ABSTRACT

Cyclooxygenase (COX-2) has been found to be overexpressed in various tumors including colorectal cancer. Cepharanthine (CEP), a bisoclarine alkaloid isolated from Stephania cepharantha Hayata, exhibits anticancer activity against several different types of cancer including oropharynx cancer, leukemia, hepatocarcinoma and cholangiocarcinoma. In this study, we investigated the anticancer effects of diclofenac, celecoxib, berberine, CEP and 5-fluorouracil against a COX-2 positive human colon cancer cell line, HT-29. Of all compounds tested, CEP was the most effective agents for controlling the growth of the cancer cells. CEP could significantly induce colon cancer cell apoptosis and effectively inhibit COX-2 mRNA expression. These findings demonstrated that CEP could potentially be used as a novel anticancer agent for COX-2-positive colon cancer cells.

บทคัดย่อ

มะเร็งหลายชนิดมีการแสดงออกของ COX-2 ที่มากกว่าปกติจากการศึกษาที่พบว่า cepharanthine (CEP) ซึ่งเป็นสารในกลุ่ม bisoclarine alkaloid ที่พบได้ในรากของต้น Stephania cepharantha Hayata มีฤทธิ์ต้านมะเร็งหลายชนิดเช่น มะเร็งช่องปากและลำคอ มะเร็งเม็ดเลือดขาว มะเร็งตับ และมะเร็งท่อน้ำดี วัตถุประสงค์ของการทดลองนี้เพื่อศึกษาฤทธิ์ต้านมะเร็งของ diclofenac, celecoxib, berberine, CEP และ 5-fluorouracil ต่อเซลล์ HT-29 ซึ่งเป็นเซลล์มะเร็งลูกจูงเป็นเซลล์มะเร็งลูกจูงที่มีการแสดงออกของ COX-2 จากผลการทดลองแสดงให้เห็นว่า CEP สามารถยับยั้งการเจริญของเซลล์มะเร็งได้ดีกว่า diclofenac, celecoxib, berberine, และ 5-fluorouracil โดย CEP สามารถชักนำให้เซลล์มะเร็งตายแบบอะพอพโทซิสและสามารถยับยั้งการแสดงออกของยีน COX-2 การทดลองแสดงให้เห็นว่า CEP อาจจะเป็นยาที่มีประสิทธิภาพในการรักษาโรคมะเร็งลูกจูงเป็นเซลล์มะเร็งลูกจูงที่มีการแสดงออกของ COX-2.

Key Words: Cepharanthine (CEP), Apoptosis, Colon cancer

คำสำคัญ: เซฟราเรนทีน อะพอพโทซิส มะเร็งลำไส้ใหญ่
Introduction

Colorectal cancer is one of the most common cancers in both man and woman (Ferlay et al., 2012). The majority of colorectal carcinomas are adenocarcinomas, which originate from the epithelial cells of the colorectal mucosa. The most common treatment for colorectal cancer is surgical resection, followed by adjuvant therapy with 5-fluorouracil, oxaliplatin, leucovorin or radiation. Although chemotherapy has been widely used, its use often limited due to drug resistance and serious side effects. Therefore, a novel compound that has potent anticancer activity and minimal side effects is urgently needed. Evidence has shown that patients with inflammatory bowel diseases including ulcerative colitis (UC) and Crohn’s disease (CD) have a significant higher risk of developing colorectal cancer (Askling et al., 2001).

Cyclooxygenase (COX) enzyme has played a key role in the biosynthesis of prostaglandins and thromboxane from arachidonic acid. There are two major COX isoforms, including COX-1 and COX-2. COX-1 is constitutively expressed in many tissues and plays an important role in tissue homeostasis, while COX-2 is induced by inflammatory stimuli and involved in pathological processes (Wang, Dubois, 2011). COX-2 overexpression has been detected in 84.9% of colon carcinoma and 57.9% of adenomas, suggesting a critical role of COX-2 in colorectal cancer development (Wu et al., 2003). Several studies have found that COX-2 overexpression could increase accumulation of prostaglandins (PGs) particularly, PGE₂ which promotes colorectal tumor development by stimulating angiogenesis, cell proliferation and apoptosis evasion. Previous study showed that HT-29 cells overexpressing COX-2 were resistant to apoptotic cell death (Limami et al., 2011).

Epidemiological studies have been suggested that long-term treatment with aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs), or selective cyclooxygenase 2 (COX-2) inhibitors may reduce the risk of colorectal cancers (Giovannucci et al., 1994), suggesting that COX-2 is an important target in the treatment of colorectal cancer.

