Celastrol Suppresses Tryptophan Catabolism in Human Colon Cancer Cells as Revealed by Metabolic Profiling and Targeted Metabolite Analysis

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Celastrol is well known for its anti-cancer effects, yet its specific mechanisms against colon cancer are still not fully elucidated. In this study, cytotoxic effect of celastrol against HCT116 colon cancer cells was investigated based on cell viability assay and flow cytometry assay, and the possible mechanism was explored using a strategy combining metabolic profiling and targeted metabolite analysis based on ultra performance liquid chromatography (UPLC)/MS. Celastrol was found to inhibit the growth of colon cancer cells and induce apoptosis. Metabolomics analysis revealed characteristic changes in metabolic profiles of the colon cancer cells, revealing altered levels of amino acids, carnitine, and lipid markers. Most interestingly, with the assistance of targeted metabolite analysis, tryptophan (Trp) level was significantly increased whereas kynurenine (Kyn) level was decreased in colon cancer cells after celastrol treatment, together with markedly declined Kyn/Trp ratios. Western blot analysis revealed that expression of indoleamine 2,3-dioxygenase (IDO), the enzyme catalyzing Trp to generate Kyn, was dramatically inhibited in colon cancer cells after celastrol treatment, with a dose-dependent manner. These results suggest that suppression of IDO expression and tryptophan catabolism may be part of the mechanisms of celastrol in its cytotoxic effect against HCT116 colon cancer cells. This study provided scientific basis for further development of celastrol on treating colon cancer.

Key words celastrol; colon cancer; metabolomics; tryptophan; indoleamine 2,3-dioxygenase

Colorectal cancer (CRC) is the third leading cancer among men and the second among women globally. With the changes of lifestyle and dietary patterns, the incidence of CRC has been rapidly increased in China. Currently, commonly used chemotherapy for CRC includes 5-fluorouracil, bevacizumab, cetuximab and so on. However, adverse reactions of 5-fluorouracil include bone marrow suppression and gastrointestinal toxicity. Dermatologic toxicity of cetuximab is also common. Therefore, research and development of new drugs for treating CRC are of great interest.

Celastrol, an active ingredient extracted from Trypterygium wilfordii Hook F., is well known for its anti-inflammatory, antioxidantive, and anti-cancer effects. Currently, a few molecules or signal pathways were reported to be involved in the anti-cancer mechanisms of celastrol. For example, celastrol down-regulated phosphatidylinositol 3-kinase (PI3K)/Akt/nuclear factor-kappaB (NF-κB) signaling pathway in osteosarcoma and ovarian cancer cells; celastrol was reported to suppress the invasion and metastasis of colon cancer cells through down-regulating the expression of CXCR4 chemokine receptor, yet other mechanisms of celastrol against colon cancer are still not fully elucidated.

By characterizing the endogenous metabolites in biological systems using liquid chromatography-tandem mass spectrometry (LC-MS) or GC-MS, metabolomics can be used to monitor the metabolic alterations in the intervened pathways in tumor. Compared with normal cells, cancer cells have high enzyme activities and high energy metabolism, resulting in specific metabolic characteristics. For example, cancer cell metabolism is more dependent on glucose aerobic glycolysis, which is known as the “Warburg effect.” Cell metabolomics can thus highlight the disturbed pathways in cells and facilitate exploration of mechanisms of bioactive compounds.

On HCT116 human colorectal cancer cell line, anti-proliferative activities of Chamaecyparis obtusa (CO) leaf extract was revealed, where the activation of c-Jun N-terminal kinases was found to play a key role; another cell metabolomics study discovered some important metabolic pathways in the anti-tumor effect of flexiblebide, a marine natural compound. In our previous work, celastrol was found to intervene the energy, amino acid and nucleic acid metabolism in human cervical cancer cells. In this study, the cytotoxic effects of celastrol against HCT116 colon cancer cells were investigated and the potential mechanisms were elucidated using metabolic profiling together with targeted metabolite analysis.

