Berberine and Evodiamine Synergically Exert Anti-colorectal Cancer Effects via P38/MAPK/MAPKAPK2/HSP27 Signaling Pathway

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Abstract

Objective: Combinatorial natural products have high application potential for treatment of complex diseases owing to their synergistic effects and multi-targeting effect. However, studies have not explored the therapeutic effect and the synergetic mechanisms of action combinations of natural products. The present study aimed sought to evaluate the synergistic antitumor effects of a combination of Berberine and Evodiamine, and explore the drug effect on proliferation, migration, invasion of HCT116 and RKO human colorectal cancer cells.

Results: The effect of berberine and evodiamine at a specific paired dose (BER30 μ M, EVO 0.8 μ M) was explored. A combination of berberine and evodiamine had no effect on activity and proliferation of HCT116 and RKO cells. The combination regulates the cell cycle of HCT116 and RKO cells at different cell phases. Berberine mainly blocked the cell cycle at G0/G1 phase, whereas evodiamine induced cell cycle arrest at G2/M phase. The results showed that the combined effect of berberine and evodiamine does not offset each other, but plays a synergistic role in regulation of colon cancer cell cycle. Western blot analysis showed that the combination of berberine and evodiamine regulated cell cycle by downregulating expression of cdc25c and upregulating expression of p21. The combination significantly inhibited cell migration and invasion by regulating EMT related proteins, upregulating expression of E-cadherin and downregulating expression of N-cadherin. The combination of berberine and evodiamine significantly inhibited phosphorylation of P38 MAPK in HCT116 and RKO cells, and further inhibited phosphorylation of the downstream MAPKAPK2 and HSP27, thus playing a synergistic anti-colon cancer role.

Conclusion: Berberine and Evodiamine exhibit synergistic antitumor effects by suppressing cell proliferation, inducing cell cycle arrest and inhibiting EMT by modulating P38MAPK /MAPKAPK2/HSP27 pathway.

Significance of the study: To illustrate the potential mechanism of formula-based combination of natural products, and explore the potential applications of the combination and possible antitumor therapeutic targets.

Introduction

ZuoJinWan (ZJW) is a Chinese medicinal formula comprising two herbal medicine¹, *Coptidis Rhizoma* (CR, the rhizome of *Coptis chinensis* Franch. Huanglian in Chinese) and *Euodiae Fructus* (EF, the fruit of *Euodia rutaecarpa* (Juss.) Benth. Wuzhuyu in Chinese) at a ratio of 6:1 (w/w). The two components of ZJW have therapeutic effects against various tumors^{2,3}. ZJW is a formula-based combination of natural products which is widely used to treat gastrointestinal disease owing to the potential synergistic effects⁴. Moreover, ZJW has been widely explored as a multi-drug therapy in gastrointestinal cancers, such as colorectal cancer, gastric cancer and liver cancer owing to the diverse biological activities and anti-tumor

effect^{5,6,7}. However, the mechanisms of the anti-tumor effects of the formulation have not been elucidated.

The antitumor activity of ZJW is attributed to presence of Evodiamine and Berberine. Evodiamine is a tryptamine indole alkaloid isolated from *Euodiae Fructus*, and is an antitumor drug compound with multiple targets^{8,9,10}. Evodiamine suppresses proliferation, induces apoptosis, and activates autophagy of human colon cancer cells^{11,12,13,14}. Moreover, Evodiamine inhibits proliferation of the stem cell and non-stem-cell populations in human cancer cells by modulating WNT and NOTCH signaling¹⁵. Berberine is an isoquinoline alkaloid isolated from *Coptidis Rhizoma*. It exhibits anticancer activities by suppressing colon cancer cell migration, inhibition of cellular proliferation by inducing cell cycle arrest reversing of EMT process, apoptosis, activation of autophagy, and altering gut microbial balance thus inhibiting inflammation^{16,17,18,19,20}. Furthermore, Berberine interrupts the paracrine Hedgehog signaling pathway activity to inhibit colorectal tumorigenesis *in vivo* and *in vitro*^{21,22}. A multi-center, randomized, controlled and double-blind clinical study explored the effect of Berberine on prevention of recurrent colorectal adenoma in China and the findings showed that berberine was highly effective in reducing the risk of recurrent adenomas.

