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Cytotoxic effects of dimethyl-celecoxib (DMC) on bone tumor cell line MG63 and cell line HEK293

Tahereh Naji[1]*, Maryam Fatehi[2], Rahim Ahmadi[3], Morteza Ghahramani[2], Mahla Khalaji[2]

1 Associate professor, Department of Basic Sciences, faculty of pharmacy and pharmaceutical sciences, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.
2 Department of Basic Sciences, faculty of pharmacy and pharmaceutical sciences, Tehran Medical Sciences, Islamic Azad University Tehran, Iran.
3 Assistant of Professor, Department of Physiology, Hamedan Branch, Islamic Azad University, Hamedan, Iran.
Corresponding Author: naji_t@iaups.ac.ir / tnaji2002@gmail.com Tel:09123704676
Fax: 02122602059

Tahereh Naji
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Abstract

Purpose: Dimethyl-celecoxib (DMC), is a celecoxib (CXB) derivative has no inhibitory function on cyclooxygenase-2 (COX-2) and displays antitumor properties. This substance can be helpful in advancing the treatment of COX-2-indipentent cancers. In this study, we assayed the efficacy of DMC on MG63 bone tumor and Human embryonic kidney (HEK293) cell lines.

Methods: The cellular viability, nitric oxide content, and Inducible nitric oxide synthases (iNOS) gene expression were measured respectively with MTT (The MTT assay is a colorimetric assay for assessing cell metabolic activity.) Griess reaction, and real-time Reverse transcription polymerase chain reaction (RT-PCR) procedures. IC50 was determined by MTT assay, and iNOS gene expression was evaluated by RT-PCR. Also, monoxide nitrogen production was monitored by a Griess test and finally one-way Anova (Analysis of Variance (ANOVA) is a statistical formula used to compare variances across the means (or average) of different groups.) and T-test were used to analyze the data.

Results: The Results clearly showed that Dimethylcelloxib at concentrations of: 62.5, 125, 250, 500 µg /ml at 48 h significantly decreased the survival rate of MG63 and HEK-293 tumor cells ($P<0.001$). It was found that iNOS gene expression decreased significantly ($P<0.001$) and production of monoxide nitrogen molecule had a significant increase ($P<0.01$ and $P<0.001$). Conclusion: Our research showed that Since Dimethylceloxib reduced iNOS gene expression, it is expected to decrease nitrogen monoxide, but this drug showed its cytotoxic effects through increased nitrogen monoxide production.

Keywords: Bone tumor, Dimethyl-celecoxib, HEK293, MG63

1. Introduction

In recent years, physicians have witnessed a remarkable shift in the connection between cancer and bone in bone metastasis incidence. Attempts have been led in osteooncology (1). Thus, there is a great demand to detect novel therapeutic procedures for the treatment of bone cancer diseases. So far, effort has been made to identify the cancer regulators, leading to cyclooxygenase-2 (COX-2), which acts as a key inflammatory regulator (2). It overexpresses in a variety of cancers and plays a role in tumor aggressiveness by contributing to the survival and proliferation of cancer cells. The distinct suppressors for COX-2 including the “coxib” family are known as potent candidates for various cancer treatments (3). These suppressors can also sensitize tumors to photodynamic therapy, radiotherapy, or chemotherapy (4).

Despite these favorable impacts, the chronic application of the suppressors has been shown to be related to acute side effects, such as enhanced risks of myocardial or stroke infarction and gastric ulcers. Furthermore, the antitumor features of the suppressors do not necessarily depend on 2-cyclooxygenase. Taken all together, these data suggest that precautions have to be taken for chronic application of cyclooxygenase-2 in the treatment of various cancers such as bone cancers diseases. Non coxib celecoxib analogs such as 2, 5-dimethyl-celecoxib (DMC) present promising approaches for cancers therapy without any generic side effect (5). A variety of research has revealed that DMC decreases the growth of cancer cells more effectively than COX-2 suppressors (6). These anticancer attributes are related to the blockage of tumor angiogenesis and invasion, cell cycle changes, as well as apoptosis induction (7). The apoptosis induction has been documented to be related to AKT (protein kinase B)/PI3K signaling suppression and survival signaling downstream paths (kB nuclear factor) (8), and more recently to endoplasmic reticulum stress, displaying that DMC induces apoptosis by various mechanisms. The mitochondrial incidents occurring within the apoptosis process depend on the activation of proapoptotic proteins Bax and Bak, which could be hindered by heterodimerization with antiapoptotic proteins including Bcl-xL and Bcl-2 .DMC can decline the expression of Bcl2 gene (9).

