Dual inhibition of COX-2/5-LOX blocks colon cancer proliferation, migration and invasion in vitro

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Abstract. Inflammation is emerging as a new hallmark of cancer. Arachidonic acid (AA) metabolism, the family of cyclooxygenases (COXs) and lipoxygenase (LOX) play important roles in AA-related inflammatory cascades. In 94 colorectal cancer samples collected from the Han population, the immunohistochemical results indicated that 68% of the patients with colorectal cancer had a co-expression of both COX-2 and 5-LOX, while both displayed low expression in the matched normal tissues. In cell lines, three colorectal cancer cell lines exhibited high expression of COX-2 and 5-LOX. During stable silencing of the expression of COX-2 or 5-LOX in LoVo cancer cells, we found that downregulation of either COX-2 or 5-LOX significantly diminished the growth, migration and invasion of the colon cancer cells and specifically, downregulation of COX-2 could elicit upregulation of 5-LOX protein and vice versa. The above results suggested that the simultaneous blocking of COX-2 and 5-LOX activity may bring more potential benefits in managing the progression of colon cancer. Therefore, we sought to explore the effectiveness of a dual COX-2/5-LOX inhibitor darbufelone on the proliferation, migration, invasion and apoptosis of colon cancer cells, as well as the underlying mechanism of action. The results indicated that darbufelone significantly decreased the proliferative and invasive abilities of the colon cancer cells, in a dose-dependent manner. During the study of the related mechanisms, we found an upregulation of p27 and downregulation of cyclin D1 as well as CDK4 after darbufelone treatment, which indicated that darbufelone could arrest the cell cycle of LoVo cells at the G0/G1 phase. Furthermore, the activation of caspase-3 and -9, upregulation of Bax and downregulation of Bel-2 demonstrated the occurrence of apoptosis by darbufelone. Finally, darbufelone also prevented the migration and invasion of LoVo cells, which may be ascribed to the upregulation of E-cadherin and ZO-1. In summary, our data suggest that the inhibition of both COX-2/5-LOX may be an effective therapeutic approach for colon cancer management, particularly for those patients with high expression of COX-2/5-LOX.

Introduction

Colorectal cancer is responsible for a large proportion of cancer morbidity and mortality, particularly in the developed countries, which affects over a quarter of a million people each year (1,2). Surgical resection is the traditional therapeutic method to manage colon cancer; however, 25% of patients with metastatic disease have a five-year survival rate of only 10% (3). Existing chemotherapy has reached a plateau of effectiveness in improving the hope of prolonged survival. Therefore, it is critical to seek more precise and creative strategies which are beyond the current concept of chemotherapy.

The role of inflammation has been a hallmark in the development of cancer over the past decades (4,5). Several families of mediators mediate the process of inflammation, and the arachidonic acid (AA) cascade represents one of the most important families among them. AA is mainly metabolized via the cyclooxygenase (COX) and the lipoxygenase (LOX) pathways, leading to the formation of prostanoids and leukotrienes (LTs), respectively (6). A series of epidemiological and clinical studies have demonstrated a 40-50% reduction in the relative risk of colorectal cancer in humans who take non-steroidal anti-inflammatory drugs (NSAIDs) (7,8). In contrast, the selective COX-2 inhibitor celecoxib has demonstrated anticancer activities in vivo and in vitro (9-11). Likewise, inhibition of 5-LOX is also related to cancer cell viability, proliferation...
and cell migration (12). Hence, exploring the agents that target the AA cascade may bring more benefit to cancer chemotherapeutic strategies (13). The growing evidence of research has shown that small-molecule inhibitors, which target COX-2 and 5-LOX, could impede the occurrence of colorectal cancer to some extent (11,14) or directly kill colorectal cancer cells (15). While there is still a lack of systematic research on these two targets in the progression of colorectal cancer, previous studies have already acknowledged the role of COX-2 or 5-LOX in the proliferation of cancer. However, the sequencing steps in cancer progression, such as invasion and metastasis, were found to be less involved. Furthermore, it is not clear to what extent the colorectal cancer patients in the Han population can benefit from the simultaneous suppression of COX-2/5-LOX.

