Tropical Journal of Pharmaceutical Research January 2020; 19 (1): 51-56
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i1.8

Original Research Article

Imidazole-thiazolidinone inhibits oesophageal cancer cell proliferation via induction of apoptosis and cell cycle arrest at S phase

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Sent for review: 14 June 2018 *Revised accepted: 11 December 2019*

Abstract

Purpose: *To investigate the effect of imidazole-thiazolidinone on oesophageal cancer (OC) cell proliferation, and the mechanism of action involved.*
Methods: *Human OC cells (HCE-6 and KYSE-1170) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin solution at 37 ˚C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air. After attaining 60 - 70 % confluency, the cells were treated with serum-free medium and graded concentrations of imidazole-thiazolidinone (up to 160 µM) for 24 h. Normal cell culture without imidazole-thiazolidinone served as control. Cells in logarithmic growth phase were selected and used in this study. Cell proliferation and apoptosis were assessed using 3 (4,5 dimethyl thiazol 2 yl) 2,5 diphenyl 2H tetrazolium bromide (MTT), and flow cytometric assays, respectively. The levels of expression of apoptosis-related proteins were determined using Western blotting.*
Results: *Treatment of HCE-6 and KYSE-1170 cells with imidazole-thiazolidinone for 48 h led to significant and dose-dependent reduction in their proliferation, as well as significant and dose-dependent increase in the number of apoptotic cells ($p < 0.05$). Light microscopy revealed significant reduction in HCE-6 cell count, detached cells, reduced cell size and irregular cytoplasmic vacuoles. Imidazole-thiazolidinone treatment significantly and dose-dependently decreased HCE-6 and KYSE-1170 cell migration, and arrested HCE-6 cell cycle at S phase ($p < 0.05$). In HCE-6 cells, imidazole-thiazolidinone treatment significantly and dose-dependently upregulated the expressions of cleaved caspase-3/8/9 and bax, but down-regulated bcl-2 expression significantly and dose-dependently ($p < 0.05$). However, metalloproteinases 2 and 9 (MMP-2 and MMP-9) expressions in HCE-6 and KYSE-1170 cells were significantly and dose-dependently down-regulated by imidazole-thiazolidinone treatment ($p < 0.05$).*
Conclusion: *The results obtained in this study suggest that imidazole-thiazolidinone suppresses OC cell proliferation via induction of apoptosis and arrest of cell cycle at S phase.*

Keywords: *Imidazole-thiazolidinone, Oesophageal cancer, Metastasis, Cell cycle arrest, Apoptosis*

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INTRODUCTION

Oesophageal cancer (OC), a common malignant tumor among the Chinese, arises from the oesophagus [1]. Symptoms often include difficulty in swallowing and weight loss. Oesophageal cancer (OC) is ranked eighth in the hierarchy of malignant tumors, and is the sixth leading cause of cancer-related deaths worldwide [2]. Histologically, OC is of two types: squamous cell cancer (SCC) and adenocarcinoma [3]. Squamous cell carcinoma (SCC) is prevalent among Asians, while adenocarcinoma is common among Europeans [2-4]. Risk factors for OC include smoking, high alcohol consumption, excessive consumption of red meat, and obesity [4]. The insidious nature of OC makes its diagnosis difficult [5]. Surgery, chemotherapy and radiotherapy are strategies commonly employed for OC treatment [6, 7]. These treatment options are accompanied by poor prognosis and the patients have five years survival of less than 15 % [7]. Therefore, there is urgent need for the development of novel and effective treatment for OC.

Heterocyclic compounds are widely used as synthons in drug discovery [8]. Imidazole is the structural component of several complex natural products and therapeutic molecules [9]. Compounds bearing imidazole ring exhibit varied biological and pharmacological properties such as antimicrobial, antitumor, antiviral, and antimalarial effects [10]. Studies have shown that 4-nitroimidazole-containing compounds such as pretomanid and delamanid inhibit the growth of *Mycobacterium tuberculosis* [11]. Delamanid is an FDA-approved drug for the treatment of patients with multidrug resistant tuberculosis (MDR-TB) [11]. Pretomanid is presently under clinical trial for the treatment of tuberculosis (TB) [11]. The aim of this study was to investigate the effect of imidazole-thiazolidinone on OC cell proliferation, and the mechanism involved.

EXPERIMENTAL

Materials

Human OC cells (HCE-6 and KYSE-1170) were obtained from the Chinese Academy of Sciences. Fetal bovine serum (FBS) and DMEM were purchased from Gibco (USA). Annexin V/FITC kit and flow cytometer were obtained from BD Biosciences (USA). Trizol reagent was a product of Invitrogen: Thermo Fisher Scientific, Inc. (USA). Bicinchoninic acid (BCA) assay kit was purchased from Sangon Biotech Co., Ltd. (China), while polyvinylidene fluoride membranes were purchased from Roche Diagnostics (Switzerland). Enhanced Chemiluminescence (ECL) detection system was a product of Thermo Fisher Scientific Ltd. (USA).

