Original Research

The effect of celecoxib and its combination with imatinib on human HT-29 colorectal cancer cells: Involvement of COX-2, Caspase-3, VEGF and NF-κB genes expression

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Abstract: It has been shown that combination of imatinib (IM) with other agents may have some advantages in avoiding toxicity and resistance caused by this drug. The selective cyclooxygenase-2 inhibitor, celecoxib (CX), has been known to have antitumor and chemo-sensitizing effect in the treatment of colorectal cancer. In this study, we investigated the effectiveness of CX and its combination with anticancer agent IM on human colorectal cancer HT-29 cell and their probable molecular targets. Cultured HT-29 cells were exposed to IC50 dose of CX, IM, and their combination (half dose of IC50) for 24 hours to assess their effect on proliferation inhibition by MTT assay. The caspase-3 activity was estimated in HT-29 cells with colorimetric kit. COX-2, Caspase-3, VEGF and NF-κB genes expression was also investigated using real-time PCR method. Combined treatment with IM and CX, resulted in a significant (P<0.05) decrease in cell viability and increased caspase-3 enzyme activity. Decreased COX-2 gene expression has been found in CX and combined treated group. Significant increase in Caspase-3 gene expression has been shown in IM and combined treated cells. In conclusion, the present in vitro study with colon cancer cell line demonstrated that CX and its combination with IM improved the anticancer activity of each component. Caspase-3 and COX-2 dependent molecular targets seem to be involved in mediating the anti-proliferative effects of IM and CX combination. Of course, the other molecular pathways are also likely to play the role and should be explored in future studies.

Key words: Imatinib, celecoxib, colorectal cancer, COX-2, Caspase-3, VEGF, NF-κB.

Introduction

Colorectal cancer is one of the most prevalent human malignancies worldwide (1). Imatinib mesylate (Glivec) is a selective tyrosine kinase inhibitor, inhibiting kinases of BCR-Abl, c-kit, and platelet-derived growth factor receptor (PDGFR) (2). This drug has FDA approval for the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors (GIST) with positive c-kit expression (3). Several studies have shown that using various combinations of chemotherapeutic agents exert greater efficacy than a single agent (4-6) and may be useful in decreasing chemotherapy associated side effects.

The use of non-steroidal anti-inflammatory drugs (NSAIDs) in colon cancer treatment and prevention has attracted attention due to their anti-proliferative and apoptosis-promoting properties (7). According to in vitro and in vivo studies, cyclooxygenase2 (COX-2) inhibitor celecoxib (CX), can reduce the risk of colorectal cancer (8). In addition, this drug reduces the count of colorectal polyps in patients with familial adenomatous polyposis (9) and can enhance the antitumor efficacy of chemotherapeutic agents (10, 13). Besides, nuclear factor κB (NF-κB) which is important in inflammatory reaction and cell cycle control has been shown to regulate the expression of sets of genes involved in tumorigenesis (14-17). Also vascular endothelial growth factor (VEGF) is most strongly associated with tumor growth and metastasis (18). According to previous studies, CX down- or up-regulates the expression of VEGF and NF-κB in other tumors cells (19-23). The activity of NF-κB in colon cancer cell lines is abnormally high (24) therefore, inhibition of NF-κB signaling pathway by COX-2 inhibitors including CX may improve the response of colon cancer cells to chemotherapy. For this purpose, we assessed the effects of CX alone and its combination with IM on cell viability and COX-2, Caspase-3, VEGF and NF-κB genes expression in colorectal cancer HT-29 cell line.

Materials and Methods

Cell culture and drug treatment

The HT-29 human colorectal cancer cell line was ob-
tained from Iranian Biological Resource Center (IBRC). Cells were grown in DMEM medium with stable glutamine (pAA) supplemented with 10% FBS (pAA) and 100 units/mL of penicillin.

IM and CX were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) and Sigma-Aldrich Co. (St. Louis, MO, USA), respectively. Stock solutions of the drugs (20 mM) were prepared in dimethyl sulfoxide (DMSO) and diluted in DMEM before use. The final concentrations of drugs was selected on the basis of their IC50 values obtained in our previous work which were 7 μM for IM and 30 μM for celecoxib (26). HT-29 cells were treated with IC50 dose of IM (7 μM) and CX (30 μM) or their combination (half dose of IC50) for 24 hr. DMSO was used as the vehicle to deliver drugs and its concentration in control wells was equal to test wells.

