μM (gray bars) or 2.5 μM (black bars) of cepharanthine (CEP), or their combinations for 24 h. The levels of mRNA for (A) cyclin A, (B) cyclin D, (C) cyclin E, and (D) p21 in the treated cells was determined using qPCR. The data are presented as mean ± SEM from three independent experiments. * $P < 0.01$, ** $P < 0.01$, *** $P < 0.001$ vs. 0.2% DMSO vehicle control; # $P < 0.05$, ## $P < 0.01$ vs. CEP alone; † $P < 0.05$, †† $P < 0.001$ vs. CLX alone.

cyclins, a synergistic effect of CLX and CEP on increased levels of mRNA for p21 was found (Fig. 7D). Only CLX at 40 μM increased p21 mRNA levels significantly, but all combinations increased p21 mRNA levels. In the presence of 1.25 μM CEP, treatment with either 20 or 40 μM CLX increased p21 mRNA levels by approximately 4 and 9 times, respectively, the levels seen after treatment with vehicle control. In the presence of 2.5 μM CEP, treatment with either 20 or 40 μM CLX also increased p21 mRNA levels significantly. Taken together, these results

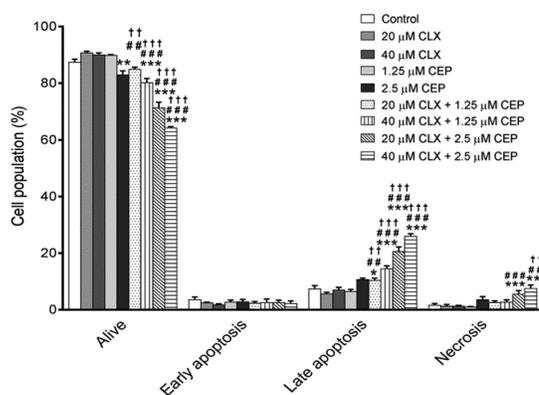


Figure 8. Effects of celecoxib, or cepharanthine, or their combination on apoptosis in HT-29 cells. Cells were treated with 20 or 40 μM celecoxib (CLX), 1.25 μM or 2.5 μM cepharanthine (CEP), or their combinations for 24 h. The treated cells were fixed, and stained with Annexin V-FITC/PI and analyzed using a flow cytometer. The data are presented as mean ± SEM from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. 0.2% DMSO vehicle control; ## $P < 0.01$, ### $P < 0.001$ vs. CEP alone; † $P < 0.01$, †† $P < 0.001$ vs. CLX alone.

suggest that downregulation of *CCNA2*, the gene for cyclin A2, and upregulation of *CDKN1A*, the gene for p21 are targets of the synergistic interaction between CLX and CEP in the induction of cell cycle arrest in HT-29 cells.

Celecoxib and cepharanthine had a synergistic effect on the apoptosis of HT-29 cells

Treatment with 20 or 40 μM CLX alone or 1.25 μM CEP alone did not induce significant apoptosis of HT-29 cells (Fig. 8). However, treatment of the cells with their combinations induced apoptosis significantly compared with treatment with either drug alone. Treatment of HT-29 cells with 20 μM CLX combined with 1.25 μM CEP or 40 μM CLX combined with 1.25 μM CEP increased numbers of late apoptotic cells, respectively, by 10% or 15% of the number induced by treatment with vehicle control. Similarly, treatment of the cells with 20 or 40 μM of CLX combined with 2.5 μM CEP triggered apoptosis significantly compared with apoptosis after treatment with either drug alone. In the presence of 2.5 μM CEP, 20 or 40 μM CLX increased numbers of late apoptotic cells, respectively, by approximately 2 and 2.5 times the number after treatment with the vehicle control. Neither CLX nor CEP treatment of HT-29 cells altered BAX mRNA levels, whereas all combinations increased BAX mRNA levels significantly compared with the expression after treatment with vehicle control (Fig. 9A). Remarkably, treatment with 20 μM CLX combined with 2.5 μM CEP increased BAX mRNA levels by approximately 2.1 and 1.7 times those seen after treatment with the vehicle control and CEP alone, respectively. Similarly, treatment of the cells with 40 μM CLX combined with 2.5 μM CEP increased BAX mRNA levels significantly compared with the levels found after treatment with vehicle control or either drug alone. In contrast to BAX, only treatment of the cells with 20 μM CLX combined with 2.5 μM CEP increased BAK mRNA levels significantly compared with the vehicle control and each drug alone. For antiapoptotic proteins, we found that Bcl-xL mRNA levels were unaffected by treatment with a single drug. However, a significant increase in Bcl-xL mRNA