Combined anticancer effects of Evodiamine and Berberine has been reported in colorectal cancer, gastric cancer, liver cancer, and breast cancer^{23,24,25}. Combination of Berberine and Evodiamine is widely explored as a multi-drug therapy owing to the diverse biological activities and anti-cancer effects^{26,27}. However, the mechanism underlying the synergistic effect of the combination has not been elucidated.

The aim of the present study was to evaluate the therapeutic efficacy of the formula-based combination of traditional Chinese medicine active components and explore the potential mechanism and therapeutic targets of this formulation. The effect of a combination of Berberine and Evodiamine in HCT116 and RKO human colorectal cells were explored *in vitro*. The results indicated that combination of Berberine and Evodiamine induces cell cycle arrest and cell migration by modulating epithelial-mesenchymal transition thus exerting anti-tumor effect. These findings provide a basis for application of natural products and potentially offer clinical benefits by determination of therapeutic targets based on the synergistic effects components of traditional Chinese medicine.

Results

1. Combination of Berberine and Evodiamine synergistically inhibits proliferation of HCT116 and RKO cells

In the present study, cells were treated with or without different concentrations of berberine, evodiamine and cBE for 24h and CCK8 assay was conducted to explore the anti-proliferative effects of berberine As shown in Fig. 1a, evodiamine As shown in Fig. 1b and a combination of berberine and evodiamine(cBE). The results of orthogonal experiment showed that treatment of cells with BER (30μM) or EVO (0.8μM) did

not significantly inhibit cell proliferation. As shown in Fig. 1c and d. However, a combination of the two cBE significantly inhibited viability of HCT116 and RKO cells. As shown in Fig. 1e and f, Cell viability results were analyzed by clone formation experiment to further explore the interaction between cBE and cells. A 10-day colorectal survival analysis (average number of clones calculated three times) showed that treatment of HCT116 and RKO cells with BER (30 μ M) and EVO (0.8 μ M) individually did not inhibit cell proliferation. On the contrary, cBE significantly reduced the number of colons in HCT116 cells and RKO cells. As shown in Fig. 1g and h, This finding indicates that cBE (BER 30 μ M, EVO 0.8 μ M) synergistically inhibits proliferation of human colorectal cells.

2. A combination of berberine and evodiamine induces cell cycle arrest but does not regulate autophagy in HCT116 and RKO cells.

PI staining followed by flow cytometric analysis was performed to determine the proportion of cell cycle phases to explore whether the synergistic effect of BER and EVO on cell viability was by affecting cell cycle distribution. As shown in Fig. 2a, Cells were treated with BER (30 μ M), EVO (0.8 μ M) and cBE (BER 30 μ M, EVO 0.8 μ M) for 24 hours, The results showed that exposure of BER(30 μ M) to the resting or initial cell cycle phase (G0/G1), increased the percentage of cells (from 47.21–51.23%;), and some cells were blocked in G0/G1 phase. In addition, treatment of cells with EVO (0.8 μ M) increased the percentage of G2/M phase cells from 24.48–70.36%, and induced cell cycle arrest at G2/M. As shown in Fig. 2b, Treatment of cells with cBE (BER 30 μ M, EVO 0.8 μ M) showed that the effect of cell cycle arrest was not reduced compared with that of individual drugs, and the two drugs played a synergistic role.

Administration of cBE (BER 30 μ M, EVO 0.8 μ M) synergistically regulated the cell cycle and the effect of the two drugs effect did not overlap. The two drugs were effective in different cell phases, and played a synergistic role. Furthermore, expression levels of proteins related to cell cycle, including CDC25A, CDC25C, P21 and CHK2 were determined by western blot. Treatment of colon cancer cells (HCT116 and RKO) with a combination of berberine and evodiamine significantly decreased the expression level of CDC25C protein whereas the level of p21 protein increased significantly. As shown in Fig. 2c and d.

