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Original Research Article

Imidazole-dione conjugate induces apoptosis and inhibits proliferation of osteosarcoma cells via activation of p65NF-κB

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Abstract

Purpose: *To investigate the effect of imidazole-dione conjugate (IMC) on proliferation of MG63 osteosarcoma cells.*
Methods: *The effect of IMC on proliferation of MG63 osteosarcoma cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, while mRNA expressions of PTEN, FasL and FasR were assayed with real-time reverse transcription polymerase chain reaction (RT-PCR). Cell apoptosis was studied by flow cytometry. The protein expression level of IκBα was determined using western blotting.*
Results: *There were reductions in the proliferation of IMC-treated MG63 cells and Saos-2 cells at IMC dose of ≥ 4 μM ($p < 0.05$). Degree of proliferation of MG63 cells on exposure to 1, 2, 4, 6, 8 and 10 μM IMC was 99, 98, 76, 59, 34 and 21 %, respectively, relative to 100 % in untreated cultures. In MG63 cell cultures, treatment with 4, 6, 8 and 10 μM IMC led to 22, 39, 62 and 69 % apoptosis, respectively, when compared with 0.9 % apoptosis in control cell cultures ($p < 0.05$). Concentration-dependent increases were observed in PTEN, FasL and FasR mRNA in IMC-treated MG63 cells. Western blot assay showed a marked increase in the level of IκBα in MG63 cells following treatment with IMC. IMC treatment also caused a concentration-dependent increase in the expression of phospho-Ser536 p65NF-κB ($p < 0.05$).*
Conclusion: *IMC exerts inhibitory effect on the proliferation of MG63 cells via up-regulation of NF-κB phosphorylation. Thus, IMC may be useful as a therapeutic agent for osteosarcoma.*

Keywords: *Imidazole-dione conjugate, MG63 osteosarcoma cells, Proliferation Phosphorylation, Translocation*

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INTRODUCTION

Osteosarcoma is diagnosed in adolescents with an incidence of about 8 per million, and it` accounts for about 2.5 % of all malignancies [1-3]. Osteosarcoma accounts for more than 60 % of all malignant bone tumors [4,5]. It has been observed that the incidence of osteosarcoma has increased over the recent years [6]. Studies have shown that osteosarcoma originates from mesenchymal tissue where it shows high invasion and migration rates, and very poor prog-

nosis [3,4]. Despite the use of chemotherapeutic agents and surgical interventions, the five-year survival for osteosarcoma patients is less than 70 % [7]. Thus, the development of newer treatment strategies for osteosarcoma is of great clinical importance.

Studies have demonstrated that osteosarcoma causes mutations in genes, leading to the development of various other types of tumors [8,9]. Various investigations have been carried out on to unravel the mechanisms involved in the pathogenesis of osteosarcoma [8-13]. Apoptosis is an important cellular processes for getting rid of unwanted cells [14,15]. Failure of apoptosis leads to cancer, and the induction of apoptosis through the use of various agents is highly important for treatment of various tumors [14,15]. Apoptosis is regulated by the activation of several factors through phosphorylation [16]. Activation of nuclear factor-kappa B (NF-κB) by phosphorylation plays important role in the regulation of apoptosis [17]. After activation, IκBα is released and is subsequently translocated to the cell nucleus where it produces its effect through various genes [17]. In the present study, the effect of IMC on the proliferation of MG63 osteosarcoma cells was investigated. In addition, the effect of IMC on apoptosis and expression of various pro-apoptotic proteins was studied so as to understand the mechanisms involved.

EXPERIMENTAL

Cell culture

MG63 osteosarcoma cell line was obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL) at a temperature of 37 ˚C. The atmosphere in the incubator contained 5 % CO_2 and 95 % air.

Effect of IMC on cell proliferation

To study the effect of IMC on MG63 cell proliferation, MTT (Sigma-Aldrich) assay was used. The MG63 cells at a density of 2 x 10^5 cells per well were seeded in 96-well culture plates in DMEM and incubated overnight. After incubation, the medium in the plates was replaced with new medium containing 1, 2, 4, 6, 8 or 10 µM IMC. The plates were incubated for 48 h, following which 50 µl MTT (5 µg/ml) was added into each well. Incubation was carried out for 2 h, after which DMSO (150 µl) was added to the plates to dissolve the formazan crystals formed. The absorbance of each well was read at 485 nm in a microplate Autoreader (Bio-Tek Instruments Inc., Winooski, VT, USA). The measurements were performed three times independently for each well.