Only a few studies have been carried out to figure out the efficacy, but it seems that 2, 5-dimethyl-celecoxib results in downregulation of cyclin B and A, which are known as key regulators in the process of G2/M transition (7).

Notably, NOS molecule is one of the molecules involved in the inflammation process and it contains three isoforms iNOS, Endothelial NOS (eNOS) and Neuronal nitric oxide synthase (nNOS). eNOS and nNOS are expressed in epithelial cells and neurons, respectively, and are calcium-dependent whereas iNOS is produced by different cells and is independent of NO. Given the documented cytotoxic effects in previous studies, we evaluated the efficacy of DMC on MG63 bone tumor cell line and HEK293 normal cell line. In this regard, the cellular viability, nitrogen monoxide content, and nitric oxide synthases (iNOS) gene expression were measured using MTT, Griess reaction, and real-time RT-PCR procedures respectively (10).

Materials and methods

Cell Culture and Reagents

The bone tumor cell line MG63 and the normal cell line HEK293 were obtained from the Cell Bank of the Pasteur Institute of Iran. These cell lines were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine (Gibco-Invitrogen), 10% (v/v) fetal calf serum (Gibco-Invitrogen), and 1% (v/v) antibiotic-antimitotic solution. The cultures were maintained in a humidified atmosphere with 5% CO2 at 37°C. Dimethyl-Celecoxib (DMC) was obtained from Aminsan and dissolved at 20 mM in 100% dimethyl sulfoxide (Sigma). Trypsin and BSA were purchased from Sigma.

MTT assay

The cytotoxic effect of Dimethyl-celecoxib (DMC) on bone tumor cell line MG63 and normal cell line HEK293 was assayed via the MTT test,

the mechanism of which is the reduction of MTT salt to formazan crystals via mitochondrial dehydrogenases in live cells (11). The outcome of this activity is the formation of soluble crystals of purple formazan soluble in dimethyl sulfoxide (DMSO). To optimize the concentration of DMC in the culture medium, the bone tumor cell line MG63 and normal cell line HEK293 were exposed to a serial dilution of DMC (500 to 1500μM) along with 0.1% dimethyl sulfoxide for seven consecutive days. After 240 minutes of incubation at 37°C, 100 ml of dimethyl sulfoxide was added and then the optical density (OD) of each well was measured using a microplate reader (Bio-Rad, Hercules.CA) at 570 nm. Finally, the percentage of cell death was calculated as follows: The percentage of cell death is calculated as (A-B)/A*100, where, A is the negative control OD, and B is the sample OD after DMC treatment (12).

Quantitative real-time RT-PCR

The mRNA level of iNOS in the studied samples was estimated using qRT-PCR technique (13). Briefly, total RNA was extracted from the studied samples through Trizol reagent and then dissolved in water containing diethyl pyro carbonate (DEPC) according to the manufacturer's instructions. Subsequently, cDNA was synthesized from 2μg of RNA and qRT-PCR was performed using ABI 7300 qRT-PCR apparatus (Applied Bio system Foster City). The PCR was set up by mixing 10μl of SYBR green PCR 10X master mix, 1 μl of cDNA,1 μM of each reverse and forward primer (Table 1), and PCR-grade water in 20 μl reaction volume. The amplification process consisted of to the following steps: step 1-denaturation for 4 minutes at 94°C; step 2- denaturation for 30 seconds at 94°C; step 3- annealing for 40 seconds at 60°; step 4- extension for 30 seconds at 72°C and step 5- analysis of melting curve. Steps 2–4 were repeated for forty cycles. Amplifications were carried out in triplicate for each gene. The specificity of the respective amplicon was verified by analyzing of its melting curve. The threshold cycle standards resulting from the above runs were used to measure the expression fold change of the studied genes using REST384 software Ver. 2.0. Normalization of gene expression was performed relative to the house-keeping gene β-actin and then plotted as relative expression change as compared to the control (12).