HCT116 and RKO cells were stained with annexin v-apc dye to explore the effect of the drugs on apoptosis of human colorectal cancer cells. cBE (BER 30 μ M, EVO 0.8 μ M) did not significantly induce apoptosis in HCT116 and RKO cells. As shown in Fig. 2e. This indicates that the synergistic effect of the drugs modulated HCT116 and RKO cell cycle arrest but did not affect autophagy of the cells.

3. A combination of berberine and evodiamine suppresses migration and invasion in HCT116 and RKO cells

The results showed that cBE inhibited the metastasis of HCT116 and RKO cells. This result was confirmed by transwell method whereby administration of BER (30 μ M). EVO (0.8 μ M) individually did not

inhibit cell invasion ability. On the contrary, administration of cBE significantly inhibited invasion ability of HCT116 and RKO cells. As shown in Fig. 3a and b. Migration ability of BER (30 μ M), EVO (0.8 μ M) and cBE (BER 30 μ M, EVO 0.8 μ M) was explored by wound healing experiment. Migration ability of HCT116 and RKO cells was low after treatment with cBE (BER 30 μ M, EVO 0.8 μ M). Treatment of cells with BER (30 μ M) and EVO(0.8 μ M) individually showed that the drugs did not inhibit cell invasion. As shown in Fig. 3c.

Further analysis was conducted to explore whether the combination of Berberine and Evodiamine regulated EMT. Expression level of EMT biomarkers, including E-cadherin and N-cadherin were determined by western blot. The results showed that expression of epithelial biomarker E-cadherin was upregulated and were mesenchymal biomarker N-cadherin expression was downregulated after treatment of cells with cBE (BER 30 μ M, EVO 0.8 μ M) compared treatment of cells with BER (30 μ M), EVO (0.8 μ M) separately. As shown in Fig. 3d. These findings imply that cBE (BER 30 μ M, EVO 0.8 μ M) synergistically restrained migration and invasion of HCT116 and RKO cells through phenotypic reversal of EMT.

4. Underlying mechanism of the synergistic effect of berberine and evodiamine on human colorectal cancer cells

Transcriptome analysis was conducted using HCT116 cells to explore the molecular mechanism of synergistic effect of Berberine and Evodiamine against proliferation, migration and invasion of HCT116 cells. A scatter plot of DEGs showed a significant difference in gene expression level between BER (30 μ M) and EVO (0.8 μ M) individually treated cells compared with the cells treated with a combination of the two drugs ($P < 0.05$). As shown in Fig. 4a. The results showed that cBE treatment was associated with a total of 3722 differentially expressed genes (DEGs) (407 upregulated genes and 3315 downregulated genes). As shown in Fig. 4b. A total of 216 differential genes in the cBE group were related to cell growth, biological adhesion and cell signaling. This finding indicates that the synergistic effect of Berberine and Evodiamine was highly associated with expression of genes implicated in cell proliferation and migration in HCT116 cells.

Further, GO and KEGG enrichment analyses were performed to explore the molecular mechanisms of the drugs. GO analysis included evaluation of biological process, molecular function and cellular component. The first 10 items of each category based on ($P < 0.05$) were selected. As shown in Fig. 4c. Representative subgroups in biological processes included cell stimulation to the outside world, rRNA processing and virus process. Molecular functions enriched after drug treatment included protein binding and poly (a) RNA binding. Enriched cell components included nucleoplasm, membrane and cytoplasm. KEGG pathway analysis showed the top 10 enriched pathways included endoplasmic reticulum protein processing, thyroid hormone signaling pathway, proteoglycan in cancer tissue and vascular endothelial growth factor signaling pathway. As shown in Fig. 4d. Notably, vascular endothelial growth factor signaling pathway was the action pathway with the highest enrichment value.