Cell culture

The HCE-6 and KYSE-1170 cells were cultured in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin solution at 37 ˚C for 24 h in a humidified atmosphere of 5 % CO_2 and 95 % air. After attaining 60 - 70 % confluency, the cells were treated with serum-free medium and graded concentrations of imidazole-thiazolidinone (up to 160 µM) for 24 h. Normal cell culture without imidazole-thiazolidinone served as control group. Cells in logarithmic growth phase were selected and used in this study.

Cell proliferation assay

The effect of imidazole-thiazolidinone on HCE-6 and KYSE-1170 cell proliferation was assessed using MTT assay. The cells were seeded at a density of 1.5 $x10^5$ cells/well in 96-well plates and cultured in DMEM for 24 h. Then, imidazole-thiazolidinone (0 – 160 µM) was added to the cells and incubated for 48 h. Subsequently, 20 µL of MTT solution (5 mg/mL) was added to the wells, followed by incubation at 37 ˚C for 5 h. The medium was finally replaced with 100 µL of 0.1 % dimethyl sulfoxide (DMSO) to completely dissolve the formazan crystals formed. The absorbance of the samples was read in a microplate reader at 562 nm. The assay was performed in triplicate, and cell proliferation © calculated as shown in Eq 1.

C (%) = (Ae/Ac)100 ……... (1)

where Ae and Ac are the absorbance values for experimental and control groups, respectively.

Assessment of morphological changes in OC cells

The HCE-6 and KYSE-1170 cells were seeded in 12-well plates at a density of 1.5 x 10^6 cells/well and cultured in DMEM for 48 h. Then, imidazole-thiazolidinone (40, 80 and 160 µM) was added to the cells and incubated for 72 h. After incubation, the cells were stained in the dark with 1 µg/mL solution of Hoechst 3325 at 37˚C for 25 min. The phosphate-buffered saline (PBS)-washed cells were subsequently examined for morphological alterations using fluorescent microscope.

Cell migration assay

Changes in migratory potential of imidazole-thiazolidinone-treated HCE-6 cells were determined using wound-healing assay. Cells in logarithmic growth phase were seeded in 12-well plates until they attained 100 % confluency, and scratches were made on the cell monolayers. The cells (2.5 x 10^5 cells/well) were then incubated with imidazole-thiazolidinone at concentrations of 40, 80 and 160 µM for 48 h at 37˚C. After washing thrice with serum-free medium, the cells were further cultured for 24 h, and then observed and photographed.

Cell-cycle analysis

The effect of imidazole-thiazolidinone on cell cycle distribution was determined using a flow cytometer. The HCE-6 cells treated with varied concentrations of imidazole-thiazolidinone (40, 80 and 160 µM) were seeded into 6-well plates and incubated for 72 h. The cells were then washed with PBS, and fixed with 70 % ethyl alcohol at 4˚C overnight. Tris-hydrochloride buffer (pH 7.5) containing 1 % RNase A was then added to the plates. The cells were subsequently stained with 50 µg/mL propidium iodide for 45 min at 37˚C in the dark and injected into the flow cytometer for analysis.

Western blotting

The HCE-6 and KYSE-1170 cells (5 x 10^8 cells/L) were incubated with imidazole-thiazolidinone for 72 h. The cells were then washed twice with PBS and lysed with 250 µL of ice-cold radio-immunoprecipitation assay (RIPA) buffer containing 0.1 % sodium dodecyl sulphate (SDS), 1X PBS, 1 % NP40, ethylenediamine tetraacetate, 0.5 % sodium deoxycholate, 1 % PMSF, protease and phosphatase inhibitors. The resultant lysate was centrifuged at 12,000 rpm for 20 min at 4 °C, and the protein concentration of the supernatant was determined using BCA method. A portion of total cell protein (10 µg) from each sample was separated on 10 % SDS-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 ^{o}C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 ◦C with primary antibodies of rabbit polyclonal anti-bax, anti-bcl-2, anti-caspase-3/8/9, anti-MMP-2, anti-MMP-9 and anti-β-actin, each at a dilution of 1 to 800. Then, the membrane was washed thrice with PBS and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1.5 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using ECL. The respective protein expression levels were normalized to that of β-actin which was used as a standard.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using SPSS (version 16.0). Groups were compared using Student's *t*-test. Statistical significance was assumed at $p < 0.05$.

