Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Cytotoxic effect of 2, 5-dimethyl-celecoxib as a structural analog of celecoxib on human colorectal cancer (HT-29) cell line

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Received February 4, 2017; **Accepted** May 10, 2018; **Published** May 30, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.7.1

Abstract: Dimethyl-celecoxib (DMC), a close derivative of celecoxib (CXB) with a low COX-2 inhibitory function, exhibits significant anti-neoplastic properties. In this study, we have investigated the effect of CXB and DMC on the human HT-29 cell line. The cellular viability, caspase-3 activity, and *VEGF*, *NF-κB*, and *COX-2* genes expressions were assessed respectively with MTT, colorimetric, and real-time RT-PCR methods. DMC, a close analogue of CXB, was more potent in inhibiting the growth of cells (IC50: 23.45 µM at 24 hr) than CXB (IC50: 30.41 µM at 24 hr). Both CXB and DMC caused a significant difference in caspase-3 activity compared to the control group. DMC significantly decreased the *NF-κB* expression. Down-regulation of the *COX-2* mRNA expression in the celecoxib-treated group was significant compared with that in the DMC-treated group. Alterations in the mRNA expression of *VEGF* were not significant between the groups. Owing to the more potent growth inhibitory effects of DMC compared to that of celecoxib, it may be important to conduct research on the anticancer application of this compound, which can reduce the side effects relating to COX2 inhibition.

Key words: Celecoxib; Colorectal; Cyclooxygenase2; *NF-κB*; 2, 5-Dimethyl-celecoxib.

Introduction

Celecoxib (CXB), a selective cyclooxygenase 2 (COX-2) inhibitor, is one of the most commonly used chemotherapeutic/chemopreventive agents in treating various types of cancers. COX-2, as an essential regulator, is engaged in many steps of cancer development like angiogenesis, apoptosis resistance, and cancer cell invasion and metastasis (1, 2). The up-regulated COX-2 expression has been shown by approximately 50% in colon adenomas and 85–90% in colorectal cancers (CRC) (3, 4). Several studies have reported the functional participation of CXB regarding the repression of different types of tumors including CRC (5-7) and other cancers like cervical (8, 9), breast (10, 11), esophageal (12, 13), and prostate (14) cancers. However, long-term and high-dose usage of CXB has been shown to be associated with some side effects (15). As many anticancer and anti-proliferative effects of CXB are independent of COX-2, non-COX-2 inhibitors like 2, 5-dimethyl-celecoxib (DMC), as a structural analog of CXB, is more likely to represent a promising alternative strategy (16). Reduction of neo-vascular formation and experimental tumor growth inhibition in various *in vivo* models have been reported by the DMC treatment (17). Anticancer activity and induction of apoptosis by DMC seem to be comparable with or more potent than CXB (15, 16, 18-21).

Owing to the evidence of the better anti-proliferative effect of DMC as well as the anti-proliferative effect of non-steroidal anti-inflammatory drugs (NSAIDS) without COX inhibition activity, the COX-2 assumption as the key factor in antitumor activity has been challenged (19). Some new COX-2-independent anti-tumor targets, such as the apoptosis-inducing factor (22), c-Myc (23),TRIAL (21), surviving (23, 24), nuclear factor-kappa B (NF-κB) (25-27), vascular endothelial growth factor (VEGF) (28, 29), and 3-phosphoinositide-Dependent protein kinase-1(17, 19) and endoplasmic reticulum Ca^{2+}-ATPases (19), has been proposed.

However, the molecular basis of the processes involved are still not fully known. In this study, we have investigated the biological effects of CXB and DMC on cell viability, apoptosis induction, and the expression of *COX-2*, *VEGF*, and *NF-κB* genes in the HT-29 cell line of CRC.

Materials and Methods

CXB, 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1yl] benzene sulfonamide and its close structural analog, DMC, where 5-aryl moiety has been altered by replacing 4-methylphenyl with 2,5-dimethylphenyl, resulting in 4-[5-(2,5-dimethylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide (17, 19), were purchased from the Sigma-Aldrich Chemical Company (USA).The drugs were dissolved in DMSO at 20 mM (stock solution) and then stored

at -20°C and added to the cell culture medium at a final concentration of the solvent (DMSO) <0.5%.