In the present study, we detected the expression of COX-2 and 5-LOX in colon cancer patients by immunohistochemistry, and the intensity of expression and the proportion of staining were calculated and analyzed. Furthermore, in order to study the role of COX-2 and 5-LOX in cell proliferation, migration and invasion, the lentivirus-mediated short hairpin RNA was specifically designed to knock down COX-2 or 5-LOX expression in human LoVo cells. In addition, we also evaluated the effectiveness of a dual COX-2/5-LOX inhibitor darbufelone on different aspects of colon cancer cells, including proliferation, migration, invasion, apoptosis, as well as exploration of the underlying mechanism. The results could further confirm the role of COX-2 and 5-LOX in the development of colon cancer treatment, and also offer an alternative therapeutic approach in cancer therapy.

Materials and methods

Patient data. Paraffin-embedded, archived colorectal cancer samples obtained from 94 patients who were histologically and clinically diagnosed with colorectal cancer, were collected from the Pudong Medical Center of Fudan University during January 2007 and December 2009. Out of the 94 colorectal cancer tissues, 52 matched adjacent non-cancerous tissues were used as controls. Prior to the usage of these clinical materials for investigation, consent from the patients and approval from the Institute Research Ethics Committee were acquired. Primary cancers of the colorectal were classified according to the pathological tumor-node-metastasis (TNM) classification (16). Clinical information of the samples is described in detail in Table I. Patients included 47 males and 47 females with ages ranging from 24 to 85 years (mean, 65.4 years).

Immunohistochemistry. Immunohistochemistry was carried out to study altered protein expression in the 94 human colorectal cancer and 52 matched adjacent non-cancerous tissues, as previously described (17,18). Commercially available antibodies against COX-2 (1:200 ab15191; lot: rabbit polyclonal immunoglobulin G) and 5-LOX (1:200 ab169755; lot: rabbit polyclonal immunoglobulin G) (both from Abcam Biotechnology, USA) were used as the primary antibody separately. Immunohistochemical kit (SP-9001 rabbit SP kit, lot: 50581654) was obtained from Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China). For each sample, one score was given according to the percent of positive cells as: no positive cells, 0; <5% of the cells, 1 point; 5-35% of the cells, 2 points; 36-70% of the cells: 3 points; >70% of the cells, 4 points. To achieve objectivity, the intensity of positive staining was also used in a four scoring system: 0 (negative staining), 1 (weak staining exhibited as light yellow), 2 (moderate staining exhibited as yellow brown), and 3 (strong staining exhibited as brown). A final score was then calculated by multiplying the above two scores. If the final score was ≥4, the tumor was considered to have high expression; otherwise, the tumor was considered to have a low expression (18).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>COX-2</th>
<th>5-LOX</th>
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<tbody>
<tr>
<td></td>
<td>Neg.</td>
<td>Pos.</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>10</td>
<td>32</td>
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<tr>
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<tr>
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<tr>
<td>Female</td>
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</tr>
<tr>
<td>C+D</td>
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</tr>
<tr>
<td>Location (colon)</td>
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</tr>
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<td>42</td>
</tr>
<tr>
<td>No</td>
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Neg., negative; Pos., positive; COX, cyclooxygenase; LOX, lipoxygenase. *P<0.05.
10% fetal bovine serum (FBS) (HyClone, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma Chemical, USA). The HEK 293T cell line was purchased from Shanghai Institute of Cell Biology (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with high glucose supplements containing 10% FBS. Cells were cultured at 37°C in a humified atmosphere of 5% CO₂ and routinely passaged with 0.25% trypsin-0.02% EDTA (Invitrogen).