5. Anti-cancer effects of a combination of Berberine and Evodiamine are mediated through P38MAPK pathway

Expression of key proteins in signaling pathways relevant to tumor cell viability, proliferation and metastasis were evaluated to determine the underlying mechanisms of the anticancer effects of the combination of berberine and evodiamine. The results showed that components of P38MAPK signal pathway were highly enriched. The results showed that a combination of Berberine and Evodiamine promoted phosphorylation of multiple nodes of P38MAPK signal pathway, including p38 MAPK, MAPKAPK2 and HSP27. To further verify that a combination of Berberine and Evodiamine an anti-colon cancer role by targeting P38MAPK/MAPKAPK2/HSP27 expression levels genes related to this pathway including p38MAPK, MAPKAPK2 and HSP27 were determined by QRT PCR. As shown in Fig. 5a. The experimental results showed that expression levels of the genes had a similar trend and amplitude with the results from western blot analysis. Expression levels of MAPKAPK2 and HSP27 proteins in HCT116 and RKO cells treated with Berberine and Evodiamine were significantly lower relative to those treated with Berberine and Evodiamine alone ($P < 0.01$). As shown in Fig. 5b. These results indicate that a combination of berberine and evodiamine plays a synergistic anti-colon cancer role by targeting p38MAPK/MAPKAPK2/HSP27 pathway.

Discussion

Complex components of Traditional Chinese medicine induce multi-targeted therapeutic anti-tumor effects thus they are used for development of formula-based combinations from natural products^{28,29,30,31}. The findings of the current study indicated that a combination of Berberine and Evodiamine significantly inhibited colon cancer cells migration and invasion compared with use of Berberine and Evodiamine treated separately. SB203085, an inhibitor of P38MAPK was administered in combination with Berberine and Evodiamine, to explore viability of the two colon cancer cells. The results showed that cell viability decreased significantly after addition of SB203085 compared with use of berberine and evodiamine separately. This indicates that combination of Berberine and Evodiamine played an anti-colon cancer role through modulation of P38MAPK signal pathway. The findings indicate that combinations of natural products components have great potential in treatment of complex diseases. These findings provide a basis for anti-tumor research and clinical application of traditional Chinese medicine.

The results demonstrated that formulation-based synergy of Berberine and Evodiamine at a specific pair dose (BER 30 μ M, EVO 0.8 μ M) significantly inhibits viability of HCT116 and RKO cells. Moreover, the combination induced anti-proliferation of the cells as shown by clonal formation experiment. Therefore, Berberine should be combined with Evodiamine to achieve the synergistic effect and improve the anti-proliferative and anti-migratory properties against colon cancer cell malignant transformation by

modulating TGF- β 1 expression. The combination regulates differentiation of colon epithelial cells by downregulating miR-42 and suppressing activity of TGF- β /Smad signaling^{32,33}.

Previous studies report that the synergistic anticancer activities of Berberine and Evodiamine are not associated with cell cycle regulation. On the contrary, the findings of the present study indicated that could the combination of the two drugs synergistically regulated HCT116 and RKO cells cycle at different cell phases. Notably, Berberine mainly blocked cell cycle at G0/G1 phase. Further, evodiamine induced cell cycle arrest at G2/M. In addition, the results showed that a combination of Berberine and Evodiamine did not antagonize each other's action effect, and cells inhibited by Berberine in G0/G1 phase were also blocked by Evodiamine at G2/M phase. Notably, the two drugs had no effect on autophagy. The results indicated that the synergistic effect plays a vital role as a "double safe catch" in regulating cell cycle arrest of HCT116 and RKO cells.

Studies report that epithelial-mesenchymal transition (EMT) is an important step in loss of intercellular adhesion and increase in motility of cancer cells thus regulating cancer cell metastasis^{34,35}. Therefore, the effect of combination of Berberine and Evodiamine on EMT was explored in the current study. The results showed that could the combination significantly inhibited cell migration and invasion compared with use of berberine and evodiamine treated individually. Notably, the combination regulated expression of EMT-related proteins. Expression of E-cadherin was upregulated whereas expression of N-cadherin was downregulated. GO and KEGG analyses indicated that vascular endothelial growth factor (VEGF) signaling pathway was highly correlated with the synergistic effect of the two drugs. Previous studies report that P38MAPK pathway is associated with high expression of VEGF ultimately promoting proliferation, migration and invasion of malignant cells. The findings of the present study further demonstrated that the combination inhibited EMT by modulating P38MAPK pathway thus suppressing migration and invasion of HCT116 and RKO cells.