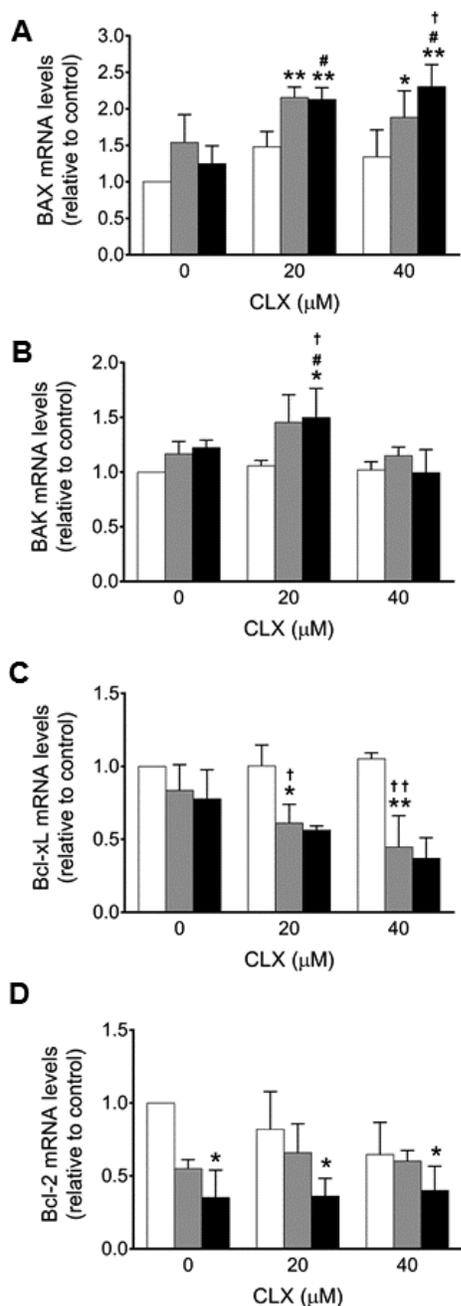


Figure 9. Effects of celecoxib, or ceftarantine, or their combination on mRNA levels of Bcl-2 family proteins in HT-29 cells. Cells were treated with 20 or 40 μM of celecoxib (CLX) alone (unfilled bars), or 1.25 μM (gray bars) or 2.5 μM (black bars) of ceftarantine (CEP), or their combinations for 24 h. The levels of mRNA for (A) BAX, (B) BAK, (C) Bcl-xL, and (D) Bcl-2 in the treated cells was determined by quantitative real time RT-PCR. The data are presented as mean \pm SEM from three independent experiments. * $P < 0.01$, ** $P < 0.01$ vs. 0.2% DMSO vehicle control; # $P < 0.05$ vs. CEP alone; † $P < 0.05$, †† $P < 0.01$ vs. CLX alone.

levels, compared with those found after treatment of HT-29 cells with vehicle control or CLX alone, were detected after treatment of the cells with 20 μM CLX combined with 1.25 μM CEP, 20 μM CLX combined with 2.5 μM CEP, or 40 μM CLX combined with 2.5 μM CEP. Notably, we did not observe synergistic effects of

CLX and CEP on levels of Bcl-2 mRNA. A significant decrease in Bcl-2 mRNA level (65% of that after treatment of HT-29 cells with vehicle control) was detected in cells treated with 2.5 μM CEP, but there was no significant difference in Bcl-2 mRNA levels between cells treated with 2.5 μM CEP, or with 2.5 μM CEP combined with 20 or 40 μM CLX.

DISCUSSION

In the present study, we found that CLX or CEP alone decreased the viability of HT-29 cells with IC_{50} values of >50 and 5.22 μM , respectively. Remarkably, the combination of CLX and CEP, at their sub- IC_{50} concentrations, synergistically had cytotoxic activity on HT-29 cells. The cytotoxic activity of CLX is strongly associated with reduced PGE_2 (Sobolewski *et al.*, 2010) and ROS production (Sullivan *et al.*, 2014). ROS and NOX-1 control COX-2 expression and PGE_2 production (Lin *et al.*, 2011; Shimizu *et al.*, 2015). We found that the drug combinations decreased PGE_2 and ROS production and downregulated *NOX1* transcription in HT-29 cells. Treatment of the cells with CLX alone decreased PGE_2 production and downregulated *NOX1* transcription, but did not have an apparent effect on ROS production. These findings were consistent with the findings of Banskota *et al.* (2015) that CLX did not inhibit tetradecanoylphorbol-13-acetate-induced ROS in HT-29 cells. Although CEP suppressed expression of *PTGS2*, the gene for COX-2, and stimulated ROS production in other studies (Fang *et al.*, 2013; Hua *et al.*, 2015; Rattanawong *et al.*, 2018), CEP did not have any apparent effect on these variables at the concentrations used in the present study. This apparent inconsistency may be a consequence of the different concentrations of CEP used in our experiments. Because we found no synergism between CLX and CEP on *PTGS2* or *NOX-1* transcription, nor PGE_2 or ROS production, it is possible that the synergistic activity of CLX and CEP, at least at their sub- IC_{50} concentrations, maybe COX-2 and ROS-independent. Our present findings are consistent with others that showed CLX to potentiate the cytotoxic effect of cisplatin in vulvar cancer cells, regardless of COX-2 expression (Kim *et al.*, 2009).

