Evodiamine exerts anticancer effects via induction of apoptosis and autophagy and suppresses the migration and invasion of human colon cancer cells

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Summary

Purpose: Colon cancer ranks as the fourth common type of cancer and is responsible for significant morbidity and mortality throughout the world. Late diagnosis and the rarity of potent and safer chemotherapeutic drugs and efficient therapeutic targets create severe obstacle in the treatment of colon cancer. This study was undertaken to examine the anticancer effects of Evodiamine against human colon cancer cells.

Methods: The proliferation rate of the SW480 colon cancer cells was monitored by MTT assay. Apoptosis was detected by Annexin V/propidium iodide (PI) and acridine orange (AO)/ethidium bromide (EB) staining. Transmission electron microscopy (TEM) was used for detection of autophagy. Cell migration and invasion was detected by wound healing and transwell assays, respectively. Protein expression was determined by western blotting.

Results: Evodiamine suppressed the proliferation of the SW480 colon cancer cells and exhibited an IC₅₀ of 10 µM. The cytotoxic effects of Evodiamine were found to be comparatively lower against the normal CDD-18Co colon cells as evidenced from the IC₅₀ of 100 µM. AO/EB staining showed that Evodiamine caused apoptosis of the SW480 cells and the percentage of the apoptotic SW480 cells increased with increase in the Evodiamine concentration as indicated by annexin V/PI staining. Evodiamine-induced apoptosis was also accompanied by upregulation of caspase-3 and Bax and suppression of Bcl-2. TEM analysis showed that Evodiamine also activated autophagy in the SW480 cells by enhancing the expression of LC3 II and Beclin 1. The wound assay showed that Evodiamine suppressed the migration of the SW480 cells. Evodiamine also reduced the invasion potential of the SW480 cells as suggested by the transwell assay.

Conclusion: The findings of the present study suggest that Evodiamine is a potent anticancer agent and may prove beneficial in the development of systemic therapy of colon cancer.

Key words: evodiamine, colon cancer, apoptosis, autophagy, migration

Introduction

Alkaloids constitute one of the largest and diverse groups of plant secondary metabolites. Over the years, alkaloids have attained great attention owing to their potent pharmacological properties. Evodiamine is an important alkaloid that is extracted from several plant species such as Evodia rutaecarpa [2,3]. This molecule has been shown to exhibit diverse bioactivities which also included its potential to stop the growth of cancer cells [4]. Evodiamine has been reported to suppress the growth of prostate cancer, drug-resistant breast cancer and bladder cancer cells to name a few [5-7]. This compound has also been shown to suppress the angiogenesis of hepatocellular carcinoma cells [8] and has been shown to inhibit the proliferation of ovarian cancer cells by arresting the cell cycle.
In human leukemia cells, Evodiamine has been shown to induce apoptosis and autophagy in gastric adenocarcinoma cells [10,11]. In yet another study, Evodiamine has been reported to enhance the sensitivity of the gastric cancer cells to radiotherapy [12]. Although the anticancer activity of Evodiamine has also been evaluated against human colon cancer cells [13], the underlying molecular mechanisms remain largely unknown. This study was therefore designed to investigate the anticancer effects of Evodiamine against human colon cancer cells together with deciphering the underlying mechanisms.

Colon cancer is the fourth prevalent cause of cancer-related mortality. It is ranked as third common type of cancer and around 1.4 million new cases of colon cancer are reported every year [14]. In 2013, around 0.7 million deaths were reported to be due to colon cancer throughout the world [15]. Although, the incidence of colon cancer has declined to some extent, it is believed to increase by 60% till 2030 [16]. Late diagnosis and the rarity of potent and safer chemotherapeutic drugs and efficient therapeutic targets create a severe obstacle in the treatment of colon cancer [17]. Herein, for the first time we report the anticancer effects of Evodiamine against the SW480 colon cancer and also explore the underlying mechanisms.

**Methods**

**MTT cell viability assay**

For assessment of cell viability, the SW480 cells were treated with 0-200 µM of Evodiamine for 24 h and then incubated with MTT (500 µg/mL) for 4 h. Dimethyl sulfoxide (10%) was then added to dissolve the blue formazan crystals formed (cellular oxidoreductase enzymes reduce the tetrazolium dye MTT into its insoluble formazan). Finally, the optical density (OD) was taken at 570 nm and cell viability taken as the percentage of the control.