Previous studies report tumor-killing abilities of the two drugs. However, the results in the study the results showed that apoptosis of cells was not promoted by the effective combination concentration, thus the role in promoting apoptosis at other concentrations should be further explored. Moreover, only *in vitro* experiments were conducted in the current study, and *in vivo* experiments were not performed to verify the results. In addition, Berberine can kill other tumor types by promoting apoptosis of tumor cells. Moreover, Evodiamine promotes apoptosis of tumor cells (reported by others). However, a combination of these drugs did not promote apoptosis of cells. Therefore, further studies should explore whether the combination is more effective in promoting apoptosis at other concentrations.

Colorectal cancer (CRC) with a multistep process is a leading cause of high cancer-related morbidity and mortality, with approximately 940,000 deaths reported worldwide in 2020³⁶. It is characterized by complex etiology, processes and therapeutic targets. Although significant advances for prevention and treatment of colon cancer using traditional Chinese medicine have been made, development and application of drugs are mainly limited by unavailability of empirical studies on targets and mechanisms of drug action in formulations such as ZJW. The results of the present study indicated the formula-based

combination of natural products exhibits synergistic antiproliferation, blocking cell cycle effects, and inhibition of EMT process by modulating p38/MAPK/MAPKAPK2/HSP27 signaling pathway. The findings of the current study provide a basis for development of effective therapies based on the synergistic effect of traditional Chinese medicine strategy for treatment of colorectal cancer.

Materials And Methods

Cell lines and cell culture

HCT 116 (Human Colorectal Carcinoma Cells) and RKO (Human Colorectal Adenocarcinoma Cells) cell lines were purchased from Beyotime Institute of Biotechnology, Chengdu, China. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Australia), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, USA) under a humidified atmosphere of 5% CO₂ at 37°C.

Cell viability assay

Viability of colorectal cancer cells (HCT116, RKO) was determined by CCK8 assay. Cells were seeded in 96-well plates at a density of 10⁴ cells per well. Different concentrations of Berberine (0, 10, 20, 30, 35 µM) were added and incubated for 24h, and different concentrations of Evodiamine (0, 0.1, 0.4, 0.8, 1 µM) were added and incubated for 24h. The mixture of the two different concentrations was treated with colon cancer cells to evaluate the mixing index. Cell viability was determined by incubating cells with Cell Counting Kit-8 (CCK-8) working solution (10ul/well) for 1 h. SPECTROMAX M5 microplate reader was then used to measure the absorbance at 450 nm.

Colon formation assay

Cells were seeded in 12-well plates at a density of 2×10³ cells/well and incubated in DMEM containing 10% Fetal Bovine Serum for 24 h. Cells were then resuspended in DMEM with 10% Fetal Bovine Serum, 100 U/ml penicillin and 100 µg/ml streptomycin and cultured in 5% CO₂ at 37°C for 10 days. Cells were centrifuged and the supernatant was washed with PBS then fixed with 4% paraformaldehyde for 15 minutes at room temperature. Cells were washed twice with PBS, then stained with crystal violet solution (0.1%) for 15 min. Relative cell confluence was calculated by Image J software. Each experiment was performed in triplicate.

Flow cytometry

Flow cytometry was performed to detect the cell cycle and apoptosis of colorectal cancer. Cells were seeded in six-well plates at a density of 1×10⁶ cells/well. After incubation for 12 hours, cells were dispersed as single cell suspension by trypsin and fixed using ice-cold 75% (v/v) ethanol overnight. Cells were then washed twice with ice-cold PBS, then stained with 20 µg/ml PI (Beyotime Biotechnology, Chengdu, China) and incubated with 8 µg/ml RNase (Beyotime Biotechnology, Chengdu, China) for 30 min at room temperature under dark conditions.

Apoptosis assay kit (cat. no. BMS500fi-300; Invitrogen.) was used for apoptosis analysis following the manufacturer's protocol. Cell cycle and apoptosis of colorectal cancer cells was then evaluated using Gallios Flow Cytometer (Beckman Coulter). Cell cycle distribution and apoptotic cells were quantified based on the DNA content in the cells and the sub-G1 cell population using Mod Fit LT software (Version 3.0).

