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Original Article

Imatinib and its combination with 2,5-dimethyl-celecoxibinduces apoptosis of human HT-29 colorectal cancer cells

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Abstract

Mono-targeting by imatinib as a main antitumor agent does not always accomplish complete cancer suppression. 2,5-dimethyl-celecoxib (DMC) is a close structural analog of the selective cyclooxygenase-2 (COX-2) inhibitor, celecoxib, that lacks COX-2 inhibitory function. In this study, we aimed to show the apoptotic effects of imatinib in combination with DMC in human HT-29 colorectal cancer (CRC) cells. HT-29 CRC cells were treated with IC_{50} dose of imatinib (6.60 μM), DMC (23.45 μM), and their combination (half dose of IC_{50}) for 24 h. The caspase-3 activity was estimated with colorimetric kit. The caspase-3 gene expression was evaluated by real-time PCR method. There was a significant up-regulation in caspase-3 enzyme activity and caspase-3 expression by imatinib and its half dose combination with DMC as compared to control. As a summary, the results of this study strongly suggest that half dose combination of imatinib with DMC induced apoptosis as potent as full dose imatinib in human HT-29 CRC cells, while minimizing undesired side effects related to imatinib mono-therapy. This study also pointed towards possible caspase-dependent actions of imatinib and DMC.

Keywords:Imatinib; Dimethyl-celecoxib; Apoptosis; Gene expression; Colorectal cancer cell line.

INTRODUCTION

Colorectal cancer is one of the major causes of morbidity and mortality worldwide and chronic inflammation is accepted to have a significant effect in the promotion and progression of this disease (1). It is well-known that chemotherapy plays significant role in the treatment of colorectal cancerdepending on the stage of the disease. The most common chemotherapy drugs using for this cancer include capecitabine (2), fluorouracil (3), irinotecan (4), oxaliplatin (5). Imatinib is a potentially novel drug for the treatment of adenoma formation and cancer progression in patients predisposed to develop colorectal cancer (6). This agent as a protein tyrosine kinase inhibitor is appeared to have apoptotic and anti-proliferative actions in several cancers including colorectal cancer (7,8). HT-29 cell line showed responsiveness to imatinib by means of a reduced proliferation or induction of apoptosis (9). This drug often has side effects like nausea, vomiting, diarrhoea, neutropenia, and anemia (10). Thus, imatinib is not usually considered a main medicine and is more frequently used in combination with other drugs like bevacizumab and cyclophosphamide as a therapy for advanced colorectal cancer (11).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are another option which have been used to diminish the toxicity of conventional chemotherapeutic drugs (12).

Of course, the use of NSAID for example celecoxib, rofecoxib, valdecoxib, and parecoxib are associated with increased side effects due to inhibition of cyclooxygenase-2 (COX-2) (13-15).

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2,5-dimethyl-celecoxib (DMC) as a close derivative of celecoxib is able to faithfully mimic all of celecoxib's numerous anti-cancer effects that have been examined so far, despite its inability to inhibit COX-2 (16,17). DMC has been reported to show *in vitro* anti-tumor efficacy as strongly as celecoxib and both drugs are potent inducers of apoptosis (18,19). This is an assumption that DMC can be a good choice for cancer treatment in the future.

In this study, we have investigated the combination effects of half dose imatinib and DMC on HT-29 human colorectal cancer cell line. According to our knowledge, this study is the first to investigate the combinatory anticancer effects of imatinib and DMC for colorectal cancer treatment.

MATERIALS AND METHODS

Cell culture and drug treatment

The human CRC cell line HT-29 (purchased from Iranian Biological Resource Center, Tehran, Iran) was routinely grown in Dulbecco's Modified Eagles Medium (DMEM) (ATOCEL, Australia) supplemented with 10% fetal bovine serum (FBS) (ATOCEL, Australia) and penicillin / streptomycin (ATOCEL, Australia). Imatinib (Cayman Chemical Co., USA) and DMC (Sigma-Aldrich Co., USA) were freshly dissolved in dimethyl sulfoxide DMSO (the drug vehicle) to yield a stock solution of 20 mM. The concentration of drugs was selected on the basis of their IC_{50} values obtained by3-(4,5-dimethy-lthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay in our previous work which were 6.60 µM for imatinib and 23.45 µM for DMC (20). Briefly, 100 µL medium including 5×10^3 HT-29 cells were seeded in each well of a 96-well plate. At 24 h after seeding, the cells were washed with phosphate-buffered saline (PBS) and treated with 7µM imatinib, 24 µM DMC alone and their half dose combinations; imatinib (3.5 µM) + DMC (12 µM). An equal volume of DMSO was added to the control wells.