**shRNA construction and lentivirus production.** The target sequences for COX-2/5-LOX mRNA were chosen according to the RNAi Consortium (TRC) shRNA library (Broad Institute). Two complementary single-strand DNA oligonucleotides of each shRNA were chemically synthesized by Shanghai Sangon Biotechnology Co., as follows: COX-2F, 5'-CCGGGTCTATTAAACCTCTATCTCGAGATAGAGCAGTTAAAAATTCAGCTTTCTGCAAACTATGGATTGCTGATCAGCAACACTATTTCTCGAGATAGAGCTTGTTGCTGATCAGCAACACTATTTCTCGAGATATGTTGTGCTGATCCTGATTTTTG-3' and 5-LOX, 5'-CCGGGTCTATTAAACCTCTATCTCGAGATAGAGCAGTTAAAAATTCAGCTTTCTGCAAACTATGGATTGCTGATCAGCAACACTATTTCTCGAGATATGTTGTGCTGATCCTGATTTTTG-3'. The annealed double-stranded oligonucleotides of each shRNA were ligated into pLKO-TRC-GFP shRNA vector by T4 ligase (Takara, Japan), which was digested by restriction enzymes AgeⅠand EcoRⅠ(Shanghai New England Biolabs, USA). The constructed plasmid was transformed into E. coli DH5α competent cells (Sangon Biological Engineering Technology, China) for plasmid amplification. To confirm the right insertion, the positive colonies were selected and further identified by DNA sequencing.

The second generation of the lentiviral vector system was generously provided by Professor Hongbin Ji (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences). Lentiviruses were produced after co-transfection of HEK293T cells with pLKO-TRC-GFP and the related shRNA vector, Δ8.91 and pVSV-G (10:10:1) using the X-tremeGENE HP DNA transfection reagent (Roche, Swiss). The culture supernatant containing the lentiviruses was harvested at 48 and 72 h after transfection. LoVo cells were passaged to a 60-mm dish at a density of 1x10⁶ cells, which were further infected with the viruses and 2 µg/ml Polybrene (Sigma, USA). Approximately 48 h post-infection, the medium was replaced with fresh completed medium containing 2 µg/ml puromycin (Gene Operation, USA). LoVo cells stably infected with the lentivirus that could survive from the pressure of puromycin, were ready for the following assays, and designated as LoVo-Vector (empty pKO-TRC-GFP vector), sh-COX-2 and sh-5-LOX cells.

**Western blotting.** LoVo cells from the different groups were washed with PBS and lysed in ice-cold SDS lysis buffer composed of 0.6 M Tris-HCl (pH 6.8), 10% SDS and protease inhibitor cocktail (Sigma). Samples were incubated on ice for 10 min and collected with 2X SDS loading buffer. Protein was separated by SDS-polyacrylamide gel electrophoresis, and transferred to a PVDF membrane (0.22 µm; Millipore, USA). After blocking with 5% non-fat milk dissolved in TBS-T buffer (10 mM Tris base, pH 7.5, 100 mM NaCl, 0.1% Tween-20) for 1 h at room temperature, the membrane was incubated with the primary antibody overnight at 4°C and then washed three times with TBS-T, and then incubating with the secondary antibody (1:3,000; Cell Signaling Technology, CST; Shanghai, China) for 1 h at room temperature. The blot was exposed to ECL blotting system after 3x10 min of TBST washing, which was further scanned by the Chemiluminescent Western Blot Scanner (LI-COR, Inc., Lincoln, NE, USA). Protein expression was quantified by densitometric analysis with ImageJ software (version 1.40g). The primary antibodies for COX-2, 5-LOX, E-cadherin, and ZO-1 were purchased from Becton-Dickinson (Shanghai, China), and incubated with the PVDF membrane at a 1:500 dilution. Active-caspase 3/9, cyclin D1, CDK4, p27 and β-tubulin were from Cell Signaling Technology (Shanghai, China), and incubated at a 1:1,000 dilution. Bcl-2 and Bax were from Bioworld Technology (Nanjing, China), and incubated at a 1:500 dilution.

**Cell proliferation assay.** LoVo cells from the control, sh-COX-2 and sh-5-LOX groups were plated at a density of 5x10³ cells/well into 96-well plates (Corning Inc., USA) and allowed to attach overnight. After incubation at different times (24, 48, 72 and 96 h), the plate was removed, and the MTT assay was performed as follows. MTT (20 µl) (5 mg/ml) was added to the wells, and the plate was incubated at 37°C for another 4 h. The supernatant was discarded and 200 µl dimethylsulfoxide (DMSO) was applied to dissolve the formazan. The absorbance value optical density (OD) was detected at 560 nm wavelength with a microplate reader (Thermo, USA), with 630 nm as reference wavelength. The inhibition rate (%) = (1 - OD_{drug group}/OD_{control group}) x 100%. In addition, the inhibitory effects of darbufelone, celecoxib and zileuton on LoVo cells were also determined by MTT assay. In brief, LoVo cells with a density of 1x10⁴ cells/well were incubated with different final concentrations of the above compounds for 24 h. Then MTT assays were performed as described above. All of the above experiments were repeated three times.