Wound healing assay

Cells (1×10^6 cells/well) were seeded in six-well plates. A 200 μ l sterile pipette gun was used to gently scrape the cells from the bottom of the culture dish when the cells grew to 95% confluence. Cells were then washed several times with PBS to remove cell debris, and cultured in serum-free medium containing the drug for 24 hours. Wound healing was observed under an inverted microscope.

Transwell migration and invasion assays

Migration and invasion rates of colorectal cancer cells were determined using Transwell Cell Culture Inserts (Corning, Inc.) according to the manufacturer's instructions. Cells were incubated on the upper part of the chamber at a density of 1×10^4 cells/well in 200 μ l serum-free medium. The bottom part of the chamber was filled with 500 μ l medium containing 10% FBS. Migration assay was conducted with no coating on the bottom of the top chambers, whereas invasion assay was performed with Matrigel coating at the top chambers. Cells were incubated for 48 h at 37°C then fixed with 4% PFA for 10 min and stained with crystal violet for 30 min. The number of cells on the membrane was counted from four randomly selected visual fields under a fluorescence microscope.

RNA-sequencing

Total RNA was extracted from the tissue using TRIzol® reagent according to the manufacturer's instructions (Invitrogen) and genomic DNA was removed from the samples using DNase I (TaKara). RNA quality was determined using 2100 Bioanalyzer (Agilent) and quantified using the ND-2000 NanoDrop (NanoDrop Technologies). RNA-seq transcriptome libraries were prepared using TruSeq™ RNA sample preparation Kit from Illumina (San Diego, CA), using 1 μ g of total RNA. Messenger RNA was isolated with polyA selection by oligo(dT) beads and fragmented using fragmentation buffer. cDNA synthesis, end repair, A-base addition and ligation of the Illumina-indexed adaptors were performed according to Illumina's protocol. Libraries were then size selected for cDNA target fragments of 200–300 bp on 2% Low Range Ultra Agarose followed by PCR amplified using Phusion DNA polymerase (NEB) for 15 PCR cycles. Paired-end libraries were sequenced by Illumina NovaSeq 6000 sequencing (150bp*2, Shanghai BIOZERON Co., Ltd) after quantification with TBS380. Bioinformatics analyses and methods for mutation interpreters are presented in detail in the Supplementary Methods.

Quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent and reverse transcribed into cDNA using the First-Strand cDNA synthesis kit (TIANGEN, Beijing). Gene expression analysis was performed using SYBR Green kit (TIANGEN, Beijing). Primer sequences of all detected genes were synthesized by Sangon Biotech

(Shanghai, China). Data were analyzed using the $2^{-\Delta\Delta CT}$ method, with GAPDH as the housekeeping gene.

Western blotting

Trypsin digested cells (0.25%) of human colorectal cancer cells in the logarithmic growth stage were collected and a single cell suspension obtained. The suspension was evenly spread in a 6-well plate. Cells were incubated with the drug for 12h, then protein lysate (containing protease inhibitor) was added and cells were lysed in an ice bath for 2h. Cells were centrifuged at a rotating speed of 3000 R/min and a temperature of 4 °C for 20min. The supernatant was obtained for extraction of total protein. Protein concentration was determined using BCA kit(Biotechnology, Chengdu, China) according to the manufacturer's instructions. Cell samples were adjusted to obtain the same protein concentration. Cells (15ul) were obtained from each group for SDS-gel electrophoresis. Proteins were transferred to a PVDF membrane. The membranes were blocked with 5% skim milk for 1 h at room temperature, and then incubated with the corresponding primary antibody and secondary antibody. Antibody dilution ratio was obtained according to the manufacturer's instructions. Protein bands were observed and photographed in the gel imaging system using enhanced chemiluminescence method. Relative expression level of proteins was obtained by analyzing the data using Image Lab software.