Nitric oxide assay

Nitric oxide assay was performed by using Griess reaction (14). To measure the concentration of nitrite and total nitrate (NOx), 100 μL of serum was deproteinized and then 100 μL of vanadium chloride (III) (8 mg/ml) was added to convert the nitrates to nitrite. Then 100 μL of mixture (1 to 1) of sulfonamide and naphthyl ethylenediamine dihydrochloride (NEDD) was incorporated and incubated for 30 minutes at 37°C. After reaction and dye formation, light absorption from dye material was read at 540 nm by ELISA reader and the concentration of samples was estimated using standard curve (15).

Table 1. Primer sequences of iNOS and GAPDH

primers	Forward (5′-3′)	Reverse (5′-3′)
iNOS	GTGCCCTGCTTTGTGCG	TCCTCCTGGTAGATGTGGTCCT
GAPDH	CCCACTCCTCCACCTTTGAC	CATACCAGGAAATGAGCTTGACAA

Statistical Analysis

All data for this experiment have been presented as mean ± SD. We used one-way analysis of variance (ANOVA) and a t-test, and experiments selected 0.05 as statistically significant.

Results

To investigate the cytotoxic effects of 2, 5-Dimethyl-celecoxib (DMC) on the MG63 tumor cell line, different concentrations of DMC were prepared and exposed to the cells for 48 hours. In terms of the MG63 tumor cell line, statistical analysis showed that DMC at concentrations of 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 mg/l for 48 hours reduced cell viability compared to the control group by 26.26, 25.86, 26.42, 93.68, and 88.03 µg/ml, respectively. The difference was significant at concentrations of 500, 250, 125, and 62.5 mg/l (P<0.001) (Figure 1).

According to nonlinear regression and Excel software, the IC50 of DMC was determined as 40.7 mg/ml for MG63 tumor cell line. In terms of the HEK cell line, it appeared that DMC at concentrations of 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 mg/l for 48 hours decreased t cell viability compared to the control group by 4.6, 5.1, 5.5, 8.4, 96.1, and 105.1, respectively. There was a significant difference at concentrations of 500, 250, 125, and 62.5 mg/l (P <0.001) (Figure 2).

Based on the nonlinear regression and Excel software, the IC50 of DMC was determined as 46.8 mg/ml for HEK cell line. To evaluate the effects of DMC on iNOS expression in MG63 bone tumor cell line and HEK normal cell line, they were treated with IC50 DMC and then the change in iNOS expression was determined.