Cell viability
Cell proliferation was analyzed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide [MTT, Cayman Chemical Co. (Ann Arbor, MI, USA)] in triplicate. Briefly, HT29 cells (5000 cells/well) were seeded in 96-well plates. At 24 hr after seeding, cells were treated with mentioned concentrations of drugs. Then 10 μl of MTT reagent was added per well 24 hr following treatments and the plates incubated at 37°C for another 3 hr. The reaction was stopped by removal of MTT-containing media. Thereafter, 100 μl of crystal dissolving solution was added to solubilize formazan crystals. Absorbance at 570 nm was recorded using an ELISA micro-plate reader (Stat-Fax 2100, Awareness Technology Inc.). The percent of cell viability was calculated as the absorbance ratio of treated samples compared with the untreated control.

Measurement of caspase-3 enzyme activity
The caspase-3 colorimetric assay kit (Abnova, Taiwan) was used to determine the caspase-3 enzyme activity. Briefly, HT-29 cells were seeded in 24-well tissue culture plates. After 24 hr the medium was changed and cells were treated with mentioned concentrations of drugs for 24 hr. The medium was removed and the cells were collected by centrifugation at 14,000 rpm for 5 min. Then, 50 μL of cell lysis buffer (10mM Tris–HCl pH 7.6, 150 mMNaCl, 5 mM EDTA, 1% Triton X-100) was added to the cells to resuspend them. After the lysis, cells were kept on ice for 10 min. Then, they were centrifuged at 10,000 rcf for 1 min. Protein concentration was assayed with a Bradford Protein Assay kit. Into each well of a 24-well plate, 50 μg protein was diluted by adding Cell Lysis Buffer to make a volume of 100 μL. 2X Reaction Buffer 50 μl (containing 10 mM dithiothreitol) was added to each well. 5 μL of the 4 mM DEVD-pNA substrate (200 μM final concentration) was added and the plates were incubated at 37°C for 2 h. The sample plate was read at 400 or 405 nm with a microtitre plate reader.

RNA extraction & cDNA synthesis
Total RNA was isolated from treated cells using the RNX-plus™ kit (CinnaGen Inc, Iran) according to the protocol provided by the manufacturer. Briefly, 1 ml of RNX-plus was added to a tube containing 105 homogenized cells, and the mixture was incubated at room temperature for 5 min. Chloroform was added to the solution and centrifuged at 12000 rpm for 15 min. The upper aqueous phase was then transferred to new tube and an equal volume of isopropanol was added. The mixture was then centrifuged at 12000 rpm for 15 min and the resulting pellet was then washed in 70% ethanol and dissolved in DEPC-treated water. The purity of the extracted RNA was confirmed by measuring the ratio of optical density at 260 nm to that at 280 nm and its integrity was examined by electrophoresis on agarose gel.

Total RNA was used to generate single-stranded cDNA with 2-step RT-PCR kit (Vivantis, Malaysia) by the following reaction: Purified total RNA was mixed with 1 μl of random Hexamers primer (50ng/μl), 1 μl dNTPs mix (10mM) and up to 10 μl of water. This mixture was added to 10 μl of a cDNA synthesis mix that included 2 μl of buffer M-MuLV (10X) and 100 unit M-MuLV reverse transcriptase. The mixture was incubated at 42°C for 60 min, and then for 5 min at 85°C. The synthesized cDNA was directly used as template for real time RT-PCR.

Real time RT-PCR
Primer design was done with regard to primer dimer formation, self-priming formation, and primer melting temperature, and was checked with Gene runner software. Blast search in the published sequence database Gene Bank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed the primers to be gene specific (Table 1). Human beta actin gene was used as a housekeeping gene.

