INTRODUCTION

Colorectal cancer (CRC) 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 µM in combination with CEP at 1.25, 2.5, or 5 µM displayed synergistic cytotoxic effects with a combination index <1. Combinations of 20 or 40 µM of CLX with 1.25 or 2.5 µM 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.

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

Parawee Lerdwanangkun¹, Piyanuch Wonganan¹, Robin James Storer², Wacharee Limpanasithikul¹*¹

¹Department of Pharmacology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.
²Office of Research Affairs, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

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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 µM in combination with CEP at 1.25, 2.5, or 5 µM displayed synergistic cytotoxic effects with a combination index <1. Combinations of 20 or 40 µM of CLX with 1.25 or 2.5 µM 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.

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 E₉ (PGE₉), 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 (Greenough 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,
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 5x10⁵ cells/ml.

**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:

\[
CI = \frac{D_{\text{combination}} \cdot D_{\text{alone2}}}{D_{\text{alone1}} \cdot D_{\text{combination2}}}
\]

Where:
- \(D_{\text{alone1}}\): IC₅₀ of the first drug.
- \(D_{\text{alone2}}\): IC₅₀ of the second drug.
- \(D_{\text{combination1}}\): The concentration of the first drug that gives 30% of cell inhibition.
- \(D_{\text{combination2}}\): The concentration of the second drug that gives 30% of cell inhibition.

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 µl 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-isolated 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 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⁻ΔΔCT method.

Statistical analysis

The data are presented as mean ± standard error of the mean (SEM) of three independent experiments. Groups were compared using a one-way analysis of variance (ANOVA) 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 IC₅₀ > 50 µM (Fig. 3A). In contrast to CLX, CEP decreased the viability of HT-29 in a concentration-dependent manner with an IC₅₀ of 5.22 ± 0.28 µM (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 IC₅₀, 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 IC₅₀ values of CLX (48.18 ± 1.53 µM) and CEP (3.08 ± 0.22 µM) 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
</tr>
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<tbody>
<tr>
<td>GAPDH Forward</td>
<td>5'-ATGCCATGGACTGTTGCTATAGT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-AAGGTGACACTTACGGTGTGT-3'</td>
</tr>
<tr>
<td>CKD1 (p21) Forward</td>
<td>5'-CCTGCGACCTGTCCTGTTG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GGGTTTGGATGTTGTTGTT-3'</td>
</tr>
<tr>
<td>CCND1 (Cyclin D1) Forward</td>
<td>5'-TTGGTGGAGTGGTCGACACC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-ATGCCGAACCTACGGTGTG-3'</td>
</tr>
<tr>
<td>CCNE1 (Cyclin E1) Forward</td>
<td>5'-TCCCTGGATGTTGACTGTCG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CCTGGACACCTACGGTGTG-3'</td>
</tr>
<tr>
<td>CCNA2 (Cyclin A2) Forward</td>
<td>5'-TGTTGGAGCAGCTAATGCTA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CCCTGGACACCTACGGTGTG-3'</td>
</tr>
<tr>
<td>PTGS2 (COX-2) Forward</td>
<td>5'-AGGAAGTCCAATGTCCAGCC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TCATGTGTGTGGAGAGCGTCAA-3'</td>
</tr>
<tr>
<td>NOX1 Forward</td>
<td>5'-GCGTTTGGAGTGGTAGAAATCT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GCGTTTGGAGTGGTAGAAATCT-3'</td>
</tr>
<tr>
<td>BCL2 Forward</td>
<td>5'-CCCTGAGCATCTACGGTTTG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CTACTGCTTTAGTGAACCTTT-3'</td>
</tr>
<tr>
<td>BCL2L1 (Bcl-xL) Forward</td>
<td>5'-TTGGGACAATGGACTGTTGGA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GCGTTTGGAGTGGTAGAAATCT-3'</td>
</tr>
<tr>
<td>BAX Forward</td>
<td>5'-AGGAAGTCCAATGTCCAGCC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TCATGGATGGACTTACGGT-3'</td>
</tr>
<tr>
<td>BAK Forward</td>
<td>5'-ATGCTGACCACTTACGGTGTG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TCATGGATGGACTTACGGT-3'</td>
</tr>
</tbody>
</table>

Table 1. The sequencing primers used for qPCR.
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 PGE$_2$ 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 40 µM) significantly decreased PGE$_2$ 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 PGE$_2$ 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 PGE$_2$ production by HT-29 cells, the combination of 40 µM CLX and 1.25 µM CEP significantly decreased the level of PGE$_2$ 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 PGE$_2$ production by HT-29 cells compared with the effect of treatment of HT-29 cells with either agent individually.

<table>
<thead>
<tr>
<th>CLX</th>
<th>1.25 µM CEP</th>
<th>2.5 µM CEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI</td>
<td>CI effect</td>
<td>CI effect</td>
</tr>
<tr>
<td>5</td>
<td>0.964</td>
<td>Nearly additive</td>
</tr>
<tr>
<td>10</td>
<td>0.720</td>
<td>Moderate synergistic</td>
</tr>
<tr>
<td>20</td>
<td>0.694</td>
<td>Synergistic</td>
</tr>
<tr>
<td>40</td>
<td>0.610</td>
<td>Synergistic</td>
</tr>
</tbody>
</table>

CI = combination index.
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).

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
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 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
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 Bel-2 mRNA. A significant decrease in Bel-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 Bel-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 IC50 values of >50 and 5.22 µM, respectively. Remarkably, the combination of CLX and CEP, at their sub-IC50 concentrations, synergistically had cytotoxic activity on HT-29 cells. The cytotoxic activity of CLX is strongly associated with reduced PGE2 (Sobolewski et al., 2010) and ROS production (Sullivan et al., 2014). ROS and NOX-1 control COX-2 expression and PGE2 production (Lin et al., 2011; Shimizu et al., 2015). We found that the drug combinations decreased PGE2 and ROS production and downregulated NOX1 transcription in HT-29 cells. Treatment of the cells with CLX alone decreased PGE2 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 PGE2, or ROS production, it is possible that the synergistic activity of CLX and CEP, at least at their sub-IC50 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 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-IC50 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 adenosquamous carcinoma 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 Bel-xL mRNA levels significantly. Furthermore, combinations of 20 or 40 µM of CLX with 2.5 µM CEP downregulated transcription of BCL2 moderately, whereas a combination of 40 µM CLX with 2.5 µM 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 Bel-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 Bel-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 (Vosudden 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 Bel-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 Bel-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 Bel-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.

REFERENCES