Effect of IMC on apoptosis

The effect of IMC on apoptosis in MG63 cells after IMC treatment was determined using flow cytometric analysis. The cells were incubated separately for 48 h with 4, 6, 8 and 10 µM IMC, and then harvested. They were thereafter washed two times with PBS and subsequently fixed with 70 % ethanol at a temperature of -20 ˚C for 22 h. Then, the cells were stained with propidium iodide (PI) and Annexin V-FITC (BD Pharmingen, San Diego, CA, USA) in accordance with the instructions of the manufacturer. Flow cytometric analysis was carried out with FACSCalibur system linked to CELLQuest software version 3.3 (Becton Dickinson, San Jose, CA, USA).

Real-time reverse transcription polymerase chain reaction (RT-PCR)

The MG63 osteosarcoma cells were cultured in 100-mm dishes at a density of 2 x 10^5 cells in DMEM. The cells were incubated for 48 h with IMC or DMSO (control). Thereafter, total RNA was extracted from the cells using RNeasy Plus Mini kit (Qiagen, Waco, TX, USA). The RNA was reverse-transcribed to cDNA at a temperature of 37 ˚C for 20 min and 85 ˚C for 5 sec using PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.), while qPCR was carried out with SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd.) connected to LightCycler 480 system (Roche Diagnostics, Basel, Switzerland). The reaction mixture consisted of cDNA (2 µl), SYBR Premix Ex Taq II (12 µL), 1 µL of each primer, and 8 µL of diethyl pyrocarbonate water. Initial denaturation for 2 min at 95 ˚C was followed by amplification of PTEN, FasL, FasR and GAPDH over 40 cycles at 92 ˚C for 25 sec, 65 ˚C for 45 sec and 70 ˚C for 45 sec. The reactions were carried out in triplicate using GAPDH as internal control.

Western blot analysis

After treatment for 48 h with IMC, total protein was extracted from MG63 osteosarcoma cells using radioimmuno-precipitation buffer assay (Beyotime Institute of Biotechnology, Haimen, China) and $PhCHSO_2Cl$. The protein was quantified using bicinchoninic (BCA) method in line with commercially available kit (Beyotime Institute of Biotechnology. Then, 30-µg protein

samples were subjected to 8 % SDS-polyacrylamide gel electrophoresis. The proteins were subsequently transferred by electroblotting to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were incubated overnight at 4˚ C with primary antibodies against phosphorylated proteins. Thereafter, the membranes were washed with PBS and 0.05% Tween-20, and incubated for 1 h at room temperature with horse radish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (dilution, 1:20,000). Visualization of the blots was achieved with a chemiluminescence detection kit (Amersham; GE Healthcare Life Sciences, Chalfont, UK), with β-actin as an internal reference.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM, n = 3). Statistical analysis was performed using one-way analysis of variance and Scheffe's post hoc test. All statistical analyses were carried out with SPSS 19.0 software (IBM Corp., Armonk, NY, USA). Statistical significance of differences was fixed at $p < 0.05$.

RESULTS

IMC inhibited proliferation of MG63 and Saos-2 cells

The results in Figure 1 show that IMC reduced the proliferations of MG63 and Saos-2 cells in a concentration-dependent fashion. No marked inhibitory effects on proliferations of MG63 and Saos-2 cell proliferation were observed at 1 and 2 µM IMC. The reduction in MG63 cell proliferation by IMC was significant as from 4 µM IMC. The proliferation of MG63 cells was reduced to 99, 98, 76, 59, 34 and 21 % on exposure to 1, 2, 4, 6, 8 and 10 µM MIC, respectively, relative to 100 % proliferation in untreated cell cultures. Similarly, in Saos-2 cells, exposure to 1, 2, 4, 6, 8 and 10 µM of IMC decreased proliferation to 98, 97, 72, 54, 29 and 20 %, respectively (Figure 1).

IMC induced apoptosis of MG63 cells

Exposure of MG63 cells to IMC caused induction of apoptosis in 48 h (Figure 2). In MG63 cell cultures, treatment with 4, 6, 8 and 10 µM IMC led to 22, 39, 62 and 69 % apoptosis, respectively, while only 0.9 % apoptosis was observed in control cell cultures.