Cepharanthine (CEP), a natural compound isolated from Stephania cepharantha Hayata possess many pharmacological effects such as anti-inflammation, anti-retrovirus, anti-oxidant and anticancer (Rogosnitzky, Danks, 2011). CEP could inhibit production of pro-inflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6) and nitric oxide via suppressing NF-κB (Kudo et al., 2011). Many studies also showed that CEP has anticancer activity against several types of cancer such as oropharynx cancer, leukemia, hepatocarcinoma and cholangiocarcinoma (Furusawa et al., 1998; Wu et al., 2001; Seubwai et al., 2010). It has been reported that CEP inhibit tumor growth through multiple mechanisms, including increasing host immune response (Ebina, 1990), inducing cancer cell to undergo apoptosis (Harada et al., 2001; Biswas et al., 2006.) and stimulating cell cycle arrest (Chen et al., 2012). Although there were reports regarding to cytotoxicity of CEP against several tumors, the anticancer activity of this compound has not been evaluated in COX-2- positive colon cancer cells.

Objective of the study

The aim of this study were to determine anticancer effect of diclofenac, celecoxib, berberine, cepharanthine, and 5-fluorouracil against COX-2-
positive HT-29 human colon cancer cells and investigate the mechanism(s) underlying the anticancer effect of cepharanthine.

Methodology

Cell culture

Human colorectal cancer cell line HT-29 was obtained from American Type Culture Collection (ATCC) (Rockville, MD). The cells were maintained in complete Dulbecco’s modified Eagle’s medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO2 incubator.

Cytotoxicity assay

Cell viability was evaluated by the ability of mitochondrial reductase enzyme in living cells to reduce resazurin into resorufin (Vega-Avila, Pugsley, 2011). Cells were seeded in a 96-well plate at a density 5x10^3 cell/well and incubate overnight at 37°C, 5% CO2. Cells were then treated with berberine, celecoxib, cepharanthine, diclofenac, 5-fluorouracil or 0.2% DMSO (vehicle control) in complete DMEM medium at a concentration of 0.01, 0.1, 1, 10, 50 or 100 μM for 48 hours. Then, 15 μL of resazurin solution (0.05 mg/ml) was added to each well and incubated at 37°C for another 5 hours. The colorimetric was quantified by measuring the absorbance at 570 and 600 nm using a microplate reader (Thermo). The percent of cell viability was calculated using the following equation: (Abs. sample/Abs. control) x100. The values of half inhibitory concentration (IC50) were calculated using the fitted line by GraphPad prism software.

Quantitative real time PCR

HT-29 cells were seeded in a 6-well plate at a density of 5 x10^3 cell/well and incubated overnight. The culture medium was then replaced with fresh complete medium containing 2.5, 5, 10 or 20 μM CEP and incubated for 6 or 24 additional hours. At the end of treatment, the total RNA was extracted using TRizol (Invitrogen, USA) and mRNA was reversed transcribed using Improm-II™ Reverse Transcription system (Promega, USA) according to the manufacturer’s instructions. Real-time PCR was carried out using SYBR Green qPCR super mix universal (Invitrogen, USA) with the following primers specific for COX-2.: 5’-CCCTG AGCATCTACGGTTTG-3’ (forward), 5’-TCGCATACTCTGTGTGTCC-3’ (reverse) and for GAPDH: 5’-AAGGTCGGAGTCAACGGATTTGGT-3’ (forward) and 5’-ATGGCATGGACTGTGGTCATGAGT-3’ (reverse). GAPDH was used as an internal control. DNA amplifications were carried out using a StepOnePlus™ Real-Time PCR with the following cycling conditions: 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 30s. The fold change in COX-2 gene expression after CEP treatment normalized to GAPDH and relative to the expression in vehicle treatment was calculated using the 2^ΔΔCT method.

Apoptosis assay

HT-29 cells were seeded in a 6-well plate at density of 3x10^5 cell/well and incubated overnight. The cells were then treated with CEP at different concentrations (2.5, 5, 10 or 20 μM) in culture sample/Abs. control) x100. The values of half inhibitory concentration (IC50) were calculated using the fitted line by GraphPad prism software.
were then stained with 1 μl Annexin V FITC (Invitrogen, USA) and 1 μl of 0.05 μg/ml PI (Santa Cruz Biotechnology, USA) for 15 min at room temperature in dark. The stained cells were analyzed using flow cytometry (BD LSR II, Biosciences). Four populations of cells can be distinguished, including viable cells (annexin V-, PI-), early apoptotic cells (annexin V+, PI-), late apoptotic cells (annexin V+, PI+) and necrotic cell (annexin V-, PI+), which are located in the lower left, lower right, upper right, and upper left quadrants of the cytograms, respectively.