Cell line and culture

Human HT-29 CRC cells (Iranian Biological Resource Center, Tehran, Iran) were routinely grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin (ATOCEL, Australia) at 37°C in a humidified incubator containing 5% CO_2. For experimental incubations, the cells were plated at a density of 5×10^3 cells/ml and allowed to attach overnight. The cells were subsequently exposed to increasing concentrations of 0–100 µM CXB and DMC for 24 hr. The controls were treated with a DMSO as a vehicle control at a concentration equal to that of the drug-treated cells. The HT-29 cell line used in this experiment was free of any mycoplasma infection.

MTT assay

The effect of CXB and DMC on the viability of the HT-29 cell line was determined by the MTT assay (Cayman Company, USA). Briefly, a 100-µl medium, including 5×10^3 HT-29 cell suspensions, was plated in each well of a 96-well plate. The cells were treated with different concentrations of CXB and DMC (20, 40 and 60µM). We added an equal volume of DMSO to the control wells. After 24 hr, the cells were washed with phosphate-buffered saline (PBS) and treated with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT). The plates were incubated in the dark for 3 hr. The supernatants were removed and 100 µl of the crystal dissolving solution was added to each well. A microtiter plate reader (Awareness Technology Inc., USA) was used for measuring the absorbance of the produced color at 570 nm. The viability percentage was calculated as: Cell viability (%) = (absorbance of test / absorbance of control) × 100. The inhibitory concentration (IC50) value of each drug was determined using the Compusyn software.

Measurement of caspase-3 enzyme activity

The enzymatic activity of caspase-3 was measured by the caspase-3 colorimetric assay kit (Abnova, Taiwan). In this regard, HT-29 cells were seeded in 24-well tissue culture plates. After 24 hr, the medium was changed and the cells were treated with IC50 doses of the drugs for another 24 hr. After discarding the medium and detaching the cells by trypsin, the cells were harvested by centrifugation at 300 g for 5 min. Next, the cells were re-suspended in 50 µL of the cold lysis buffer (10mM Tris–HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100). The lysed cells were placed on ice for 10 min and centrifuged at 10,000 g for 1 min. The protein concentration was then measured with the Bradford protein assay kit. In each well of a 24-well plate, 50µg of proteins were diluted by adding the cell lysis buffer to make a total volume of 100 µl. The 2X reaction buffer 50 µl (containing 10 mM dithiothreitol) was then added to each well. The process was finalized by adding the Asp-Glu-Val-Asp-chromophore p-nitroanilide (DEVD-pNA) 5µL 4 mM substrate (200 mM final concentration) and incubating the plates at 37°C for 2 h. The sample plate was read at 405 nm with a microtiter plate reader.

RNA extraction and reverse transcription PCR analysis

To analyze the expression of *VEGF*, *NF-κB*, *COX2,* and *b-actin* mRNAs, the cells were treated with each drug at their IC50 doses. The total cellular RNA was isolated from cells using the Total RNX-Plus Solution Kit (CinnaGen Co., Iran) according to the manufacturer's protocol, followed by the assessment of the RNA concentration by the UV spectrophotometer. cDNA was prepared from 2 µg of RNA using the 2-step RT-PCR Kit with M-MuLV RT/Taq DNA Polymerase, 100app-RTPL12 (Vivantis, Malaysia) according to the manufacturer's instructions. The sequences of designed primers for real-time RT-PCR are listed in Table 1.

Real-time PCR assay of *COX-2*, *VEGF*, and *NF-κB* mRNA levels

Quantification was done by RT-qPCR using the AccuPower®2X Green StarqPCR Master Mix (Sybr Green-based methods, Bioneer Co.) The housekeeping gene β-actin was used as an internal control. The RT-q PCR was performed using a standard protocol recommended by the manufacturer, while the analysis was conducted in the Bio-Rad iQ5 cycler Sequence detection system (Bio-Rad Laboratories Inc.). The fit point method was employed for data analysis. The relative amounts of *COX-2*, *VEGF*, and *NF-κB* mRNA were standardized against *β-actin* by using the delta Ct method. Afterward, the relative mRNA expression of these genes in HT-29 cells, treated with CXB and DMC, was compared to that of the control cells. The data was analyzed by the Bio-Rad iQ™5 optical system software using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Mean ± SE was used for presenting the results of

Table 1. The sequences of the primers used in real-time RT-PCR method.