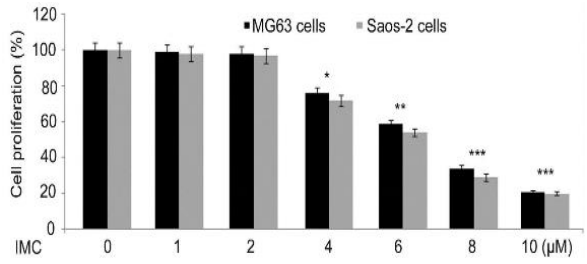

Figure 1: Effect of IMC exposure on proliferation of MG63 and Saos-2 cells. Cultures of MG63 and Saos-2 cells were exposed to 1, 2, 4, 6, 8 and 10 µM IMC and incubated for 48 h. Changes in proliferation were determined with MTT assay. Measurement of proliferation was performed in triplicate; $*p < 0.05$, $**p < 0.02$, $***p < 0.01$, versus control cells

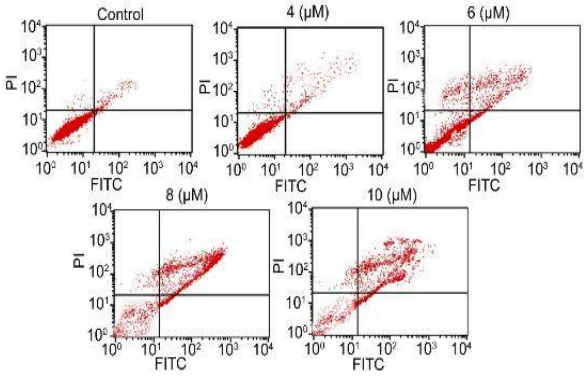

Figure 2: Effect of IMC treatment on apoptosis in MG63 cells. The cell cultures were incubated with 4, 6, 8 and 10 µM IMC for 48 h. Flow cytometry using Annexin V and propidium iodide (PI) was used for determination of cell apoptosis

Exposure of MG63 cells to IMC altered the mRNA expressions of PTEN, FasL and FasR

Results from RT-PCR showed that treatment of MG63 cells with IMC led to concentration-dependent increases in the mRNA expressions of PTEN, FasL and FasR mRNA (Figure 3). The IMC-induced increases in mRNA levels of PTEN, FasL and FasR were maximum at 10 µM IMC.

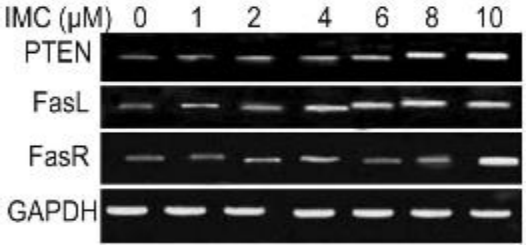

Figure 3: Effect of IMC on the mRNA expressions of PTEN, FasL and FasR in MG63 cells. The cell cultures were exposed to 1, 2, 4, 6, 8 and 10 µM IMC, and were analysed for PTEN, FasL and FasR mRNA levels using RT-PCR. The level of GAPDH mRNA was used as internal control

IMC increased Iκ-Bα level in MG63 cells

Western blot assay showed a marked increase in the level of IκBα in MG63 cells treated with IMC. The increase in IκBα was maximum at IMC dose of 10 μM. Similarly, exposure of MG63 cell cultures to TNF-α at concentration of 10 ng/mL increased IκBα level.

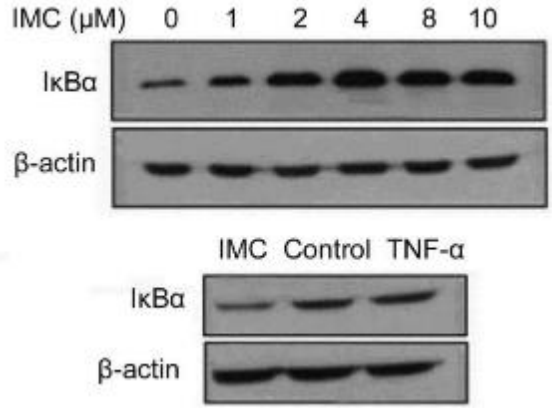

Figure 4: Effect of IMC on the expression of IκBα in MG63 cells. The cells were incubated with 1, 2, 4, 6, 8 and 10 μM IMC and TNF-α (10 ng/mL), and then analysed for IκBα expression using western blot assay

IMC up-regulated NF-κB activation

Treatment of MG63 cells with IMC caused a concentration-dependent increase in the expression of phospho-Ser536 p65NF-κB (Figure 5). The expression of phospho-Ser536 p65NF-κB was maximum at 10 μM IMC. However, there were no significant changes in the expression of phospho-Ser529 in MG63 cells on incubation with 1, 4, 6, 8 and 10 μM IMC (Figure 5).