Statistical analysis

All data are presented as mean ± standard error of mean (SEM). Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by LSD post hoc test using SPSS statistics 21 software. Difference is considered significant if \( P \leq 0.05 \).

Results

Effect of cepharanthine on the COX-2 mRNA expression in HT-29 cells

COX-2 has been found to be overexpressed in various tumors including colorectal cancer (Eberhart et al., 1994). Several studies have shown that cepharanthine (CEP) possess anti-inflammatory activity by inhibiting production of pro-inflammatory cytokines (tumor necrosis factor (TNF)-\( \alpha \), interleukin (IL)-1\( \beta \), IL-6) and nitric oxide (Okamoto et al., 2001; Murakami et al., 2000). Moreover, it was recently reported that inhibition of COX-2 expression is involved in radio sensitization of CEP in cervical adenocarcinoma cell line (Fang et al., 2013). Therefore, the effect of CEP on COX-2 mRNA expression in HT-29 cells was evaluated in this study. We initially determined the basal level of COX-2 mRNA in two human colon cancer cell line, HT-29 and Colo-205 cells. As shown in Figure 1A, COX-2 mRNA was detected only in HT-29 cells. Quantitative real time PCR analysis showed that COX-2 mRNA level in HT-29 cells was 2000 fold higher than that in Colo-205. Treatment with 5 or 10 μM CEP significantly decreased COX-2 mRNA expression level only at 6 h after incubation (p<0.05) (Figure 1B). We however did not observe any change in COX-2 mRNA level after treatment with CEP at 24 h after incubation (Figure 1B).
Effect of CEP on COX-2 mRNA expression in HT-29 cells.

Basal level of COX-2 mRNA in HT-29 and Colo-205 were determined using RT-PCR. B) COX-2 mRNA expression in HT-29 cells after treatment with CEP at 2.5, 5, 10 and 20 μM CEP for 6 and 24 h.* P<0.05, **P < 0.01 with respect to vehicle control (0.2% DMSO).

Effect of dilofenac, celecoxib, berberine, cepharanthine, and 5-Fluorouracil on cell viability of HT-29 cells

We then evaluated anticancer effects of diclofenac (a NSAID), celecoxib (a selective COX-2 inhibitor), berberine and CEP (alkaloids exhibiting both anti-inflammatory and anticancer activities) and 5-fluorouracil (5-FU) (an anticancer drug) against COX-2-positive HT-29 cells using resazurin assay. At 48 h of treatment, diclofenac at 50 and 100 μg/ml and celecoxib and berberine at 50 and 100 μM significantly inhibited the growth of HT-29 cells (Figure 2A-C) while the cytotoxic effects of CEP and 5-FU were detected at 10-100 μM in a concentration-dependent manner (p< 0.05)(Figure 2D and E). The IC50 value of diclofenac was 71.88 ± 5.04 μg/ml while they were 73.39± 3.58, 6.21± 1.68, and 54.73± 3.86 μM for celecoxib, CEP and 5-FU, respectively, suggesting that CEP exhibits the potent anticancer effect against COX-2-positive human colon cancer cells.

Effect of cepharanthine on apoptosis induction of HT-29 cells

Most of the currently chemotherapeutic drugs induce cancer cells to undergo apoptosis. We therefore examined the apoptosis induction effect of cepharanthine in HT-29 cells using annexin V-FITC and PI double staining. At 24 h after incubation, CEP at 2.5, 5, 10 or 20 μM could significantly induce apoptotic cell death in a concentration-dependent manner (Figure 3). Treatment of 20 μM of CEP reduced cell viability about 3 times with respect to the vehicle control group. The number of viable cells were 94.54±1.43% for the vehicle control group and 35.95±5.72% for the 20 μM CEP group. It however should be noted that the percentage of late apoptotic cells are greater than early apoptotic cells. Treatment of CEP at 5, 10 and 20 μM could induce cells to undergo late apoptotic about 11.25±2.66, 30.47±1.68 and 30.36±2.80%, respectively. These results suggest that CEP effectively induced COX-2-positive HT-29 cell death via apoptosis induction.
Figure 2  Cytotoxic effect of diclofenac, celecoxib, berberine, cepharanthine and 5-fluorouracil against COX2-positive HT-29 human colon cancer cells. The cells were treated with 0.01, 0.1, 1, 10, 50 and 100 \(\mu\)M or \(\mu\)g/ml (for diclofenac) for 48 h. Cell viability was determined using resazurin assay. Each value is expressed as the mean ± SEM. \(n=3\). ***\(P< 0.001\) with respect to vehicle control (0.2% DMSO). Data are representative of three independent experiments.