RESULTS

Effect of imidazole-thiazolidinone on OC cell proliferation

As shown in Figure 1, treatment of HCE-6 and KYSE-1170 cells with imidazole-thiazolidinone for 48 h led to significant and dose-dependent reductions in their proliferation ($p < 0.05$). The proliferation of HCE-6 cell was reduced from 94 to 33 %, while KYSE-1170 cell proliferation decreased from 96 to 37 %.

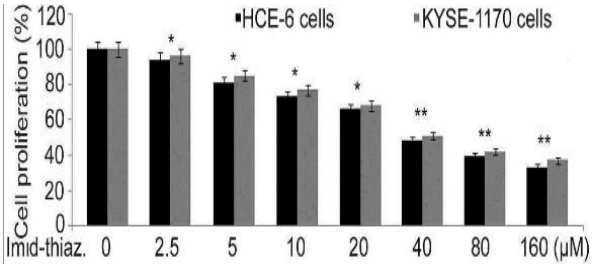

Figure 1: Effect of imidazole-thiazolidinone on OC cell proliferation. $^{*}p < 0.05$ and $^{**}p < 0.01$, when compared with control cells

Morphological alterations in HCE-6 cells after treatment with imidazole-thiazolidinone

Light microscopic examination revealed significant reductions in HCE-6 cell count, detached cells, reduced cell size and irregular cytoplasmic vacuoles after treatment with imidazole-thiazolidinone. However, the control cells were polygonally shaped, with marked borders and exponential growth. There were obvious apoptotic changes in HCE-6 cell cultures treated with graded concentrations of imidazole-thiazolidinone. These results are shown in Figure 2.

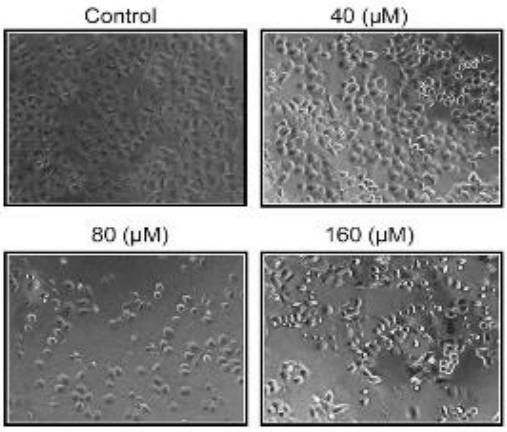

Figure 2: Effect of imidazole-thiazolidinone on HCE-6 cell morphology (x 200)

Effect of imidazole-thiazolidinone on OC cell migration

Imidazole-thiazolidinone treatment significantly and dose-dependently decreased the migrations of HCE-6 and KYSE-1170 cells ($p < 0.05$). In HCE-6 cells, migration decreased from 51.32 to 6.31 %, while that of KYSE-1170 cells was reduced from 56.14 to 11.87 % (Figure 3).

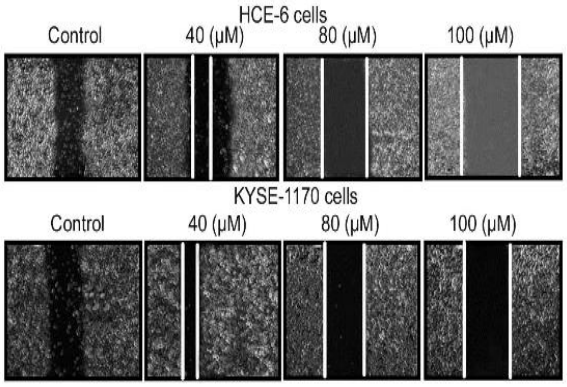

Figure 3: Effect of imidazole-thiazolidinone on OC cell migration

Effect of imidazole-thiazolidinone on HCE-6 cell cycle arrest

Imidazole-thiazolidinone treatment significantly and dose-dependently increased the population of HCE-6 cells in S phase ($p < 0.05$). The HCE-6 cell population in S phase increased from 13.56 to 27.14 %. The HCE-6 cell population in G1/G0 and G2/M phases were significant reduced, relative to control group ($p < 0.05$). Treatment of HCE-6 cells with imidazole-thiazolidinone significantly arrested HCE-6 cell cycle at S phase. These results are shown in Figure 4.