**AO/EB staining assay**

The SW480 cells (0.6×10^6) were cultured in 6-well plates. Following incubation of around 12 h, the SW480 cells were subjected to treatment with Evodiamine at 0, 5, 10 and 20 µM concentration of Evodiamine for 24 h at 37°C. A 25 µl of cell culture were put onto a glass slide and stained with a 1 µL solution of AO/EB. The slides were cover-slipped and examined under fluorescence microscope.

**Annexin V/PI staining assay**

ApoScan kit was used to determine the apoptotic SW480 cell percentage. In brief, SW480 cells (5×10^4 cells per well) were treated with 0, 5, 10 and 20 µM of Evodiamine for 24 h. This was followed by staining of these cells with annexin V-FITC or PI. The percentage of apoptotic SW480 cells at each concentration was then determined by flow cytometry.

**Transmission electron microscopy (TEM)**

The SW40 cells were treated with 0, 5, 10 and 20 µM of Evodiamine for 24 h at 37°C. The cells were subjected to fixation in a solution of 4% glutaraldehyde 0.05 M sodium cacodylate. The cells were then postfixed in 1.5% OsO4, and dehydrated in alcohol. They were then prepared for flat embedding in Epon 812 and observed using Zeiss CEM 902 electron microscope.

**Wound healing assay**

Briefly, the SW480 cells were treated with 10 µM of Evodiamine for 24 h. Then, Dulbecco’s Modified Eagle’s Medium (DMEM) was removed and cells were washed with phosphate buffered saline (PBS). A sterile pipette tip was employed to scratch a wound in each well, the cells were washed again and a picture was taken. The plates were cultured for 24 h and a picture was taken again with an inverted microscope.

**Transwell assay**

The effects of Evodiamine on the invasion ability of SW480 cell was determined by transwell chambers (8 mm pore size, Corning, NY, USA) with Matrigel. Two hundred ml of SW480 cell culture were placed onto the upper chambers and only DMEM medium was placed in the bottom wells. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded via the chambers were fixed with methanol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200x magnification.

**Figure 1.** A: The structure of Evodiamine. B: MTT assay showing the growth inhibitory effects of Evodiamine on the colon SW480 cells and CCD-18Co normal cells. The experiments were performed in triplicate and shown as mean ± SD (p<0.05).
Western blot analysis

The Evodiamine-treated SW480 cells were harvested with centrifugation. These cells were then lysed in lysis buffer containing protease inhibitor. Around 45 µg of protein from each sample were subjected to separation through electrophoresis via SDS-PAGE gels followed by transferring to polyvinylidene difluoride (PVDF) membranes. Thereafter, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally, the band signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalisation.

Statistics

Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test, using SPSS software package v9.05 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean±standard deviation of 3 independent experiments, and p<0.05 was considered to indicate a statistically significant difference.

Results

Suppression of colon cancer cell growth by Evodiamine

The cell viability assay showed that Evodiamine (Figure 1A) caused a significant decline in the viability of the SW480 cells in a dose-dependent manner. The IC_{50} of Evodiamine was 10 µM against the SW480 cells, indicative of the potent anticancer effects of the compound (Figure 1B). Interestingly, the cell viability assay showed that Evodiamine exhibited 10-fold higher IC_{50} value against the normal CDD-18Co cells with an IC_{50} of 100 µM, suggestive of the cancer cell specific growth inhibitory effects (Figure 1B).

Induction of apoptosis in colon cancer cells by Evodiamine

The AO/EB staining results showed that Evodiamine increased the orange and red colour fluorescent cells, indicative of apoptotic cell death (Figure 2). Annexin V/PI staining showed that the apoptotic cell percentage was 1.20, 8.03, 16.44 and 22.62% at.

Figure 2. Evodiamine induced apoptosis in SW480 cells in a concentration-dependent manner as depicted by AO/EB staining. The experiments were performed in triplicate. Arrows indicate apoptotic cells which emit yellow and orange fluorescence. The number of these cells increases with drug dose.

Figure 3. The percentage of the apoptotic SW480 cells increased with increase in the concentration of Evodiamine as shown by Annexin V/PI staining. The experiments were performed in triplicate. The apoptotic cell percentage increased with increasing Evodiamine dose.