Target genes		Primer sequences
COX-2	Forward	5'- GGAACACAACAGAGTATGCG-3'
	Reverse	5'- AAGGGGATGCCAGTGATAGA -3'
VEGF	Forward	5'- AGGAGGAGGGCAGAATCATC -3'
	Reverse	5'- GGCACACAGGATGGCTTGAA -3'
NF-κB	Forward	5'-GGAGATCGGGAAAAAGAGC-3'
	Reverse	5'- GACTCCACCATTTTCTTCCTC-3'
β-actin	Forward	5'-CTGGAACGGTGAAGGTGACA-3'
	Reverse	5'-TGGGGTGGCTTTTAGGATGG-3'

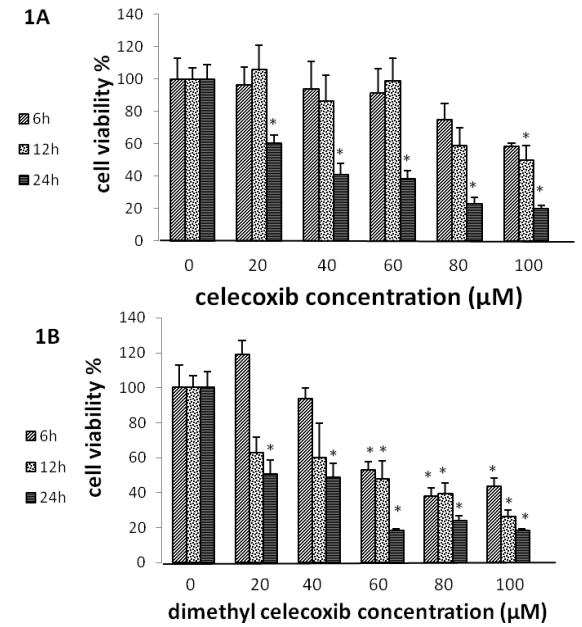

Figure 1. The effects of celecoxib and dimethyl-celecoxib on the proliferation of HT-29 cell line. The data was shown as mean ± SEM, and the viable cells of controls were used as 100%. Statistical significance (*P< 0.05) was determined by one-way ANOVA with Tukey's HSD post hoc analysis.

each experiment that was repeated at least three times in triplicate. The significance of the differences between the experimental groups was analyzed using the one-way ANOVA in the SPSS statistical software package (SPSS 16). P values lower than 0.05 were considered significant.

Results

Effect of CXB and DMC on inhibition of HT-29 growth *in vitro*

In this study, we used the human HT-29 cell line and investigated the effects of CXB and DMC on its growth and survival. As shown in Figure 1, the treatment of CXB and DMC produced time- and dose-dependent growth inhibition in the HT-29 cell line. DMC (60, 80 and 100 µM) caused a significant decrease in the cell viability at 6, 12 and 24 hr, whereas CXB showed this effect only at the highest concentration (100µM) after 12 and 24 hr of drug exposure and had no effect following 6 hr of the treatment. DMC had more inhibitory effects on the growth of cells (IC50: 23.45 µM at 24 hr) than CXB (IC50: 30.41 µM at 24 hr).

Caspase-3 activity of HT-29 cells treated with CXB and DMC

The effect of CXB and DMC (at their IC50 doses) on caspase-3 activity is shown in Figure 2. The treatment of CXB and DMC produced caspase-3 activity induction in the HT-29 cell line. Both CXB and DMC caused a significant difference in caspase-3 activity compared to the control group (P=0.003) & (P=0.001) respectively.