**Scratch assay.** The migration ability of the LoVo cells was assessed by scratch assay as follows. When the cells grew to 100% confluence on a 6-well plate, scratches were performed using a sterile 200-µl pipette tip, and the medium was discarded and washed once with PBS to remove the floating cells. For minimizing the interference of cell proliferation, RPMI-1640 medium with 5% FBS was used instead of 10% FBS. After different times of incubation (12, 24 and 36 h), the plate was removed from the incubator and images were captured under a microscope. The average wound closure rate was calculated using Image-Pro Plus 6.0 software.

The effectiveness of darbufelone on the migration of LoVo cells was also determined by scratch assay. In consideration of the cell growth inhibitory effects caused by darbufelone, lower final concentrations (5, 10 and 20 µM) were applied in the scratch assay, which was performed as described above. All of the above experiments were repeated three times.

**Matrigel invasion assay.** A 24-well plate with an inner chamber (Corning, Inc.) was used for the invasion assay. The membranes with 8 µm pores were precoated with Matrigel (Becton-Dickinson, USA), which was mixed with RPMI-1640...
medium without FBS at a dilution of 1:5. After a 5-h incubation and hydration with RPMI-1640 medium for 0.5 h, the LoVo cells of the control, sh-COX-2 and sh-5-LOX groups at a density of 2x10^4/chamber were cultured in the inner chamber with RPMI-1640 with 1% FBS and 0.1% bovine serum albumin. Then, 500 µl of RPMI-1640 medium containing 10% FBS was placed in the lower chamber. After incubation at 37˚C for 36 h, cells on the upper surface of the filters were removed by a cotton swab. In addition, the chamber was kept at room temperature for 30 min and then immersed in 0.5% crystal violet containing 1% methanol for another 30 min. The crystal violet was washed with PBS for three times. Cells on the lower chamber were counted under a microscope in four fields randomly.

The effects of darbufelone on invasion of the LoVo cells were also evaluated by invasion assay. LoVo cells were incubated with 5, 10 and 20 µM darbufelone in the inner chamber (the setting of the concentration was based on the same reasons described in the scratch assay). Other procedures were in consistent with the above description of the invasion assay. All of the above experiments were repeated three times.

Flow cytometry. For cell cycle analysis, the LoVo cells were seeded on a 6-well plate at a density of 5x10^5 and were then starved for 24 h for growth synchronization. After that, the cells were incubated with darbufelone at the concentrations of 0, 20, 40 and 60 µM for 24 h. After incubation, the cells were harvested by trypsinization, washed two times with PBS, and were then fixed at 4˚C with 75% alcohol overnight. The supernatant was removed by centrifugation, and the cells were incubated for 30 min in DNA-staining solution containing 50 µg/ml propidium iodide (PI), 0.1% Triton X-100 and 50 µg/ml RNAase at room temperature. DNA content analysis was then performed on a BD Accuri™ C6 flow cytometer (Becton-Dickinson).

For the apoptosis analysis, Annexin V-FITC/PI double staining was performed using an apoptosis detection kit (Becton-Dickinson). After treatment with 0, 20, 40 and 60 µM darbufelone for 24 h, the LoVo cells were harvested by trypsinization (without EDTA), and then the cells were incubated with 5 µl FITC-labeled Annexin V for 15 min and PI for 5 min in 300 µl of binding buffer in the dark, respectively. After incubation, another 200 µl of binding buffer was added to the cell suspension. The cell preparations were then analyzed by a BD Accuri™ C6 flow cytometer. The fluorescent compensation was made through single staining PI or Annexin V-FITC in LoVo cells.

Statistical analysis. Data are expressed as mean ± SD. Statistical analysis was performed using a two-tailed Student's t-test or one-way ANOVA followed by the Tukey's test using GraphPad Prism 5. Statistical significance was verified at P<0.05.