MATERIALS AND METHODS

Chemicals and Reagents McCoy’s 5A medium was purchased from HyClone Thermo scientific (Beijing, China). Fetal
bovine serum (FBS) was obtained from GIBCO BRL (Grand Island, NY, U.S.A.). Celestrol and tryptophan standards were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (purity > 98%, Shanghai, China). Kynurenine standard was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). 5-Fluorouracil was purchased from Shanghai Xudong HaiPu Pharmaceutical Co., Ltd. (Shanghai, China).

**Cell Culture** Human colon carcinoma cell line (HCT-116 cells) was purchased from Chinese Academy of Sciences (Shanghai, China). Cells were cultured in McCoy’s 5A medium with 10% FBS, 100 µg/mL streptomycin and 100 µg/mL penicillin. Cells were incubated at 37°C in a 5% CO₂-humified air atmosphere incubator, and cells were passaged every 2–3 d when the cell density reached about 80%.

**Cell Viability Assay** Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was used to determine cell viability. We seeded each well of 96-well plate with ca. 7000 HCT116 cells, and then cultured them in the incubator for 24 h. Next, celestrol (0.00, 0.75, 1.50, 2.00, 2.50, 3.00, 4.50 µM) and 5-fluorouracil (0.00, 0.77, 1.54, 7.69, 38.44, 192.19, 960.95 µM) were added into each well with three replicates of each concentration, respectively. After drug treatment for 24, 48, and 72 h, medium was replaced by 110 µL fresh McCoy’s 5A medium containing 10 µL CCK-8 reagent and incubated for 1 h. Thermo Varioskan MicroplateReader (Thermo Fisher Scientific, U.S.A.) was used to determine the absorbance at 450 nm (A450).

**Apoptosis Analysis by Flow Cytometry** The Annexin-V/PI method was used to examine the cell apoptosis by flow cytometry. We seeded each well of 6-well plate with ca. 50000 HCT116 cells, after 24 h incubation, celestrol (0.00, 0.75, 1.50, 2.00, 3.00, 4.50 µM) and 5-fluorouracil (0.00, 0.77, 1.54, 7.69, 38.44, 192.19, 960.95 µM) were added into each well with three replicates of each concentration for 24 h. All HCT116 cells were collected with trypsin (without ethylenediaminetetraacetic acid (EDTA)) and washed twice with phosphate-buffered saline (PBS) to avoid the interference of medium. PI and Annexin VDetection Kit (BD Biosciences, CA, U.S.A.) were then used to stain cells for 15 min at room temperature in the darkness. These samples were analyzed by BD FACSCalibur™ Flow Cytometer (BD Biosciences).

**Metabolomics Analysis**

1. **Cell Quenching and Sample Preparation**

   Four groups, i.e., control group, celestrol-treated group (2 µM), celestrol-treated group (3 µM), and positive drug-treated group (5-fluorouracil, 38.44 µM) were used for metabolomics study. Six replicates of each group were prepared. All cells were seeded into the culture dish (10 cm) with a cell density of ca. 3000000. After culturing for 24 h, celestrol (2 and 3 µM) and positive control drug (38.44 µM) were added into the dish for 24 h, respectively. After overnight incubation, the medium was removed and the cells were rapidly washed twice with 2 mL PBS, then 1 mL 50% cold methanol (−80°C) was added into the dish for cell quenching and metabolite extraction. After that, the cells were scraped from the dish using a cell scraper, and the solution containing cells was transferred into the 5 mL centrifuge tubes. The dish was then washed with 1 mL 50% cold methanol (−80°C) and the solution was also transferred into the 5 mL centrifuge tubes. All the samples were stored in the freezer for 30 min at −80°C, and then sonicated for 5 min and centrifuged for 15 min at 13000 rpm to obtain the supernatant. The supernatant was evaporated under N₂ stream at room temperature, and the residue was dissolved in 50% methanol solution. Metabolite measurements were normalized to cell count as previously described. Quality control (QC) samples were prepared by pooling equal volumes of each analyzed sample.