Measurement of caspase-3 enzyme activity

The caspase-3 colorimetric assay kit (Abnova, Taiwan) was used to determine the caspase-3 enzyme activity of the cells. Briefly, after 24 h treatment of HT-29 cells with mentioned drugs, the medium was removed and cells were collected by centrifugation at 14,000 rpm for 5 min. Then, 50 µL of cell lysis buffer (10 mM Tris–HCl pH 7.6, 150 nM NaCl, 5 mM EDTA, 1% Triton X-100) was added to the cells. After the lysation, cells were kept on ice for 10 min and centrifuged at 10,000 Rcf for 1 min. Protein concentration was determined with the Bradford method (21). Samples were dyed with coomassie brilliant blue G-250 (Serva Electrophoresis GmbH, Heidelberg, Germany), and the measurements were performed at 595 nm using a BioTek spectrophotometer (USA). Into each well of a 24-well plate, 50 µg proteins were diluted by adding cell lysis buffer. 50 µL 2× reaction buffer (containing 10 mM dithiothreitol) was added to each well then 5 µL DEVD-*p*NA 4 mM 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 microtiter plate reader.

RNA extraction and cDNA synthesis

For extraction of total RNA about 5×10^5 HT-29 treated cells were harvested in PBS and centrifuged at 12000 rpm for 15 min. Total cellular RNA was isolated from cells using the total RNX-Plus solution kit (CinnaGen Co., Iran) according to the manufacturer's protocol. 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. Complementary DNA (cDNA) was prepared from 2 µg of total RNA using the 2-steps RT-PCR kit with M-MuLV RT/Taq DNA polymerase andRTPL12-100app (Vivantis, Malaysia) according to the manufacturer's instructions. We designed caspase-3 specific and β-actin primers (as an invariant housekeeping gene). The sequences of the primers and the respective conditions used are shown in Table 1. For amplification by PCR, cDNA was subjected to 30 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 59 °C, and extension for 30 s at 72 °C. The RT-PCR products were then visualized by ethidium bromide after electrophoresis on 2% agarose gel.Also, the synthesized cDNA was directly used as template for real time RT-PCR using Bio-Rad iQ5 cycler sequence detection system (Bio-Rad Laboratories Inc.).

Table 1. Sequences of primers used to evaluate expression of β-actin and caspase-3 genes in HT-29 cell line.

Target gene	Primer sequence	Product size (bp)	T (°C)
β-actin-forward	5′-CTGGAACGGTGAAGGTGACA-3′	161	59.3
Reverse	5′-TGGGGTGGCTTTTAGGATGG-3′		
Caspase-3-forward	5′-AGAACTGGACTGTGGCATTGAG-3′	191	59.9
Reverse	5′-GCTTGTCGGCATACTGTTTCAG-3′		

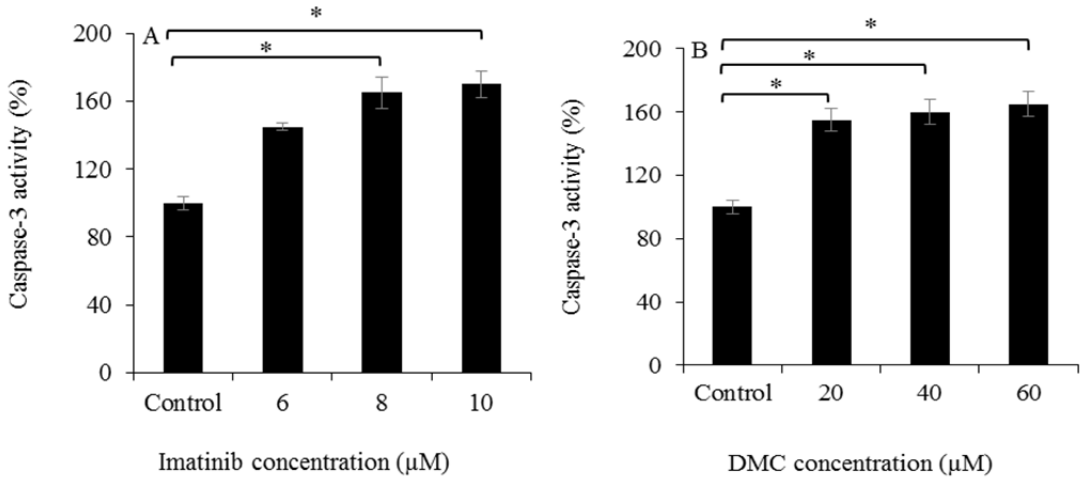

Fig. 1. Effect of (A) imatinib and (B) DMC on HT-29 cell apoptosis. HT-29 colorectal cancer cells were treated with 4, 6, and 8 μM of imatinib or 20, 40, and 60 μM of dimethyl celecoxib (DMC) for 24 h. Apoptosis induction was measured by caspase-3 assay compared to untreated cells (control). The data are depicted as means ± SEM. Asterisks indicate sample that is significantly different compared to other samples using analysis of variance. (*P< 0.05).