The effect of CXB and DMC on *VEGF*, *NF-κB*, and *COX-2* expression

The quantitative real-time PCR analysis demonstrated that both drugs (at their IC50 doses) had an insignificant effect on the reduction of the *VEGF* level. DMC

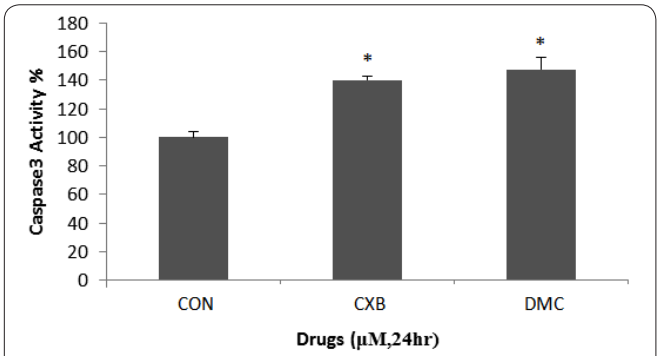

Figure 2. The effects of CXB and DMC on HT-29 cell apoptosis in comparison with DMSO (control). The data was presented as the mean ± SE and the mean number of untreated control cells was set at 100%.

significantly decreased the *NF-κB* expression in HT-29 CLC compared to the control group (P=0.016) (Figure 3). CXB down-regulated the expression of *COX-2* mRNA significantly in comparison with the DMC (P=0.011).

Discussion

In this study, we reported that the treatment of HT-29 CLC with DMC dose and time-dependently inhibited cell viability. Also, the growth inhibitory effects of DMC (IC50 of 23.45 µM) were noticeably more pronounced than what was observed with CXB (IC50 of 30.41 µM) in HT29 CLC. Caspase 3 activity was increased significantly by both analogues. CXB and DMC have been shown to inhibit the cell viability in different tumor cells such as hepatoma (30), head and neck squamous cell carcinoma (31), glioblastoma, and multiple myeloma cells (32). Also, the effective impact of DMC has been shown on the cell growth inhibition compared to that of CXB on other cells in several studies (19, 20, 25). These effects are modulated through different mechanisms in various cell lines.

COX-2 is involved in producing prostaglandins during inflammatory and oncogenic processes(33); it has been well studied especially in the context of colorectal cancer pathogenesis. COX-2, whose transcription is activated by NF-κB, also promoted angiogenesis (34, 35). Our results show that DMC has a low inhibitory function on COX-2 inhibition. Hence, it is proposed that the celecoxib may exert part of its effect through COX2-independent mechanisms as its close analogue (DMC) affects NF-kB without affecting the COX-2 inhibition.

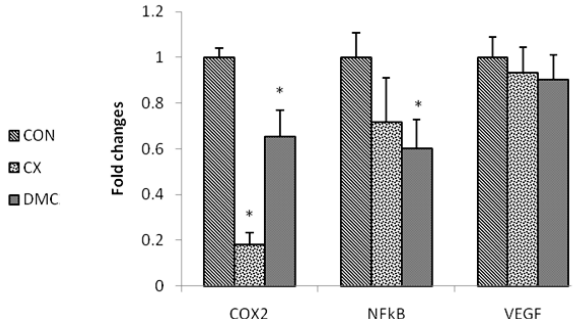

Figure 3. The effects of CXB, and DMC on inhibition of the *NF-κB*, *VEGF*, and *COX-2* expression in comparison with DMSO (control). The data was depicted as means ± SE. The P value was compared to untreated group (*P <0.05).

Significant reduction in the *NF-κB* expression was one of the COX-2 independent mechanisms for the cytotoxic effect of DMC on HT-29 CLC. NF-kB plays a key role in the control of multiple steps of tumor progression including angiogenesis, cell differentiation and migration, and apoptosis (36). According to the role of NF-kB in producing resistance to chemotherapy, its inhibition enhanced the response of tumor cells to chemotherapeutic agents (37). In our study, DMC at its IC50 dose decreased the *NF-κB* level and the degree of such reduction was more than what had been achieved by CXB at their corresponding IC50 doses.

The repression of *NF-κB* transcriptional activity (27) and the down-regulation of *NF-κB* have been shown in multiple myeloma cells by DMC (25), while western blot analysis revealed increases in the nuclear expression of the *NF-κB* in human cervical cancer cells (26). It needed to be confirmed by future studies as one of the possible mechanism of the action of DMC on HT-29 CLC.