2. **UPLC-MS Analysis**

   Ultra-performance liquid chromatography coupled with quadrupole/time-of-flight mass spectrometer (Agilent 1290 Infinity LC system and Agilent 6538 UHD Accurate-Mass Q-TOF mass spectrometer) was used in metabolomics analysis. A Waters ACQUITY HSS T3 column (2.1 × 100 mm, 1.8 µm, Waters, Milford, MA, U.S.A.) was used as the stationary phase at 50°C. The mobile phase was consisted of water containing 0.1% formic acid (Phase A) and acetonitrile containing 0.1% formic acid (Phase B). Gradient elution conditions was initially 5% B and held for 2 min, then 5% B was increased to 95% B from 2 to 17 min, finally 95% B was held for 2 min. The column equilibration time is 5 min. Flow rate was 0.3 mL/min. The Q-TOF mass spectrometer was operated with a capillary voltage of 4.0 kV, drying gas flow of 11 L min⁻¹, and a gas temperature of 350°C. The nebulizer pressure was set at 45 psi. The fragmentor voltage was 120 V and skimmer voltage was 60 V. The mass range was set at m/z 100–1100. The positive ionization mode was used in this study.

3. **Data Processing and Multivariate Data Analysis**

   The acquired LC-MS data was converted into mzdata format by Agilent Masshunter Qualitative Analysis B.06.00 software (Agilent Technologies, U.S.A.). Data deconvolution was performed using XCMSOnline (https://xcmsonline.scripps.edu/). Centroiding, deisotoping, filtering, peak recognition, and integration were performed to generate a multivariate data matrix of sample identity, ion identity (retention time and m/z), and ion abundance. The variables presenting in at least 80% of the samples were kept. SIMCA-P11 software (Umetrics, Kinnelon, NJ, U.S.A.) was used to perform multi-dimensional statistical analysis. The major latent variables were modeled using principal component analysis (PCA) and partial least-squares-discriminant analysis (PLS-DA). The ions with variable influence on projection (VIP) values higher than 1.5 and p values less than 0.05 were selected to be discriminating ions and then identified by comparing with the authentic standards based on their MS/MS fragmentation patterns and retention times. Metabolite levels (fold changes) were calculated as the mass response (area) ratios between two groups.

**Protein Expression Analysis**

Western blot was performed as previously described using antibodies including IDO (Indoleamine 2,3-dioxygenase) (DSI4E) Rabbit mAb (diluted 1:1000, Cell Signaling Technology, Inc.) and anti-β-Catenin (BD Biosciences cat# 610153, San Jose, CA, U.S.A.). Detailed experimental steps refer to https://www.cellsignal.com/product/protocol.jsp?id=500184354. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (diluted 1:1000) served as loading control.

**Data Analysis** Experimental values were expressed as mean ± standard deviation (S.D). Statistical analysis was performed using Prism 6.0 (GraphPad Software, Inc., San Diego, CA, U.S.A.) with two-tailed Student’s t-test or Mann–Whitney test. A p-value below 0.05 was considered statistically significant.


RESULTS

Effects of Celastrol against Human Colon Cancer Cells

As shown in Fig. 1 and Fig. S1, based on cell viability assay, HCT116 cells were exposed to 0.00–4.50 µM celastrol and 0.00–960.95 µM 5-fluorouracil for 24, 48, and 72 h, respectively. Celastrol was found to significantly inhibit the growth of HCT116 cells (Fig. 1), and celastrol treatment significantly inhibited HCT116 cells growth in 24 h. Cell apoptosis assay was used to evaluate the effects of celastrol on HCT116 cells by Annexin-V/PI method. The results showed that celastrol significantly induced apoptosis (Fig. 2), total cell apoptosis rates at 0.75, 1.50, 2.00, 3.00, and 4.50 µM doses were 6.73±0.10, 10.75±0.21, 17.58±0.44, 25.39±1.05, and 30.51±0.83%, respectively.