Real time PCR of caspase-3 mRNA

Real time RT-PCR was performed by using AccuPower®2× GreenStar qPCR master mix(Bioneer, South Korea) in a total volume of 25 μL according to the manufacturer's instructions. The reactions were prepared in a 96-well optical plate for 10 min at 95 °C followed by 40 cycles of 20 s at 95 °C and 45 s at 59 °C. In addition, a no-template control was used to test the potential contamination and primer dimer formation. The relative expression of each mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, where Ct is the threshold cycle (22). Relative expression levels of mRNA were normalized to β-actin and analyzed for statistical significance with one-way analysis of variance using SPSS 16 statistical analysis software.

Statistical analysis

The results were expressed as the mean ± SEM 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. The level of significant difference was set at P< 0.05. The fold differences of gene expression normalized to the control was presented graphically in the form of histograms, using Microsoft Excel computer program.

RESULTS

Caspase-3 activity of HT-29 cells treated with imatinib, DMC, and their combinations

As shown in Figs. 1A and 1B, imatinib and DMC produced dose-dependent caspase-3 activity induction in HT-29 cell lines. Based on our results, 4 μM imatinib showed insignificant difference compared to control treatment. However, treatment at 6 and 8 μM increased caspase-3 activity significantly (P< 0.01), (Fig. 1A). Treatment with 20, 40, and 60 μM DMC for 24 h showed significant difference compared to control treatment (Fig. 1B). Treatment with 6 μM imatinib had no significant difference in comparison to 8 μM imatinib and also there was no significant difference between DMC-treated groups (40 and 60 μM). Therefore, to study the combined

effects of these drugs, we used lower effective doses of them (half dose of IC_{50} values that previously approved with MTT assay (20)).

The most potent apoptotic effects in combination treatment were observed at a concentration of 3.5 µM imatinib and 12 µM DMC which was statistically significant compared to control (Fig. 2). Treatment with imatinib (3.5 µM) and DMC (12 µM) for 24 h increased apoptosis to 76% versus control ($P<$ 0.001). The combined treatment showed more apoptosis induction than each of the drugs alone compared to control (50% for 7 µM imatinib and 38% for 24 µM DMC).

Effect of imatinib in combination with DMC on caspase-3 gene expression

Based on the preliminary results, a combination of 3.5 µM imatinib and 12 µM DMC were used for complementary tests. According to real time RT-PCR results, there was an approximately 2-fold and 1-fold increase of caspase-3 mRNA as a result of 7 µM imatinib ($P = 0.05$) and 24 µM of DMC ($P<$ 0.05) treatment compared to the vehicle-treated control group in HT-29 cells, respectively. However, the combined treatment with 3.5 µM imatinib and 12 µM DMC noticeably increased the level of caspase-3 mRNA in HT-29 cells(3 fold) as compared to the untreated control group ($P<$ 0.001)(Fig. 3).These results indicate that imatinib increased caspase-3 mRNA expression more effectively when combined with DMC.

Results of present study also pointed towards possible caspase-dependent actions of imatinib and DMC.

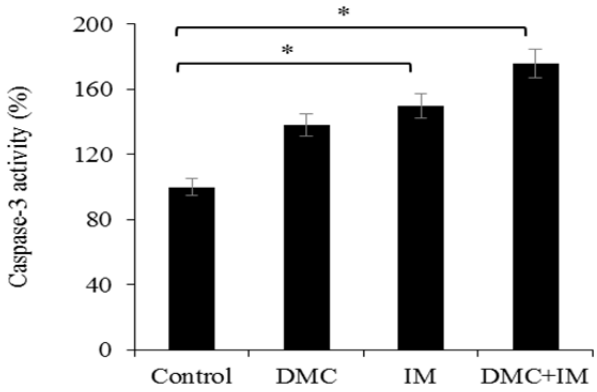

Fig. 2. Effect of imatinib in combination with DMC on HT-29 cell apoptosis. HT-29 CRC cells were treated for 24 h with 7 µM of imatinib or 24 µM of dimethyl celecoxib (DMC) alone and half dose of imatinib (3.5 µM) plus DMC (12 µM) combination. Apoptosis induction was measured by caspase-3 assay compared to untreated cells (control) ($P <$ 0.05), *$P<$ 0.05 versus control.