The present study found that treatment of HT-29 cells with combinations of 20 or 40 μM of CLX with 1.25 or 2.5 μM of CEP increased cell accumulation at the G1 phase significantly compared with the effect of cell treatment with either CLX or CEP alone. Moreover, our mechanistic studies found that compared with vehicle controls, these combinations decreased significantly the levels of mRNA for cyclin E, which activates CDK2 to form a CDK2-cyclin E complex in the late G1 phase of the cell cycle. Compared with treatment by each drug alone, cell treatment with their combinations also dramatically decreased the levels of mRNA for cyclin A, which activates CDK2 and CDK1 in S phase and G2 phase, and increased the levels of p21 mRNA. Notably, a single treatment of HT-29 cells with CLX alone also increased the levels of p21 mRNA and decreased the levels of mRNA for cyclin E and cyclin A, whereas CEP at sub- IC_{50} concentration only decreased the levels of mRNA for cyclin A. CLX and CEP have been shown to arrest cell cycle progression of not only CRC cells but also several other cancer cell lines. CLX was shown to arrest colon cancer cells at G1 phase (Buecher *et al.*, 2003; Grosch *et al.*, 2001) and chronic myeloid leukemia cells at G1/S phase (Zhang *et al.*, 2006). CLX also upregulated many cyclin-dependent kinase

inhibitors, including p21, p16, and 27, and downregulated cyclin A and cyclin B1 expression in CRC cells (Peng *et al.*, 2004). Similarly, CEP induces cell cycle arrest at G1 phase in adenocarcinoma cells, myeloma cells, and CRC cells by upregulating p21 and downregulating several cyclins (Harada *et al.*, 2001; Kikukawa *et al.*, 2008; Rattanawong *et al.*, 2018). Therefore, more pronounced effects of the combinations on cell accumulation at G1 phase, upregulation of p21, and downregulation of cyclin A2 are likely and may be associated with the synergistic cytotoxic effects of individual drugs on HT-29 cells.

In the present study, we found that at sub-IC₅₀ concentrations, a single treatment with CLX or CEP alone did not have apoptosis-inducing effects on HT-29 cells after 24 hours. However, combinations increased the numbers of late apoptotic cells significantly, suggesting that CLX combined with CEP induced HT-29 apoptosis synergistically. Our present findings demonstrated that combinations increased BAX mRNA levels and decreased Bcl-xL mRNA levels significantly. Furthermore, combinations of 20 or 40 μ M of CLX with 2.5 μ M CEP downregulated transcription of *BCL2* moderately, whereas a combination of 40 μ M CLX with 2.5 μ M CEP upregulated *BAX* transcription significantly compared with the effect of cell treatment with each drug alone. Our results were consistent with demonstrations that CLX and CEP induced the apoptosis of cancer cells by modulating the expression of Bcl-2 protein family members. CLX upregulates BAX expression in breast cancer cells (Kern *et al.*, 2006), whereas CEP upregulates expression of BAX and downregulates expression of Bcl-2 in non-small cell lung cancer cells (Hua *et al.*, 2015). Changes in the levels of cell cycle regulators also induce apoptosis. Binding of p21 with cyclin A/CDK2 complex leads to activation of caspase 3, an executioner caspase in the apoptotic pathway (Gartel *et al.*, 2002). The upregulation of p21 also correlates with induction of expression of the proapoptotic protein BAX. Moreover, the upregulation of p21 is associated with cell cycle arrest and apoptosis in p53-wild type and p53-mutant cancer cells (Vousden *et al.*, 2002). Genistein, an isoflavonoid, induced p53 mutant breast cancer MDA-MB-231 cells to undergo apoptosis by inducing p21 and BAX expression (Li *et al.*, 1999). Thus, it is possible that, in addition to Bcl-2 family proteins, the effects of the drug combination to induce apoptosis in p53 mutant HT-29 cells may also be mediated through modulation of cell cycle regulators. However, it should be noted that only mRNA and not protein expression has been tested in the present study and protein expression may be different to the relative level of mRNA expression for various reasons. Many variables, including mRNA stability, translation rate, and protein degradation influence the steady-state protein concentration (Maier *et al.*, 2009). Investigating the effect of CLX and CEP combinations on protein expression of cell cycle regulators and Bcl-2 family members may add support to the present findings. Moreover, the present study was conducted in just one cell line. Replication of the present study in various cell lines would support more general applicability of our findings.

CONCLUSION

Treatment of HT-29 cells with CLX and CEP combined inhibited cell proliferation synergistically via cell cycle arrest and apoptosis. The synergism of the drugs was associated with increases in the level of mRNA for p21 and BAX and decreases

in the levels of mRNA for cyclin A2 and Bcl-xL. Our present findings warrant using combinations of CLX and CEP to determine potential synergistic antitumor effects in animal models, which might suggest a combination is a promising approach to CRC treatment in humans.

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CONFLICT OF INTEREST

None of the authors declare any potential conflict of interest.

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