Results

Correlation between COX-2 and 5-LOX protein expression and clinicopathological features. As shown in Fig. 1, in the samples of colorectal cancer, 5-LOX and COX-2 showed strong positive expression, which was higher than the corresponding colorectal mucosa. Table I shows the relationship between the expression of COX-2 protein and clinical characteristics. The positive ratio of COX-2 was 77.7%. There were no significant correlation between the expression level of COX-2 protein and age, histological classification, histological differentiation, tumor diameter, location and invasion or distant metastasis of the colorectal cancer patients. However, the expression of COX-2 was closely associated with Dukes' stage of the colorectal cancer patients (P=0.008), invasion (P=0.03) and metastasis (P=0.01). The expression of COX-2 protein was positively correlated with stage and invasion (Table I). Expression of 5-LOX in colon cancer was also significantly higher than that in the normal colonic mucosa (70.2 vs. 46.2%; P<0.05), most of which had strong positive expression. In conclusion, 5-LOX and COX-2 in colon cancer are highly co-expressed up to a rate of 68%, and were significantly correlated with Dukes' stage, depth of invasion and metastasis (P<0.05), regardless of patient gender, age, tumor location, tumor size and degree of differentiation (P>0.05).
Correlation between COX-2/5-LOX protein expression and tumor-related characteristics. To choose a cell line which shows high COX-2 and 5-LOX expression, COX-2 and 5-LOX protein levels were detected in three colorectal cancer cell lines, SW620, LoVo and SW480. The results indicated that LoVo cells showed high expression levels of both COX-2/5-LOX and were regarded as the target cell line (Fig. 2A). Subsequently, the lentivirus-mediated delivery of short hairpin RNA was specifically designed to knock down COX-2 and 5-LOX expression in the human LoVo cells. Western blotting confirmed that sh-COX-2 decreased the expression of COX-2 to 37.71%, however, the expression of 5-LOX increased to 124.55% after COX-2 knockdown. Likewise, sh-5-LOX led to a downregulation of COX-2 to 61.22% and an upregulation of 5-LOX.
5-LOX to 167.22%. The results of western blotting indicated that the downregulation of COX-2 led to an upregulation of 5-LOX and vice versa (Fig. 2A).

Additionally, we found a positive correlation between COX-2/5-LOX expression and the tumor-related characteristics, such as proliferation, migration and invasion, by MTT, scratch and Matrigel invasion assays, respectively. We evaluated the LoVo cell proliferation after COX-2/5-LOX knockdown, which indicated a significantly lower proliferation rate at 24, 48 and 72 h and a balanced level at 96 h in the sh-COX-2 groups compared to the control group (Fig. 2B). The reason for this phenomenon could be explained on the basis of high cell density existing in the control group at 96 h, thus no further proliferation rate was detected. In the scratch assay (Fig. 2C and D) and Matrigel invasion assay (Fig. 2E), we observed that the migration and invasion of the LoVo cells were significantly inhibited after downregulation of COX-2 or 5-LOX.

These results suggest that expression of COX-2 and 5-LOX is involved in the regulation of cell growth, migration and invasion in human colon cancer cells. Particularly, the results of the western blotting also indicated that inhibition of either COX-2 or 5-LOX leads to feedback expression of another protein, thereby suppression of COX-2 and 5-LOX may be more effective for controlling the progression of colorectal cancer.

Darbufelone inhibits the proliferation of LoVo cells and down-regulates the expression of COX-2/5-LOX. To confirm the inhibitory effects of dual COX-2/5-LOX inhibitor darbufelone on LoVo cells, different concentrations of darbufelone, single target inhibitor celecoxib (COX-2 inhibitor), zileuton (5-LOX inhibitor) and their combination were included for comparison. MTT assay was performed to detect the inhibition rate of each concentration. As shown in Fig. 3A, we observed that after treatment with darbufelone for 24 h, darbufelone significantly decreased LoVo cell viability in a dose-dependent manner. The effectiveness was much more potent than celecoxib, zileuton and their combination. Furthermore, the result of the western blotting indicated that darbufelone also decreased the expression of COX-2/5-LOX dose-dependently (Fig. 3B).