Figure 4. Effect of Evodiamine on the expression of apoptosis marker proteins as depicted by Western blotting. Evodiamine treatment led to decrease in Bcl-2 expression and increase in the expressions of Bax and caspase-3. The experiments were performed in triplicate.
0, 5, 10 and 20 µM concentrations of the compound, indicating an increase in the apoptotic SW480 cells with increase in the concentration of Evodiamine (Figure 3). Western blot analysis showed that Evodiamine increased the expression of caspase-3 and Bax and decreased the expression of Bcl-2, confirming the induction of apoptotic cell death of SW480 cells (Figure 4).

**Induction of autophagy in colon cancer cells by Evodiamine**

TEM analysis showed that Evodiamine caused increase in the autophagic structures in the SW40 cells and these effects were found to be concentration-dependent (Figure 5). Western blot analysis showed that the expression of LC3 II and Beclin 1 in SW480 cells increased upon treatment with Evodiamine. Nonetheless, the expression of p62 and LC3 I was decreased, confirming the induction of autophagy in the SW480 cells (Figure 6).

**Inhibition of cell migration and invasion of the colon cancer cells by Evodiamine**

The inhibitory effects of Evodiamine were also investigated on the migration of the SW480 cells by wound healing assay. The results showed that Evodiamine remarkably decreased the migration of the SW480 cells as evidenced from the scratch width (Figure 7). The transwell assay showed that Evodiamine also suppressed the invasion of the SW480 in a dose-dependent manner (Figure 8).
Discussion

Colon cancer is a devastating disease and its incidence is expected to increase dramatically in the coming years [18]. The clinical outcome is unsatisfactory and treatment strategies have a number of flaws. The currently available chemotherapeutic agents have adverse effects and efficient therapeutic targets are lacking [19]. Consistently, this study was undertaken to investigate the anticancer effects of Evodiamine against a human colon cancer cell line. It was revealed that Evodiamine suppresses the proliferation of the SW480 cells concentration-dependently. Interestingly, the IC\textsubscript{50} of Evodiamine was approximately 10-fold lower than the normal CDD-18Co cells, indicating that this molecule targets cancer cells selectively. Previous studies have also proven the anti-proliferative effects of Evodiamine on several cancer cell types. For example, the proliferation of DU145 and PC3 prostate cancer cell lines was significantly reduced upon Evodiamine treatment [20]. Similarly, Evodiamine has been shown to suppress the growth of the chemo-resistant breast cancer cells [21]. Also, growth inhibitory effects of Evodiamine have also been reported on human lung cancer cells [22].

Next, to ascertain the underlying molecular mechanism for the anticancer activity of Evodiamine, AO/EB staining was performed and it was found that this compound caused apoptotic cell death of the SW480 cells and the percentage of the apoptotic SW480 cells increased with increase in the concentration of Evodiamine as indicated by the annexin V/PI staining. Moreover, Evodiamine also caused upregulation of caspase 3 and Bax and downregulation of Bcl-2. The apoptosis-inducing property of Evodiamine has also been proved by previous investigations wherein Evodiamine has been reported to trigger apoptotic cell death in ovarian cancer cells via activation of the JNK signalling cascade [23]. In another study, Evodiamine has been reported to induce mitochondrial apoptosis in non-small lung cancer cells [24]. The results of TEM studies showed that Evodiamine triggered the development of autophagic structures in the SW480 cells which was also accompanied with up-regulation of LC3 II and Beclin 1 expression and suppression of p62 expression. This observation is in agreement with a previous study wherein Evodiamine has been shown to prompt autophagy in murine Lewis lung carcinoma cells [25]. A previous study has shown that Evodiamine inhibits the migration and invasion of nasopharyngeal cancer cells [26]. Herein, we also observed that Evodiamine remarkably decreased the migration and invasion of the SW948 colon cancer cells.

Conclusion

The findings of the present study showed that Evodiamine suppresses the proliferation of the human colon cancer cells via induction of both apoptosis and autophagy. Moreover, Evodiamine could also suppress the migration and invasion potential of colon cancer cells. Taken together, Evodiamine may prove to be a lead molecule in the development of systemic therapy for colon cancer.

Conflict of interests

The authors declare no conflict of interests.

References