Metabolomics Analysis of Celastrol Treatment on HCT116 Cells

In order to demonstrate the mechanisms of celastrol against human colon carcinoma cells, metabolomics was subsequently used to characterize the metabolic profiles of various groups and uncover the potential metabolite markers. PCA and PLS-DA scores plot showed clear clustering of the four groups, i.e., control group, celastrol-treated group (2.00 µM), celastrol-treated group (3.00 µM) and positive drug-treated (38.44 µM) group (Fig. 3). The celastrol-treated group (3.00 µM) was further away from the control group than the 2.00 µM group. Based on the PLS-DA loadings plot, 14 ions discriminating the control and celastrol treated groups with VIP values higher than 1.5 and p values less than 0.05 were screened, which were later identified as amino acids including L-tryptophan, L-valine, L-phenylalanine, L-threonine, lipids including lysophosphatidylcholine (LPC 18:1), and carnitines including L-carnitine, oleoylcarnitine, linoleyl carnitine, 3-dehydroxycarnitine, L-palmitoylcarnitine and dodecanoylcarnitine, etc. The relative levels of the above metabolites in the four groups were shown in Fig. 4.

Quantification of Tryptophan (Trp) and Kynurenine (Kyn)

Kyn pathway, where Trp is catalyzed by IDO to generate Kyn, is closely related to the occurrence and development of tumor, mainly involved in tumor immune escape.14) From Fig. 4, Trp level was significantly increased after celas-
trol treatment; whereas Kyn was failed to be screened out as a discriminating metabolite, possibly due to its relatively low levels. Hence, we subsequently quantified Trp and Kyn levels in various groups using multiple reaction monitoring (MRM) mode of Triple Quadrupole LC/MS/MS, to accurately describe the alterations of key metabolites in Kyn pathway following celastrol treatment. LC-MS/MS quantification method of Trp and Kyn, MRM parameters, and linearity, accuracy, and precision results were described in the supplementary materials.

From the targeted quantification results of Trp and Kyn (Fig. 5), compared to control group, Trp concentration was significantly increased after celastrol treatment at 2.00 µM, and this trend was even more obvious after celastrol treatment at a higher dose (3.00 µM), consistent with the results shown in Fig. 4; at the same time, Kyn levels were decreased after celastrol treatment, also with a dose-dependent manner. Since approxi-
mately 95% of Trp is catabolized to Kyn by IDO, the above results indicate that tryptophan catabolism was probably suppressed after celastrol treatment.

**Western Blotting of Indoleamine 2,3-Dioxygenase (IDO)**

Protein Expression  Kyn/Trp ratio was reported to reflect the activity of IDO in cancer patients. In this study, Kyn/Trp ratio was markedly decreased by 26 times after celastrol treatment (2.00 M) and by 100 times with 3.00 M celastrol treatment (Fig. 6), indicating that IDO expression levels may be markedly inhibited in the colon cancer cells after celastrol treatment. As expected, according to Fig. 7, after celastrol treatment, IDO expression levels were significantly down-regulated in the HCT116 cells with a dose-dependent manner, whereas this did not happen in the 5-fluorouracil treated groups.

**DISCUSSION**

Celastrol has shown definite anti-cancer effect. In this study, based on cell viability assay and flow cytometry assay, celastrol was found to inhibit HCT116 cell growth and induce apoptosis in a dose-dependent manner. Using a strategy combining metabolic profiling and targeted metabolite analysis, possible mechanisms of celastrol against HCT116 cells were explored.

Metabolism of amino acids is found to be notably altered in cancer cells. In our study, celastrol treatment increased the levels of several amino acids, such as L-tryptophan, L-valine, L-phenylalanine, and L-threonine. Lysophosphatidylcholine acyltransferase 1 (LPCAT1) which converts lysophosphatidylcholine (LPC) into phosphatidylcholine (PC) is significantly upregulated in human colorectal cancer; in our work, celastrol up-regulated LPC (18:1) level of colon cancer cells, implying that celastrol may intervene LPC metabolism. Also, celastrol was found to upregulate the levels of some carnitines, consistent with the outcomes of the celastrol treated groups because carnitine can inhibit the proliferation of human colon cancer cells and induce apoptosis in vitro, but why the linoleyl carnitine levels were reduced after celastrol treatment remains unclear.

The most notable findings in our study are the suppressed tryptophan catabolism and markedly decreased Kyn/Trp ratios after celastrol treatment, since lower serum Trp level, higher Kyn level, and an remarkably increased Kyn/Trp ratio are regarded as the diagnostic indicator for colorectal cancer.