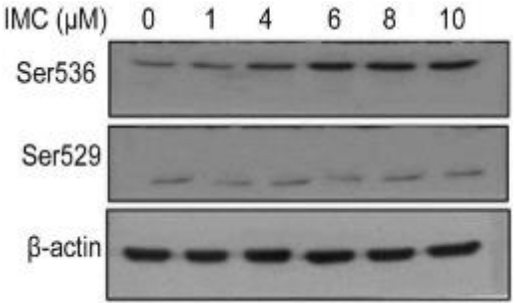

Figure 5: Effect of IMC on NF-κB activation in MG63 cells. Following incubation of the cells with IMC, western blot assay was used for determination of NF-κB activation

IMC caused NF-κB translocation to the nucleus in MG63 cells

Western blotting showed interaction between anti-p65NF-κB and 65-kDa in the cytoplasm of IMC-treated MG63 cells, as well as in control cells (Figure 6). The interaction between anti-p65NF-κB and 65-kDa was observed only in the nuclear fraction of IMC-treated MG63 cells. No such interaction was found in the nuclear fraction of control cells. The expression of phospho-Ser536 p65NF-κB was higher in the cytosolic and nuclear fractions of IMC-treated MG63 cells. Treatment of MG63 cells with TNF-α also increased the expression of phospho-Ser536 p65NF-κB in cytosolic and nuclear fractions.

Incubation of MG63 cells with IMC and TNF-α (10 ng/ml) led to expression of Eps15 in cytosolic fraction alone. The expression of Eps15 was absent in the nuclear fraction of IMC- and TNF-α treated MG63 cells, while Lamin B1 was expressed in the nuclear fraction of IMC and TNF-α treated MG63 cells, but was absent in the cytosolic fraction (Figure 6).

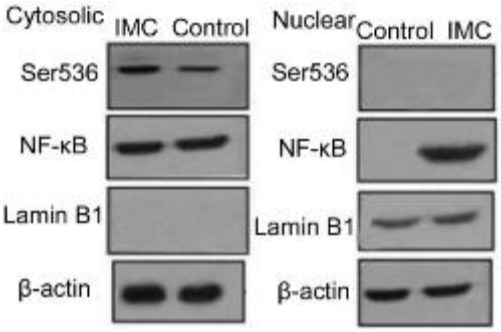

Figure 6: Effect of IMC on NF-κB translocation to nucleus in MG63 cells. After incubation of the cells with IMC or TNF-α, the expression of NF-κB was assayed in the nuclear and cytosolic fractions with western blotting

DISCUSSION

Osteosarcoma alone accounts for more than 60 % of malignant bone tumors, and it has very poor prognosis [10-12]. Over the recent years, it has been observed that the incidence of osteosarcoma has increased at alarming rate [13]. These findings indicate the immense clinical significance of developing novel treatment strategies for osteosarcoma. The present study was carried out to determine the effect of IMC on MG63 osteosarcoma cell proliferation and apoptosis. Proliferation of carcinoma cells leads to formation of cellular mass [10-12].

In the present study, IMC decreased the proliferation of MG63 osteosarcoma cells. This finding suggests that IMC may be of chemotherapeutic importance for osteosarcoma, it motivated further mechanistic investigations. Decreases in osteosarcoma cell proliferation may be due to onset of a cellular process known as apoptosis [14,15]. In the present study, flow

cytometric analysis showed that IMC treatment caused induction of apoptosis in MG63 osteosarcoma cells. The apoptotic cell population increased markedly in cultures treated with IMC, when compared to the control cells. Apoptosis is regulated by various cellular pathways, including the NF-κB pathway [18]. Apoptosis through NF-κB pathway is regulated by the up-regulation of the expressions of PTEN, FasR and FasL [18-20]. In the present study, the levels of mRNA corresponding to PTEN, FasL and FasR were markedly higher in IMC-treated MG63 osteosarcoma cells. This suggests that IMC caused onset of apoptosis in MG63 cells through up-regulation of NF-κB pathway.

Another important process in NF-κB pathway is the activation of p65NF-κB by phosphorylation of serine at 536 position [21]. It has been reported that p65NF-κB activation results from the phosphorylation of serine residue at position 536 [21]. The present study showed that IMC treatment activated p65NF-κB by phosphorylation of serine residue at 536 position. This indicates that IMC exerts its effect by phosphorylation of p65NF-κB in MG63 osteosarcoma cells. Thus, after activation, p65NF-κB was translocated to the nucleus of MG63 cells.

CONCLUSION

The findings of this study show that IMC reduces the proliferation of MG63 osteosarcoma cells by enhancement of their apoptosis and activation of p65NF-κB. Thus, IMC may be of importance in the development of new treatment strategies for osteosarcoma.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this study.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Haixiao Li, Guoping wu, Haizhao Wen and Baoxi Wang performed the experimental work, carried out the literature survey and analysed the data. Sanli Cao and Haixiao Li compiled the data. Shaohui Shi designed the study and wrote the paper. All the authors read the paper thoroughly and approved it for publication.

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