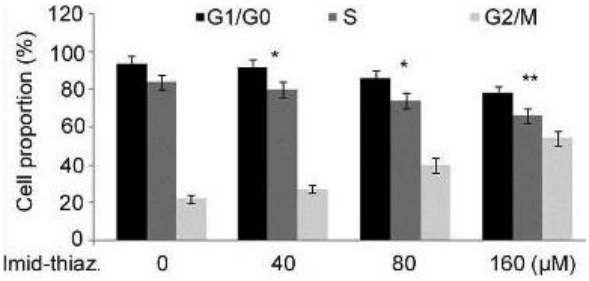

Figure 4: Effect of imidazole-thiazolidinone on HCE-6 cell cycle arrest**;** *$p < 0.05$, when compared with control cells

Expressions of apoptosis-related proteins in OC cells

In HCE-6 cells, imidazole-thiazolidinone treatment significantly and dose-dependently upregulated the expressions of cleaved caspase-3/8/9 and bax, but down-regulated bcl-2 expression significantly and dose-dependently ($p < 0.05$). However, MMP-2 and MMP-9 expressions in HCE-6 and KYSE-1170 cells were significantly and dose-dependently down-regulated by imidazole-thiazolidinone treatment ($p < 0.05$; Figure 5).

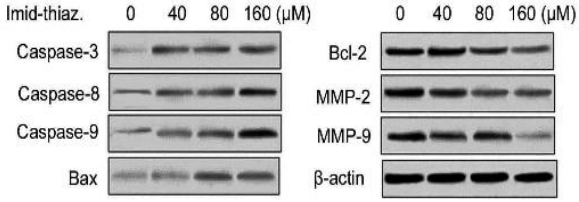

Figure 5: Effect of imidazole-thiazolidinone treatment on the levels of expression of apoptosis-related proteins in OC cells

DISCUSSION

Oesophageal cancer (OC), a common malignant tumor among the Chinese, arises from the oesophagus [1]. Symptoms often include difficulty in swallowing and weight loss. Imidazole is the structural component of several complex natural products and therapeutic molecules [9]. Compounds bearing imidazole ring exhibit varied biological properties such as antimicrobial, antitumor, antiviral, and antimalarial effects [10]. This study investigated the effect of imidazole-thiazolidinone on OC cell proliferation, and the mechanism involved.

Cancer cells are characterized by uncontrolled proliferation. Thus, regulation of cell growth is vital in cancer treatment [12].

In this study, imidazole-thiazolidinone treatment significantly and dose-dependently inhibited OC

cell proliferation. These results are in agreement with those of previous reports [13]. Studies have shown that cell cycle progression is closely associated with increased cell proliferation. Each phase of the cell cycle is regulated by checkpoint proteins which control transition from one phase to another [13]. Failure of any of the checkpoints leads to uncontrolled cell division and ultimately cancer [14].

Cell cycle arrest forms the basis for inhibition of cancer cell proliferation [15]. In this study, imidazole-thiazolidinone treatment led to arrest of HCE-6 cell cycle at S phase. In imidazole-thiazolidinone-treated cell cultures, the proportion of HCE-6 cells in S phase was markedly increased, but reduced in G1/G0 and G2/M phases. The highly regulated process of apoptosis is responsible for elimination of unwanted cells from an organism [16]. Apoptosis activation involves the secretion of caspases, with caspase-3 being the most important [17].

Activation of bax is usually accompanied by increased mitochondrial membrane permeability and cytochrome *c* efflux into the cytoplasm [17]. Cytochrome *c* plays a key role in the activation of caspase-9 which is required for induction of apoptosis [17]. Therefore, activation of the apoptotic pathway by chemotherapeutic agents is a major consideration in cancer treatment [18]. In this study, imidazole-thiazolidinone treatment significantly and dose-dependently upregulated the expressions of cleaved caspases-3/8/9 and bax, while down-regulating bcl-2 protein expression in OC cells. It is likely that imidazole-thiazolidinone regulates HCE-6 and KYSE-1170 cell proliferation via induction of apoptosis. During metastasis, cancer cells invade the lymph and blood vessels, thereby spreading to nearby organs [19]. In more than 50 % of patients, OC is detected at the metastatic stage which is characterised by poor prognosis [20]. Studies have shown that the levels of extracellular matrix proteins and degree of cell metastasis are key in cancer treatment [21]. In this study, imidazole-thiazolidinone treatment significantly inhibited the migration and invasiveness of HCE-6 cells. It also significantly and dose-dependently down-regulated the expressions of MMP-2 and MMP-9 in HCE-6 and KYSE-1170 cells.

CONCLUSION

The results obtained in this study suggest that imidazole-thiazolidinone suppresses OC cell proliferation via induction of apoptosis and arrest of cell cycle at S phase. Therefore, the compound has potentials for development into an agent for the treatment of oesophageal cancer.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

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. Qian Wang, Yuan Yuan and Mi Jiang performed the experimental work. Lihua Huang and Hongyuan Shen carried out the literature survey and analysed the data. Jie Huang compiled the data and designed the study. All the authors wrote and approved the paper for publication.

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