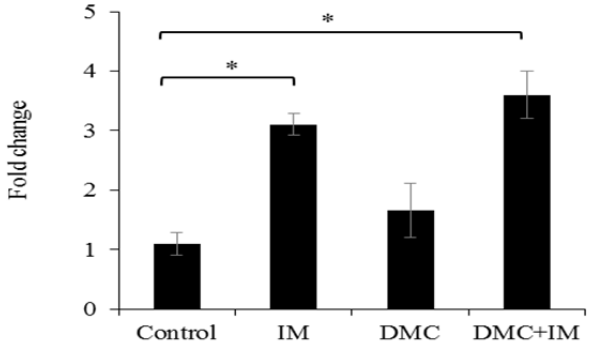

Fig. 3. Real-time quantitative RT-PCR analysis to determine the effects of imatinib (IM) (7 µM), dimethyl celecoxib (DMC) (24 µM) alone and in combination with each other (IM 3.5 µM + DMC 12µM) on caspase-3 mRNA levels with β-actin as the internal control. Vertical bars indicate the mean fold changes ± SEM for three independent experiments in each treatment group. Imatinib and its half dose combination with DMC significantly increased the expression of caspase-3 mRNA compared to untreated HT-29 cells for 24 h *$P<$ 0.05 versus control.

DISCUSSION

In the current study, we observed that imatinib and its combination with DMC significantly increased caspase-3 enzyme activity. Apoptosis effects of imatinib in combination with DMC associated with increase in caspase-3 mRNA levels in HT-29 colorectal cancer cells. Inhibition of HT-29 cell proliferation and apoptosis induction by imatinib has been shown in previous studies (8,9). Also DMC's anti-proliferative and apoptotic effects have been shown in tumor cells *in-vitro* and *in-vivo* (23). We didn't find any study about the effect of imatinib in combination with DMC on HT-29 colorectal cancer cells. Stahtea, *et al.* proved that imatinib with 5-Fluorouracil combination significantly enhances the growth inhibition of the highly tumorigenic HT-29 cells (9). Accordingly Abdel-Aziz, *et al.* showed that selenite-imatinib combination was more effective than single drugs in increasing caspase-3 activity, apoptosis induction and reducing bcl-2 expression in HCT116 cells (7). Shi, *et al*. showed imatinib with tetrandrine combination obvious synergistic effect, probably due to up-regulation of caspase-3 mRNA and protein expressions, and down-regulation of bcl-2 mRNA and protein expressions (24). Baran, *et al*. also showed apoptosis induction through loss of mitochondrial membrane potential and increases in caspase-3 enzyme activity by fludarabine and imatinib combination in human K562 chronic myleloid leukemia cells (25).

Combination therapy seems to have the potential to be more effective than single agent imatinib in the prevention of imatinib resistance (26,27) and recurrent dose interruption (28,29) which have been shown by imatinib monotherapy. Besides the use ofappropriate combination of these drugs might be effective in reducing the side effects ofimatinib (30). In the present study, we have shown comparable effect of half dose combination with full dose monotherapy. According to our findings, imatinib alone and in combination with DMC significantly up-regulatedcaspase-3 gene expression and activity. Then the enhanced potency of DMC in our study seems to be mediated, at least in part, by caspase-3-dependent mechanism. It is especially noteworthy that DMC showed augmenting effects with imatinib on apoptosis in HT-29 cells at low concentration of DMC and imatinib (half dose of IC_{50}).

Our previous study also showed that imatinib-celecoxib combination effectiveness in HT-29 colorectal cancer cells (31). DMC mimics all of celecoxib's numerous anti-cancer effects despite its inability to inhibit COX-2 (16).In the present study, imatinib in combination with DMC (half dose of IC_{50}) showed remarkable potent effects on HT-29 colorectal cancer cells which was more noticeable than imatinib in combination with celecoxib (half dose of IC_{50}). DMC (in low dose alone) and especially in combination with imatinib might not cause the serious side effects that recently developed with the prolonged use of coxibs such as celecoxib that inhibit COX-2 (13,14). The increased effectiveness of imatinib in combination with DMC should be considered as advantages in clinical practice. In contrast to celecoxib, DMC has not yet been studied in humans and evaluation of the *in vitro* mechanism should be followed by appropriate preclinical assays.

CONCLUSION

We conclude that imatinib and especially its combination with DMC has a role in apoptosis induction through caspase-3 pathway in colorectal cancer cells which was confirmed by up-regulation of the caspase-3 expression. Therefore, these findings provide further perceptions to the possible use of DMC in combination with other drugs without the undesired side effects related to mono-targeting, although results require further investigation especially before using this combination in clinical practice.

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