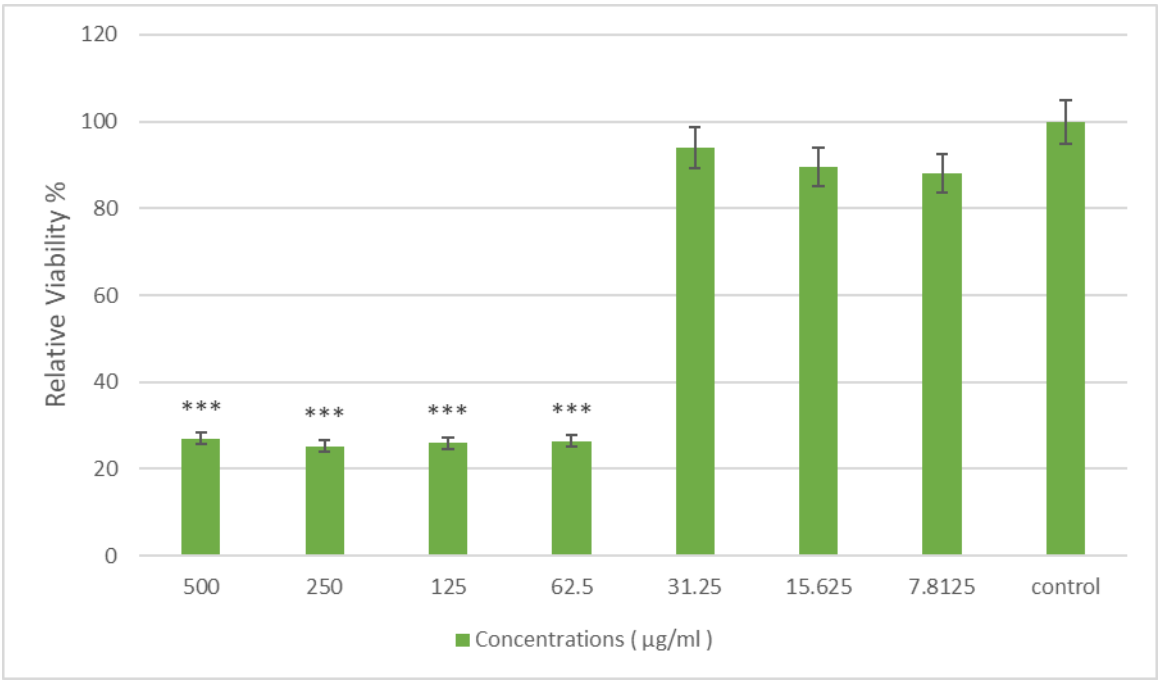

Fig 1. The effect of different concentrations of DMC on the MG63 tumor cell line by MTT in 48hours. Data are expressed as percentage of survival mean ± SEM. *** indicates a significant difference between the treated group and the control group with P<0.001.

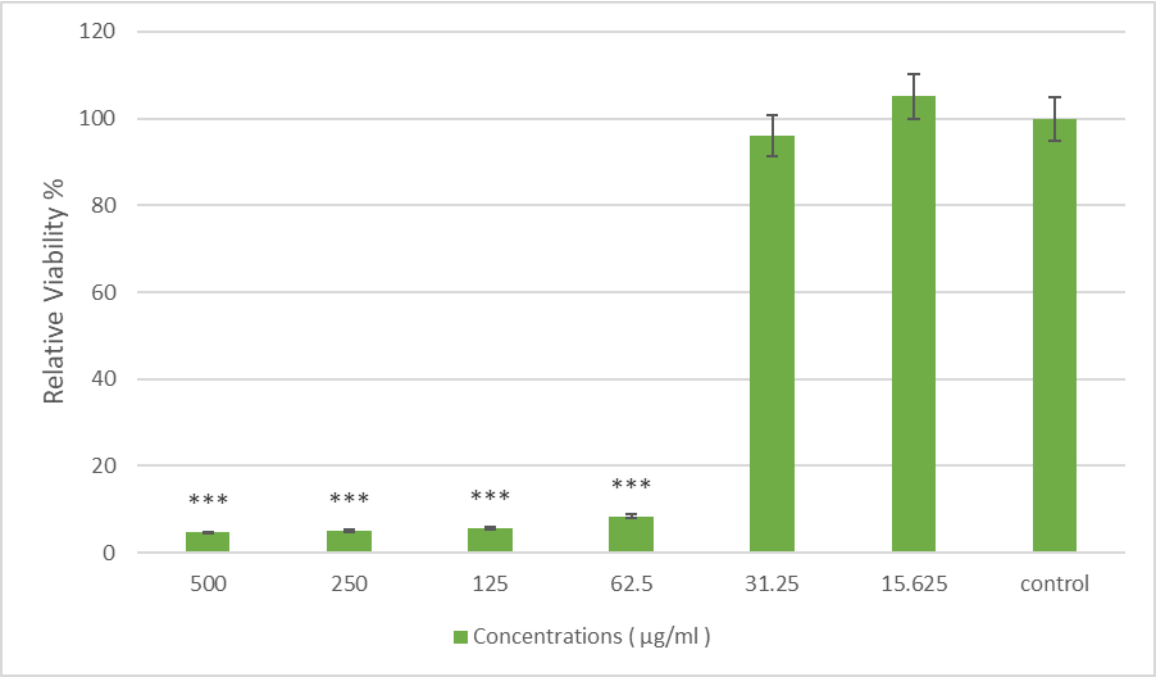

Fig .2 The effect of different concentrations of DMC on the HEK cell line by MTT in 48 hours. Data are expressed as percentage of survival mean ± SEM. *** indicates a significant difference between the treated group and the control group with P<0.001 .