Discussion

It has been that cyclooxygenase (COX)-2 and its product, PGE\(_2\), are involved in cell proliferation, angiogenesis, and inflammation, leading to development of many cancers including colorectal cancer (Sinicrope, Gill, 2004). It has been shown that COX-2 mRNA is over-expressed in almost 80% of the colorectal tumors, compared with normal colorectal mucosa (Roelofs et al., 2014). Previous study showed that HT-29 cells overexpressing COX-2 were resistant to apoptotic cell death (Limaniet al., 2011). In the present study, we therefore investigated the effect of cepharanthine (CEP) on COX-2 expression in HT-29, a COX-2 positive human colon cancer cell line. CEP could significantly inhibit COX-2 mRNA expression (Figure 1). We then evaluated the cytotoxic effects of diclofenac, celecoxib, as well as berberine and CEP against the HT-29 cells. The cytotoxicity of 5-FU, a commonly chemotherapeutic drug used for colon cancer, was also tested. We found that all test agents could effectively inhibit the growth of HT-29 cell in a concentration dependent manner, however, with different potencies (Figure 2). Of five compounds tested, CEP was the most effective anticancer agents against HT-29 cells.
Figure 3 Apoptotic induction effect of CEP in HT-29 cells. A) Representative cytograms of cell apoptosis analysis of HT-29 cells after treatment with CEP (2.5-20 μM) for 24 h. B) The percentage of different HT-29 cell populations (alive, early and late apoptotic cells) after treatment with CEP. Each value is expressed as mean ± SEM. (n=3). **P<0.01, ***P<0.001 compared to the control late apoptotic cells, ###P<0.001 compared to the control viable cells.

The IC₅₀ value of CEP was approximately 6 μM which was much more potent than 5-FU, the anticancer agent commonly used for colon cancer. Previously, the anticancer activities of diclofenac and celecoxib, at high doses, were also observed in colon cancer cells (Choa et al., 2005; Swamy et al., 2003). Mechanistic studies indicated that diclofenac exhibited its tumor suppression effect in HCT-116 cell, a colon cancer cell line, by inhibiting Wnt/β-catenine signaling pathway (Choa et al., 2005) while celecoxib could inhibit COX-2 expression, leading to nuclear accumulation of p53 in HCT-116 and HT-29 cells (Swamy et al., 2003), suggesting different mechanisms underlying anticancer effects of anti-inflammatory drugs. It has been reported that 5-FU and berberine induce cancer cells to undergo apoptosis in a p53-dependent manner (Osaki et al., 1997; Suna et al., 2009). Therefore, it is likely that p53 plays a key role in 5-FU and berberine resistance of HT-29 cells. Besides p53, it was shown that COX-2-derived PGE₂ contributes to acquired resistance of colon cancer cells, SNU-C5, to 5-FU (Choi et al., 2011). Thus, it is possible that overexpression of COX-2 may also be involved in resistance of HT-29 cells to 5-FU and the potent anticancer activity of CEP against the HT-29 cells may be associated with COX-2 down-regulation. Moreover, constitutive up-regulation of COX-2 has been found to be related to apoptosis resistance (Choi et al., 2011).
apoptotic cell death in HT-29 cells (Figure3). Additional studies to determine whether inhibition of COX-2 is related to anticancer effect of CEP are currently under active investigation in our laboratories. CEP has also shown to induce cancer cells to undergo apoptosis through several mechanisms including i) activating caspase-3 and 9 (Harada et al., 2001; Biswas et al., 2006; Seubwai, 2010), ii) stimulating pro-apoptotic signaling pathways such as JNK, ERK and p38 MAPK (Biswas et al., 2006; Harada, 2009), and iii) inhibiting expression of anti-apoptotic gene Bel-xl (Chen et al., 2013). Therefore, mechanisms other than down-regulating of COX-2 may likely be responsible for the anticancer activity of CEP in HT-29 cells as well.

**Conclusion**

The results in the present study clearly demonstrated that cepharanthine (CEP) has a potent anticancer activity against HT-29 cells, a p53-mutant and COX2-positive human colon cancer cell line. Mechanistic studies indicated that CEP effectively induced HT-29 cells to undergo apoptosis as well as inhibited COX-2 expression. These findings suggest that CEP could potentially be used as a novel anticancer agent for p53-mutant or COX-2-positive colon cancer cells which are commonly resistant to currently chemotherapeutic agent.

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