Real time RT-PCR was performed using the AccuPower® 2X Greenstar qPCR Master mix (Bioneer, Korea) in a total volume of 25 μl according to the manufacturer’s instruction. The expression of mentioned genes

Table 1. Sequences of primers used to evaluate expression of β-actin, Caspase-3, VEGF, NF-κB and COX-2 genes in HT-29 cell line.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Forward 5′-CTGGAACCGGTGAGGTTGACA-3′</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-TGCGGTGCTTTTGTAGGATG-3′</td>
<td></td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Forward 5′-AGAACTGAGGCGATGCGAG-3′</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-CGCCTGTGGCCTATTTTGACTCG-3′</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>Forward 5′-AGGAGGAGGGCCAGATATGC-3′</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-GGGCAGGCGAGGCTTGGGA-3′</td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>Forward 5′-GGAGATCGGGGAGAAAAAGAGC-3′</td>
<td>315</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-CATCCACGACCACTTTTCTCTG-3′</td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>Forward 5′-GGAACGAGGAGATGAG-3′</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-AGGGGATGCGGATAGAACTGC-3′</td>
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was analyzed using an iQ5 Real-Time PCR detection system (Bio-Rad, CA, USA). In addition, a no template control was used to test the potential contamination and primer dimer formation. The reactions were prepared in a 96-well optical plate for 10 min at 95°C followed by 40 cycles of 20 sec at 95°C and 45 sec at 59°C. A melting curve analysis was conducted to confirm the specificity of the amplification reactions. Each sample was replicated three times. The relative expression of each mRNA was calculated using the 2-ΔΔCt method, where Ct is the threshold cycle (27). Relative expression levels of mRNA were normalized to β-actin and analyzed for statistical significance.

Statistical analysis

All data were presented as mean ± standard error for at least three separate experiments for each treatment. Statistical significance of differences between mean values was analyzed by one way ANOVA followed by Tukey’s HSD post-hoc test using SPSS 16 statistical analysis software (SPSS Inc. Chicago, IL). The level of significant difference was set at P<0.05. The fold differences of gene expression normalized to control was presented graphically in the form of histograms, using Microsoft Excel computer program.

Results

Interactions between IM and CX in the inhibition of HT-29 cell viability

The percentage of viable cells detected after treatment with CX (15 µM) in combination with at half dose of IC50, 15 µM) was reduced to 40% compared to the control (P<0.05), whereas, lower effects were produced by treatments with higher concentrations of CX (30 µM) or IM (7 µM) alone (Figure 1).

IM in combination with celecoxib induced caspase-3 activity in HT-29 cells

Caspase-3 activity studies reported an increase in the activity levels in HT-29 cells treated with CX (15 µM) in combination with IM (at half dose of IC50, 3.5 µM) approximately 60% compared to the control (P<0.001) (Figure 2).

Discussion

Abounding evidence for functional participation of CX in tumor suppression has been reported in several studies including breast cancer (28, 29), esophageal cancer (30, 31), prostate cancer (32), cervical cancer (33, 34) and colorectal cancer (35, 36). Besides multiple lines of evidence indicate that CX exerts its effect in...
combination with other drugs including curcumin (37), 5-fluorouracil (38, 39), atorvastatin (40) in colon cancer cells, cisplatin (31) in human esophageal squamous cell carcinoma cells and IM in K562 leukemia cells (41). There was no study about combination of IM and CX on HT-29 colorectal cancer cells.

In this study, we demonstrated that CX, IM and their combination decreased cell viability which was significantly lower in the case of combination treatment compared to control group in HT-29 colorectal cancer cells. We also observed that IM in combination with CX significantly increased caspase-3 activity in these cells. Anti-proliferative effects of IM in combination with CX were associated with decrease in COX-2 and increase in Caspase-3 mRNA levels. Alteration in VEGF and NF-κB genes expression was not statistically significant.