The results of the evaluation of iNOS expression on the MG63 cell line treated with DMC compared to the control group are shown in Figure 3. Statistical analysis showed that iNOS expression was significantly decreased in MG63 cells treated with DMC compared with the control group (P<0.001).

The results of the one-way ANOVA analysis showed that the amount of nitric oxide produced in the supernatant of MG63 cells treated with IC50 dose of DMC (40.7 mg/ml) significantly increased compared to the control group (Figure 4).

Furthermore, the NO amount in the supernatant of HEK cells treated with IC50 dose of DMC (40.7 mg/ml) remarkably rose in contrast to the control group (Figure 5).

Discussion

Among the category of COX2 suppressors (coxibs), celecoxib (CXB) appears to be unique as it has an additional role that is independent of its well-known COX-2 inhibition ability (15).

This second role involves stimulating cell death and inhibiting cell proliferation at a much lower concentration compared to other selective COX-2 inhibitors. Interestingly, these two roles are controlled by different parts of the CXB molecule and can be separated (16). One derivative, 2, 5-dimethyl-celecoxib (DMC), retains the apoptosis-inducing and anti-proliferative effects but completely loses the COX-2 inhibitory function.

DMC has been shown to mimic all the anti-tumor effects of CXB that have been tested so far, including tumor growth inhibition and reduction of neovascularization, both in vivo and in vitro (insert appropriate references here). Given the life-threatening side effects of CXB, exploring the potential benefits of derivatives like DMC for the treatment of anticancer diseases is advantageous. It should be noted that DMC may be particularly useful in the treatment of COX-2-independent tumors, as it lacks the COX-2 inhibitory function but still retains the anticancer properties of CXBs (15).