Declarations

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Author contributions statement

Y.R: analysed the results; Writing- Original draf preparation, Writing- Reviewing and Editing;

Z.Z.Y: conceived the experiments, conducted the experiments, analysed the results; Writing- Original draf preparation, Writing- Reviewing and Editing;

C.N.N: conceived the experiments,conducted the experiments, analysed the results; Writing- Original draf preparation, Writing- Reviewing and Editing;

Y.Y: conceived the experiments,conducted the experiments;

J.Y.F: analysed the results; Writing- Original draf preparation, Writing- Reviewing and Editing;

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Figures

Figure 1

Effect of Berberine combined with Evodiamine on activity of colon cancer cells (HCT116, RKO) *in vitro*. (a) Chemical structure formula of Berberine; (b) Chemical structure formula of Evodiamine; (c) HCT116 and (d) Cell viability of RKO cells treated with Berberine 30 (uM), Evodiamine (0.8uM). (e) and (f) Berberine and Evodiamine combined (30uM + 0.8uM) for 24 hours, as determined by CCK8 method. (g,h) colon cancer cell clonal cell survival test (HCT116, RKO), the number of colonies was determined by

Image J software. * P < 0.05 and ** P < 0.01 and *** P < 0.001 and **** P < 0.0001 compared with the control group. All data are expressed as mean ± standard deviation of at least three experiments. All graphics were generated by GraphPad prism8 software. M, mol/L; CK, MOCKI.

Figure 2

Combined effects of Berberine and Evodiamine on cell cycle distribution and apoptosis of colorectal cancer cells. (a, b) HCT116 and RKO cells were incubated with berberine (30uM) and evodiamine (0.8uM), and berberine and evodiamine combined (30uM + 0.8uM) for 24 hours. DNA content of colon cancer cells was determined by flow cytometry, and cell cycle distribution was analyzed using mod fit LT software. (c, d) Expression levels of cycle related proteins (CDC25A, cdc25c, CHK2 and p21) as evaluated by western blot analysis. The protein levels were quantified by image J software. (e) HCT116 and RKO cells were incubated with berberine (30uM) and evodiamine (0.8uM), and Berberine and Evodiamine combined (30uM + 0.8uM) for 24 hours. Cells were stained with annexin v-apc. Flow cytometry used to detect cell apoptosis, and flow J software was used for analysis. Data are from at least three independent experiments. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared with the blank group.

Figure 3

Effects of Berberine and Evodiamine on migration and invasion of colon cancer cells (HCT116 and RKO) *in vitro*. (a, b, c) Cells were treated with a combination of Berberine and Evodiamine and wound healing, migration and invasion (scale, 50mm) of HCT116 and RKO cells determined. (d) Expression levels of E-cadherin and N-cadherin as determined by western blot. Data are expressed as mean $\pm$ standard

deviation. Data represent at least three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with the blank group.

Figure 4

Heat map of the relative expression level of DEGs in colon cancer cells after treatment with a combination of Berberine and Evodiamine. (a) HCT116 cells. Color intensity represents expression value, red represents upregulated genes, and blue represents downregulated genes. (b) Volcano map of DEGs. Red indicates the upregulated genes and blue indicates downregulated genes. $\log_2|\text{foldchange}| > 1$ and $P < 0.05$ were set as the cut-off value. (c) Go and (d) KEGG enrichment DEGs analysis. Terms were sorted by statistical significance, in reverse order, and the values – $\log_{10}(P \text{ value})$ were converted to color codes. The number of genes is represented by the size of points. BP, CC and MF GO terms are presented as dots, triangles and squares.

Figure 5

Berberine and Evodiamine exert synergistic anti colon cancer effect by targeting p38/MAPK/MAPK2/HSP27 pathway. (a) mRNA expression levels of P38MAPK, MAPKAPK2 and HSP27 in colon cancer cells (HCT116 and RKO) after treatment with Berberine and Evodiamine, and berberine and evodiamine (30uM + 0.8uM). (c,d) Expression levels of p38MAPK, P-P38MAPK, MAPKAPK2 and HSP27 proteins in colon cancer cells treated with Berberine (30uM) and Evodiamine (0.8uM), and

Berberine and Evodiamine (30uM + 0.8uM) for 24 hours. Protein levels were quantified by image J software. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with the blank group. Data represent at least three independent experiments.

Supplementary Files

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