IDO expression is markedly increased in variety of cancers, including ovarian, colorectal, pancreatic cancers, and non-small-cell lung carcinoma, and plasma IDO level can be used as the diagnostic indicator for colorectal cancer. In this work, celastrol treatment caused obviously decreased
Kyn/Trp ratios, indicating the down-regulated IDO activity in colon cancer cells, which was further proved by western blots analysis. According to Fig. 7, celastrol treatment led to significantly down-regulated IDO expression levels with a dose-dependent manner, whereas this did not happen in the 5-fluorouracil treated groups. How celastrol correlates with IDO, however, remains unknown and awaits further study. In a recent report, two IDO inhibitors, 1-methyl-tryptophan (1-MT) and Epacadostat (INCB024360), were found to significantly suppress the viability of two colon cancer cell lines including HCT-116 and HT-29 cells. These new findings are quite supportive to our findings and also identified the IDO/Kyn pathway as a promising preventive target for colorectal cancer.

IDO is known to play a role in cancer-mediated evasion of the immune system. IDO1 activity in specialized antigen presenting cells (APC) in tumor draining lymph nodes is postulated to promote immune tolerance and facilitate tumor escape from immunosurveillance, via direct suppression of tumor-reactive T cells and enhancement of local Treg function. At the same time, accumulated Kyn can promote apoptosis of activated Th cells and induce differentiation of immunosuppressive Treg cells, and thereby inhibit immunoreactions. Evidences indicated that celastrol regulated the genes associated with immune responses, and reduced Th17 cells but increased Treg in inflamed joints. Therefore, it is possible that via IDO pathway, celastrol plays dual roles in both inhibiting cancer cell proliferation and modifying tumor immune surveillance remains unknown, which is a very interesting question raised from and beyond this metabolomics investigation.

In their work, Thaker et al. revealed that blockade of IDO1 activity in colon cancer cells reduced nuclear β-catenin, whereas exogenous administration of IDO1 pathway metabolites led to activation of β-catenin and proliferation of human colon cancer cells, and increased tumor growth in mice, indicating that IDO1 activity directly promotes tumor growth and proliferation of the neoplastic epithelium via activation of β-catenin signaling. Notably, β-catenin was reported to mediate the apoptosis induction effects of celastrol in human colorectal cancer HT29 cells; in an ulcerative colitis-related colorectal cancer (UC-CRC) mouse model, celastrol treatment significantly prevented the upregulation of expression levels of oncologic markers including β-catenin. Taken together, celastrol may lead to down-regulation of IDO1 and then possibly intervene β-catenin signaling to induce apoptosis in cancer cells, which still awaits further investigation.

In recent years, IDO was proved to be a new and important drug discovery target for cancer, Alzheimer’s disease, depression, and other major diseases. IDO has been regarded as an attractive target for both prevention and treatment of many cancers. However, currently there are no IDO inhibitors clinically available. Several IDO1 inhibitors have entered clinical trials, including epacadostat, indoximod (1-MT) and BMS-986205. A series of naphthoquinones and related natural compounds were reported to be potent IDO1 inhibitors. In particular, IDO is an essential target for the antitumor activity of the naphthoquinone menadione. A, a related isothiazolobenzoquinone natural product, are potent inhibitors of human IDO. Therefore, screening new IDO inhibitors for cancer treatment is still a worth exploring areas. Our results suggest that regulation of the IDO/tryptophan catabolism pathway could be regarded as a chemopreventive target for colon cancer, consistent with a recent report, and suppression of IDO expression and tryptophan catabolism may be part of the mechanisms of celastrol in its cytotoxic effect against colon cancer cells.

CONCLUSION

In this study, effects of celastrol against colon cancer cells were confirmed and its mechanism was demonstrated with the assistance of metabolomics. It is likely that celastrol suppressed tryptophan catabolism in HCT116 cells, partially caused by inhibiting the expression of IDO. This is the first metabolomics report exploring the mechanism of celastrol on colon cancer cells, which could hopefully provide scientific basis for further development of celastrol in treating colon cancer.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES