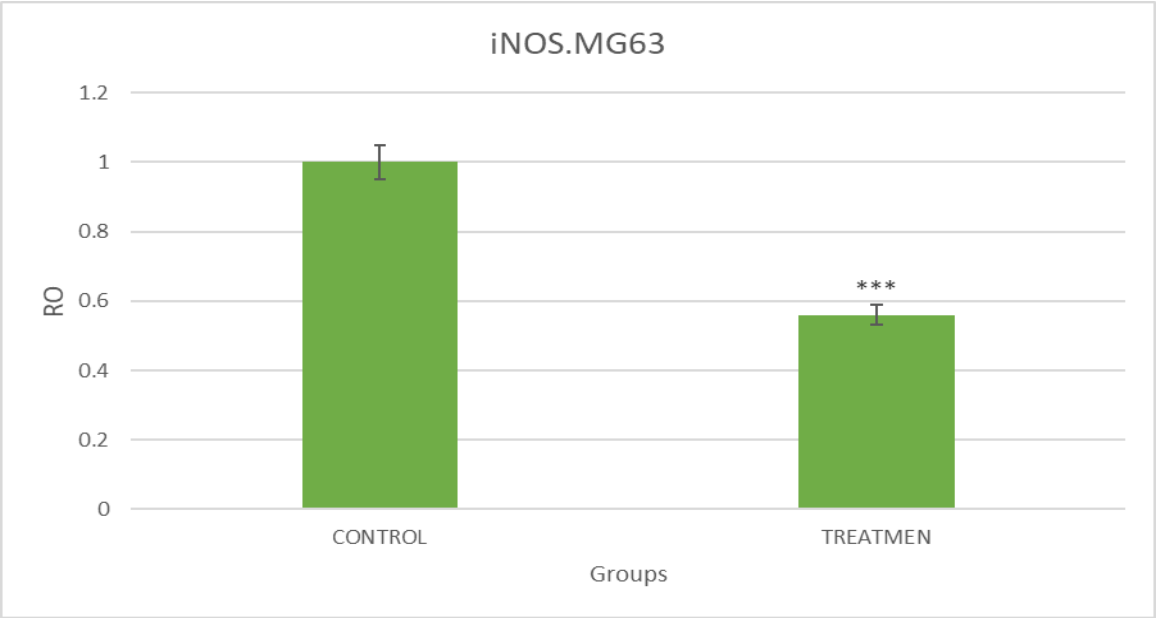

Fig .3 The effect of IC50 DMC [40.7 mg/ml] on the iNOS expression in MG63 tumor cell line by MTT in 48 hours. Data are expressed as percentage of survival mean ± SEM. *** indicates a significant difference between the treated group and the control group with P<0.001

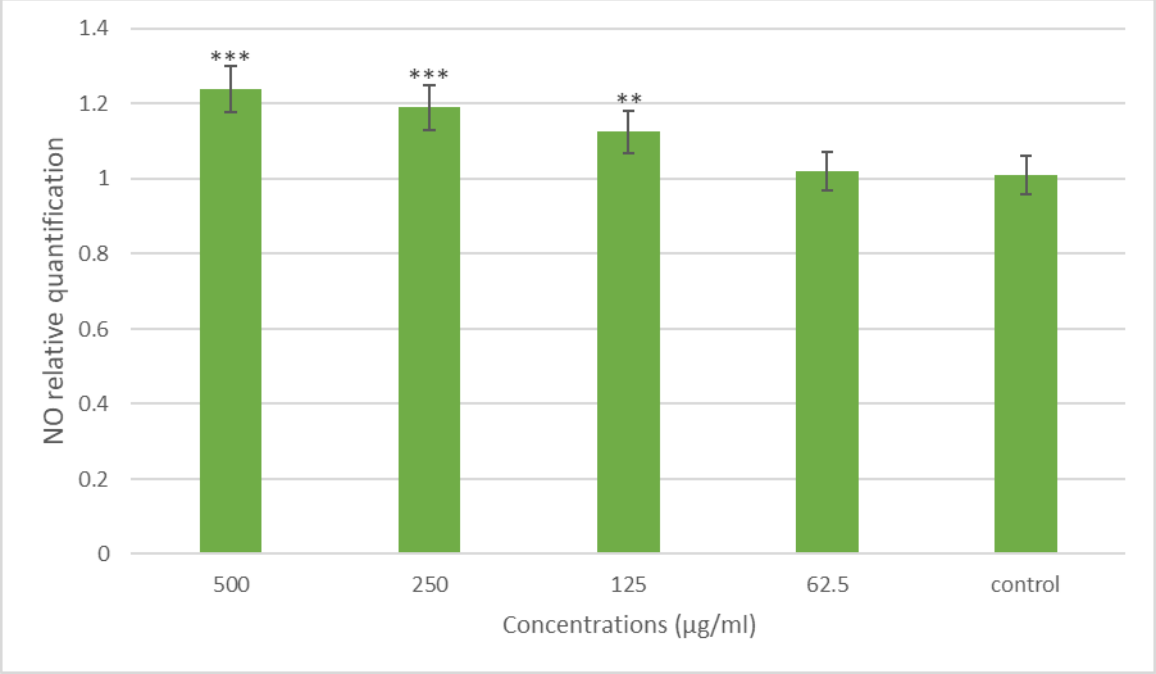

Fig .4 The effect of IC50 DMC [40.7 mg/ml] on the nitrogen monoxide amount in MG63 tumor cell line by MTT in 48 hours. Data are expressed as percentage of survival mean ± SEM. ** and ***indicate a significant difference between the treated group and the control group with P<0.01 and P<0.001, respectively.