Darbufelone decreases LoVo viability through cell cycle arrest and induction of apoptosis. The effect of darbufelone on cell cycle progression of LoVo cells was detected by PI staining. The percentage of LoVo cells in the G0/G1 phase was increased while the percentages of cells in the S and G2/M phases were decreased (Fig. 3C and D). However, we did not observe a sub-G1 peak in the flow cytometry results. This may be associated with the alcohol fixation and finally led to DNA fragment leak. Additionally, this phenomenon was potent in recognizing the effects in a dose-dependent manner.
Figure 4. Darbufelone decreases LoVo cell viability through the activation of the intrinsic apoptotic pathway. (A and B) Exposure to different concentrations of darbufelone for 24 h increased LoVo cell apoptosis and (C) western blot results indicated that darbufelone-induced apoptosis occurred in a caspase-dependent manner and promoted further activation of the intrinsic apoptotic pathway to promote the programmed cell death of LoVo cells.

Figure 5. Darbufelone decreases the invasive ability of LoVo cells by upregulating E-cadherin and ZO-1 expression. After treatment with 5, 10 and 20 µM darbufelone, (A and B) the migration and (C and D) invasion ability of the LoVo cells were evaluated. The results indicated that darbufelone decreased LoVo cell migration and invasion in a dose-dependently manner. (E) Western blotting indicated that the upregulation of E-cadherin and ZO-1 may be responsible for the effect of darbufelone on preventing suppression of migration and invasion of the LoVo cells.
The results of western blotting also indicated an upregulation of p27 and downregulation of cyclin D1 as well as CDK4 after darbufelone treatment, which indicated that darbufelone could decrease LoVo viability by G0/G1 arrest (Fig. 3E).

The results of Annexin V-FITC and PI double staining assay also indicated that darbufelone decreased LoVo viability and triggered apoptosis. As shown in Fig. 4A and B, exposure to different concentrations of darbufelone for 24 h increased the LoVo cell apoptosis. The proportion of FITC+/PI+ (early stage of apoptosis) cells increased from 20.7±3.63 to 42.13±0.35%. Likewise, FITC+/PI- cells (late stage of apoptosis) increased from 16.1±0.75 to 42.97±4.29%. These data suggested that darbufelone dose-dependently induced LoVo cell apoptosis, which coincided well with the result of the MTT assay. Furthermore, we evaluated the expression of active caspase 3/9, Bcl-2 and Bax by western blotting (Fig. 4C). These data suggest that darbufelone-induced apoptosis occurred in a caspase-dependent manner and the activation of the intrinsic apoptotic pathways may be involved in the programmed cell death of LoVo cells.

Darbufelone decreases the invasive ability of LoVo cells by upregulating E-cadherin and ZO-1 expression. In order to explore whether darbufelone has a direct effect on cell migration and invasion, in the invasion assay, based on previous results of the cell proliferation assay, a lower concentration of darbufelone was applied to the LoVo cells, which minimized the false-positive results due to the drug-induced cell inhibition and apoptosis. After treatment with 5, 10 and 20 µM darbufelone, the results indicated that darbufelone decreased LoVo cell migration (Fig. 5A and B) and invasion (Fig. 5C and D) in a dose-dependently manner. As shown in Fig. 5E, western blotting indicated that the upregulation of E-cadherin and ZO-1 may be responsible for the effect of darbufelone on the prevention of migration and invasion. Namely, coinciding well with the results of COX-2/5-LOX knockdown, the dual COX-2/5-LOX inhibitor darbufelone effectively suppressed LoVo cell proliferation, migration as well as invasion.

Discussion

Inflammation in the tumor microenvironment has been regarded as one of the hallmarks of cancer (5). Epidemiological evidence suggests that chronic inflammation is closely associated with an elevated risk of developing cancer (19,20). Additionally, the data suggest that ~20% of cancer deaths are associated with the process of unabated inflammation (21,22). In contrast, anti-inflammatory drugs could effectively decrease the risk of developing cancers, such as colon, lung and prostate cancer (7,23).