In accordance to our results, Kusunoki et al. showed that CX and its derivative inhibits proliferation and COX-2 expression in HT-29 colorectal cancer cells (42). Ninomiya et al. also demonstrated that CX reduces the growth and metastatic potential of HT-29 colorectal carcinoma in mice through COX-2 inhibition and proposed that CX has a high potential to use as a clinical agent for inhibition of hematological and lymphatic metastases of colorectal cancer (43). Besides, Zhang et al. reported that higher doses of CX (80–160 μM) significantly reduce viability and down regulate the expression of COX-2 mRNA/protein in K562 cells (41). Combination of CX and IM have also been shown to have synergistic effects in terms of anti-proliferation on K562 cells (44). Over-expression or down-regulation of COX-2 in K562 cells treated with combination of CX (10 μM) and IM (10 μM) or CX (10 μM) alone, respectively have also been demonstrated (6). In addition it has been shown that IM increased COX-2 expression in squamous cell carcinoma (45) and over-expression of COX-2 in K562 cells involved in the resistance to IM (46). Then decrease in COX-2 expression by CX and combination treatment and not by IM in our study indicates the usefulness of CX especially in combination with IM.

There are some lines of evidence suggesting that COX-2 inhibitors exert part of their anti-cancer properties without affecting COX-2 activity (47). In accordance to our results, some studies point to apoptosis as a mechanism of the effect of CX (48-50). In the study by Ninomiya et al. reduced growth and metastatic potential of colorectal carcinoma in mice, suppressed VEGF protein expression and induced apoptosis have been demonstrated by CX through COX-2 inhibition (43). On the other hand, Du et al. reported up-regulation of VEGF in HCT116 colorectal cancer cells (19). Also increased VEGF gene expression in chronic myelogenous leukemia patients (51) and decreased VEGF levels in some patients with gastrointestinal stromal tumors have been shown following IM treatment (52). In our study, IM increased but CX and its combination with IM decreased VEGF mRNA level insignificantly. If so, decreasing VEGF level by CX and its combination with IM might inhibit angiogenic process in HT-29 colorectal cancer cells.

In addition we observed that treatment with IM, CX and their combination showed a minimal (not significant) reduction of NF-κB expression in HT-29 cells. Besides, NF-κB level was lower in the cells treated with CX and its combination with IM. Consistent literature data on the effect of CX on NF-κB are available (14-16). Accordingly it has been shown that CX might prevent colorectal cancer in the early stages by down regulating NF-κB in K562 cells (53). However, because of statistically insignificant data in our study, CX induced decrease in VEGF and NF-κB levels seems to be less important and should be evaluated in the future.

As stated, observed changes in cell viability and gene expression were stronger in combined drug treatments. Since drug resistance and side effects related to IM treatment may significantly limit the effectiveness of this drug, using suitable combinations of IM along with other preventive agents like CX can be advantageous in lowering the required dose of anticancer drugs and,

Figure 3. Real-time quantitative RT-PCR analysis to determine the effects of imatinib (IM), celecoxib (CX) and their combination on Caspase-3 (A), COX-2 (B), VEGF (C) and NF-κB (D) mRNA levels with b-actin as the internal control. Vertical bars indicate the mean fold changes ± SEM for three independent experiments in each treatment group. *: P<0.05 versus control.
consequently, minimizing undesirable side effects (54).

Stahle et al. used combination of IM with 5-Fluorouracil and showed significant growth inhibition of the highly tumourigenic HT-29 cells (55). Also Zhang et al. reported that CX combined with 5-Fluorouracil could inhibit the growth of HT-29 induced xenograft carcinoma by inducing apoptosis and decreasing COX-2 expression (39). Consistent with the results, of mentioned studies we showed effectiveness of IM-CX combination in HT-29 colorectal cancer cells treatment. COX-2 inhibition and Caspase-3 activation seems to be the most important pathways which were involved in anti-proliferative effect of combined drugs in this cell line. Of course, more studies are necessary to clarify combined drugs usefulness for clinical usage and finding the best dosage of the drugs. Yamaguchi et al. used the combination of gefitinib and CX in a pilot study and showed that this combination was generally well tolerated in patients with advanced GI cancer (56).

In conclusion, the present in vitro study on colon cancer cell lines demonstrated that IM in combination with CX significantly improved the anticancer activity of each component. In this regard, our study presents Caspase-3 and COX-2 dependent molecular targets as probable mechanisms mediating the anti-proliferative effects of IM and CX combination. Of course studying other molecular targets and cell lines, could be helpful to learn more in-depth mechanism by using combined treatment with IM and CX.

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