Similar to studies in other malignant cells (21, 24, 38), we have shown that DMC at its IC50 doses induced apoptosis via the induction of caspase-3 in HT-29 CLC. The level of induction of apoptosis by DMC was slightly higher than that of CXB. Accordingly, Backhus et al. have reported that CXB and DMC, both potent inducers of apoptosis, decreased the viability and proliferation of non-small cell lung cancer cells with IC50 of 73 and 53 μm/L respectively (15).

Zhang et al. have shown the apoptosis induction independent of caspase-3 activation by the combination of DMC and ABT-737 in gastric cancer cells (22). Nonetheless, Xu et al. have reported up-regulated caspase-3 levels by CXB and cisplatin combination in drug-resistant human gastric cancer cells (39). We showed a significant decrease in cell viability and increases in caspase-3 enzyme activity and caspase-3 gene expression by the CXB and imatinib combination in our previous work. In addition, we observed that treatment with the CXB and imatinib combination showed a minimal (not significant) reduction of the *NF-κB* and *VEGF* expression in HT-29 cells(40).

Hypoxia as a major stimulator of VEGF has a strong association with malignancy and diminished therapeutic response (41). VEGF has been shown to play a role in tumorigenesis, angiogenesis, and metastasis and often serves as a negative prognostic indicator for disease-free survival of cancer patients (42). Shimada et al. (43) have found an association between increased VEGF and poor response to treatment. It has been reported that treatment by CXB contributed to the reduction of the *VEGF* expression in colon cancer cells (28). The CXB-induced *VEGF* mRNA expression in glioma cells and cancer cell lines derived from colon carcinoma has also been observed (29). Although the treatment of glioma cells with CXB and DMC did not affect VEGF secretion, it significantly inhibited the secretion of endothelin-1 as a key factor in vasoconstriction and angiogenesis (20).

It seems that the anti-angiogenesis effects of DMC in low doses are modulated via non-VEGF factors. Although the use of DMC has no effect on VEGF secretion in endothelial cells, it can reduce the expression of pre-angiogenic VEGFR2 and enhance the expression of anti-angiogenic VEGFR1 in the endothelial cells in the control group and the tumor-dependent group (44). Regarding the lack of adequate research on the anti-angiogenesis effect of DMC on colon cancer cells, more evaluation is needed in terms of the high doses effect of celecoxib and DMC on the *VEGF* expression.

Though the impact of DMC on *VEGF* in our study was insignificant, the amount of decrease was more than that was observed in CXB treatment. Therefore, VEGF might be proposed as a less efficient mechanism for action of DMC on HT-29 CLC, which needs to be investigated in more detail.

Considering the above results and according to some previous reports, it seems that the COX-2 inhibition and anti-tumor effect of CXB reside in different structural units (18). Zhu et al., in their investigation on prostate cancer cell lines, have shown that the heterocyclic system, the sulfamoyl Moiety, and hydrophobicity and bulkiness in the phenyl ring are required for inhibition of growth and apoptosis induction of these cells (45). In DMC, the 5-aryl moiety was modified by replacing 4-methylphenyl with 2, 5-dimethyl phenyl—this may be responsible for its greater potency (20).

However, DMC seems to be safer than celecoxib, especially for the treatment of COX-2-negative tumors, because it does not cause any serious cardiovascular side effects related to prolonged use of cyclooxygenase-2 inhibitors like CXB (46, 47). As the effects of CXB and DMC have been shown to be time-dependent (48), their time-related effects should be studied in the future, especially in the case of DMC that shows stronger tumor growth-inhibiting effects.

In conclusion, the results of this study demonstrate that the growth inhibition by DMC is noticeably more potent than what has been observed with CXB. Apoptosis induction appears to be involved in the mechanism of DMC action. *NF-κB* and *VEGF* changes by these drugs, as their mechanism of action should be evaluated in future studies considering the effects of time.

Acknowledgments

This study was conducted with financial support that was provided by the Urmia University of Medical Sciences. The authors sincerely thank Dr. Mehrdad Jalalian (http://www.MehrdadJalalian.com) and Dr. Akbar Afaghi Khatibi for their voluntary reviewing and providing constructive comments on the manuscript.

Conflict of interest

The Authors have no conflict of interest related to the manuscript.

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