AA is mainly metabolized via the COX and the LOX pathways, and most studies on the relationship between inflammation and cancer have focused on the COX pathways. COX-2 is a vital enzyme that catalyzes the conversion of AA to active prostanoids. Moreover, COX-2 also plays a crucial role in cancer-associated inflammation and tumor progression (24,25). In 1994, the upregulation of COX-2 was found to be implicated in human colorectal cancer (26). From then on, studies on COX-2 have focused on other types of cancers, such as breast, prostate, ovarian as well as lung cancer (27-30). Additionally, it has been proven that cancer patients with COX-2 upregulation are always closely associated with a poor prognosis and decreased survival rates (12). In contrast, AA can also be converted to other metabolites, such as LTs, which are closely associated with the 5-LOX pathway. The LOX pathway was also proven to play an important role in cancer development. Inhibition of 5-LOX activity also showed prevention of cancer cell proliferation as well as tumor metastasis (31).

COX and LOX are both downstream key enzymes of the AA cascade, suggesting that mere inhibition of COX cannot fully control the progression of cancer since cancer cells feasibly strengthen the LOX signaling pathway. Therefore, focusing on the combination of a 5-LOX inhibitor and a COX-2 inhibitor may provide a novel therapeutic approach to inflammatory diseases as well as cancer. In fact, this hypothesis has been proposed in many human cancers (32-34). Nevertheless, the related side-effects of NSAIDs, such as gastrointestinal effects and cardiovascular risk, compromise the extensive application of these drugs (35). Hence, the dual COX-2/5-LOX inhibitor may be viewed as a more effective choice to manage related diseases. The dual inhibitors attain the simultaneous inhibition of the COX/LOX pathways with more efficiency and better tolerance. Dual inhibitors do not share the same pharmacokinetic and distribution patterns as single target drugs (6).

In the present study, we first analyzed the expression of COX-2 and 5-LOX in colorectal cancer samples from a Chinese population. Consistent with the data from a Caucasian population, our results found that COX-2 and 5-LOX were both highly expressed in colorectal cancer patients; the ratio of co-expression reached up to 78%, while significantly lower expression levels of COX-2 and 5-LOX were found in normal tissues. Then, we infected the LoVo cells with a lentivirus to stably silence the expression of COX-2 or 5-LOX. The data indicated that, either downregulation of COX-2 or 5-LOX reduced cell proliferation, invasion and migration abilities to a certain extent.

In addition, we also found that when one protein weakens, it persistently causes a feedback activation of another protein. Previous studies were used to utilize the transient transfection of siRNAs to silence COX-2 or 5-LOX. The long-term observation data was minimal, therefore, our study suggested that stable silencing of either COX-2 or 5-LOX alone can produce an additional feedback for the signal pathway, maintaining a persistent activation of the AA cascade. All of the above-mentioned data indicated that the AA-related signaling pathways were potently activated in the colorectal cancer patients. Simply suppressing one node of this pathway was not enough to completely attenuate the viability of the entire pathway. In contrast, the AA signaling pathway is extremely important in the progression of colorectal cancer.

Since it was affirmed that COX-2 and 5-LOX have a significance role in colorectal cancer, we further evaluated the effects of several dual inhibitors and single target inhibitors, as well as the combination of these single-target inhibitors in colorectal cancer-related biological functions. Furthermore, we showed that darbufelone, as a dual COX-2/5-LOX inhibitor, possessed an antiproliferative effect on human LoVo cells. The effectiveness of darbufelone was much more
potent than celecoxib, zileuton and their combination. In the scratch and Matrigel invasion assays, we also observed that darbufelone effectively decreased the migration and invasion of the LoVo cells. The study of mechanisms showed that G0/G1 cell cycle arrest as well as apoptosis induction may be responsible for the decreased proliferation of the LoVo cells. In contrast, downregulation of migration-related protein ZO-1 and E-cadherin may be one of the reasons for the prevention of migration and invasion by darbufelone. All these data demonstrated that darbufelone may provide clinical benefit to colon cancer management.

Although we observed that darbufelone treatment could significantly prevent tumor-related characteristics, the underlying mechanisms appeared unclear. Further study on a mouse model combined with microarray analysis as well as immunohistochemical assay may provide clues for a better understanding of the underlying mechanisms of a dual COX-2/5-LOX inhibitor. In conclusion, our data suggest that use of the dual COX-2/5-LOX inhibitor darbufelone can be an effective therapeutic approach for the treatment of colon cancer.

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References