In the current study, DMC appeared to be potent in inhibiting the growth of MG63 tumor bone cells, with a half maximal inhibitory concentration (IC50) of 40.7 mg/ml at 48 h. It was demonstrated that CXB significantly hindered the cell proliferation of the MG-63 cell line in a concentration- and time-dependent manner within a limited range of concentrations (0-80 µg/ml). The IC50 dose of CXB for 48 h and 24 h treatment was approximately 19 µg/ml and 48 µg/ml, respectively. The expression of the iNOS gene was significantly decreased in the MG63 cell line treated with DMC compared to the control group ($P<0.001$). Niederberg et al. suggested that iNOS expression was slightly enhanced at a concentration of 200 mg/kg of CXB in their study (17). They also found that the effect of this compound on iNOS gene expression in rats was similar to what was detected in cell culture, suggesting that the mechanisms observed in vitro also occur in vivo. Furthermore, the amount of nitric oxide in the supernatant of the MG63 cell line treated with DMC significantly increased compared to the control group ($P<0.001$). Our findings suggest that DMC may exert at least part of its anticancer effects through the down-regulation of the iNOS gene and the increase in nitric oxide content. The mechanism underlying this phenomenon can be explained by the cytotoxic ability of DMC to enhance NO production and subsequently induce DNA fragmentation through NO invasion.

In previous studies investigating the mechanisms involved in the cytotoxic effects of CXB and its derivatives on cancer cells, Zhou et al (2015) observed that CXB inhibits the proliferation of the MG-63 cell line by inducing apoptosis and arresting cells in the S-phase .They suggested that CXB -induced up-regulation of RECK and down-regulation of MMP-9 and MMP-2 are associated with alteration in the local tumor microenvironment and apoptosis induction. Therefore, the authors proposed that CXB can up-regulate RECK to inhibit the expression of MMP-9 and MMP-2, thereby exerting its anticancer effects (16). In a similar study conducted by Liu et al. (2008), MG-63 cells were treated with CXB for 48 hours in a medium supplemented with serum (18). The results showed that CXB effectively inhibited cell growth in a dose-dependent manner by regulating the G1 phase of the cell cycle. Additionally, CXB induced apoptotic changes such as the formation of apoptotic bodies and DNA fragments (19). The study also demonstrated a decrease in the expression of various proteins including Bcl-2, survivin, Akt/PI3K, procaspase-3, and procaspase-9. Based on these findings, the authors suggested that CXB exerts its anti-tumor properties through mechanisms independent of COX-2, potentially involving pathways such as Bcl-2, survivin, and Akt/PI3K (20). They also hypothesized that PI3K may play a central role in the functions of CXB, as it regulates Bcl-2 and survivin. However, further research is required to fully elucidate the precise mechanism underlying the cytotoxic effects of the DMC compound on cancer cells (21).

Conclusion

In conclusion, the study suggests that DMC, a derivative of CXB, may have potential benefits in the treatment of anticancer diseases. Unlike CXB, DMC does not have the life-threatening side effects associated with COX-2 inhibitors. The study found that DMC decreased the expression of Nitric oxide synthases genes and increased nitric oxide content, indicating its potential anticancer diseases. Unlike CXB, DMC does not have the life-threatening side effects associated with COX-2 inhibitors. The study found that DMC DMC

expression of Nitric oxide synthases genes and increased nitric oxide content, indicating its potential anticancer properties. The cytotoxic capability of DMC in increasing nitric oxide production and inducing DNA fragmentation through nitric oxidase radicals may contribute to its anticancer effects. However, further research is needed to fully understand the exact mechanisms involved in the cytotoxic effects of DMC on cancer cells.

Abbreviation List

Bone tumor: Bone tumor develop when cells within a bone divide uncontrollably, forming a lump or mass of abnormal tissue

Celecoxib: Non-steroidal anti-inflammatory drug

MG63: MG-63, a cell that has fibroblast morphology isolated from the bone of a White, 14-year-old male patient with osteosarcoma

Hek293: Human Embryonic Kidney (HEK) 293 is a cell line commonly used for Biopharma and Basic Medical research and therapeutic solutions. Generated in 1973, they are derived from human embryonic kidney cells and are popular, in part because of their reliable growth in culture and for their propensity for transfection.

iNos: is one of three key enzymes generating nitric oxide (NO) from the amino acid L-arginine

Ethical Issues

Ethical approval for this study was given by the Tehran Medical Sciences, Islamic Azad University. (IR.IAU.PS.REC.1398.224).

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Conflict of interest

The Authors declare that they have no conflict of interests.

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