Tropical Journal of Pharmaceutical Research August 2019; 18 (8): 1705-1710
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v18i8.20

Original Research Article

Indole-thiophene conjugate inhibits proliferation of human cervical cancer cell lines through DNA damage

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Sent for review: 9 January 2019 *Revised accepted: 26 July 2019*

Abstract

Purpose: *To investigate the inhibitory effect of indole-thiophene conjugate (ITC) against cervical cancer cells.*
Methods: *The effect of ITC on the proliferation of cervical cells was determined using 3 (4,5 dimethylthiazol 2 yl) 2,5 diphenyltetrazolium bromide (MTT) assay. The apoptosis-inducing effect of ITC was analysed with flow cytometry, while its effect on cell invasion was assessed using Transwell assay.*
Results: *ITC inhibited proliferation of HeLa and Caski cancer cell lines, but it had no cytotoxicity against HCvEpC normal epithelial cells. Exposure to ITC at a dose of 12 µmol/L reduced the viability of HeLa and Caski cells to 22.56 and 24.78 %, respectively ($p < 0.05$). ITC treatment of HeLa cells enhanced the proportion of apoptotic cells. Exposure to ITC at a dose of 12 µmol/L led to near-complete inhibition of the invasive potential of HeLa cells. Moreover, exposure of HeLa cells to ITC downregulated the protein expressions of MMP-2 and MMP-9 ($p < 0.05$). The expressions of Bcl-2, p-ERK1/2 and p-Akt were markedly decreased in HeLa cells by ITC exposure. In addition, ITC increased Bax expression, and decreased Bcl-2/Bax ratio ($p < 0.05$).*
Conclusion: *ICT inhibits the proliferation and invasion of cervical cancer cells, and induces their apoptosis. It exhibits these effects via the suppression of Akt and ERK phosphorylation, thereby down-regulating the PI3K and MAPK pathways. Therefore, ITC may be beneficial for the treatment of cervical cancer.*

Keywords: *Thiophenes, Hetero-aromatic compounds, Metastasis, Phosphorylation, Cervical cancer*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Cervical cancer is a frequently detected carcinoma in females throughout the globe. Indeed, more than 500,000 new cases are diagnosed and about 300,000 deaths occur annually from cervical cancer [1]. Although the average 5-year survival for cervical cancer patients is 73 %, the prognosis of cases with advanced stage or recurrent cervical cancer is very poor [2]. Patients diagnosed with cervical cancer at an early stage are treated with surgical intervention and radiation therapy [3].

Patients diagnosed with local or advanced metastatic cervical carcinoma lesions are

recommended for concurrent chemotherapy and radiation therapy since cancer recurrence may not be ruled out [3]. The concurrent use of chemotherapy and radiotherapy for eradication and sensitization of tumor metastases has significantly improved the survival rate of cervical cancer patients, relative to those treated with radiotherapy alone [4,5]. However, there is no effective treatment for patients with distant organ metastasis, apart from mere supportive care. In case of cervical cancer, the most active inhibitory agent is cisplatin which has about 20 % response [6]. Paclitaxel is another active and non-platinum compound used against cervical cancer, with a response of about 17 % [7].

Thiophenes are hetero-aromatic compounds containing nitrogen atom in the five membered ring, and they comprise natural as well as synthetic compounds with varied pharmacological properties [8]. Thiophene-bearing compounds possess anticancer properties [9]. Studies have shown that compounds with thiophene ring inhibit the proliferation of various types of carcinoma cells such as pulmonary, leukemia, glioma, ovary and renal cancer cells [10]. Thiophene derivatives inhibit cancer growth through apoptosis, arrest of cell cycle and influence on polymerization of tubulin. In the present study, the inhibitory effect of indole-thiophene conjugate (ITC) against cervical cancer cells was investigated.

EXPERIMENTAL

Cell lines and culture

HeLa and Caski cancer cell lines and HCvEpC normal epithelial cells were supplied by the American Type Culture Collection, Manassas, VA, USA. Cell culture was performed in RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) containing 10 % fetal calf serum (Gibco-BRL, Grand Island, NY, USA). The medium also contained penicillin (100 U/mL) and streptomycin. The cells were cultured at a temperature of 37 ˚C under humidified atmosphere of 5 % CO_2.

Cell viability assay

The proliferation of HeLa, Caski and HCvEpC cell lines after 48 h of exposure to ITC was assessed with MTT assay. The cells were adjusted to a density of 5 x 10^6 cells/mL, and were cultured in 96-well culture plates (190 µL per well) for 24 h in an incubator under standard conditions. The cells were then exposed to ITC at doses of 2, 4, 6, 8, 10 and 12 µmol/L, or dimethyl sulfoxide (DMSO, control) for 48 h. Thereafter, MTT solution (20 µL) was added to each well of the plate and incubation carried out for 4 h. After decanting the supernatant from the plates, 150 µL of DMSO was added to each well of the plate to solubilize the formazan crystals formed. The absorbance of each well was read at 490 nm, and the readings were used for estimating cell survival. The MTT assay was done in triplicate.

Flow cytometric analysis

HeLa cells were seeded at a density of 2 x 10^6 cells per mL in 96-well culture plates. Following exposure to ITC at doses of 4, 6, 8, 10 and 12 µmol/L, or DMSO (control) for 48 h, the cells were collected and washed twice in PBS. The cells were then treated with binding buffer, and stained with Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). The cell suspension was incubated with PI for 15 min in the dark at room temperature. Measurement of the apoptotic cell proportion was carried out using FACScalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Matrigel invasion assay

HeLa cervical cancer cells were incubated for 48 h in RPMI 1640 medium containing 10 % FBS, and were then subjected to trypsinization. The cell suspension (200 µL) containing 2 x 10^5 cells per mL along with ITC at concentrations of 4, 6, 8, 10 and 12 µmol/L, or DMSO (control) was put onto a 24‐well Transwell chamber. The chamber was coated with 50 µL of Matrigel™ diluted with serum‐free medium in a volume ratio of 1:4. Into the outer cup of the chamber was put DMEM containing 10 % serum. Incubation of the cells for 24 h was followed by cleaning non-invading cells using cotton swab. The cells that invaded Matrigel and the membrane bearing 8‐µm pore were fixed for 20 min in 3 % paraformaldehyde. The cells were then stained for 20 min with crystal violet, followed by cell count in five randomly selected visual fields at a magnification of x400.

Western blot analysis

HeLa cells were exposed to ITC at doses of 4, 6, 8, 10 and 12 µmol/L or DMSO (control). The cells were then harvested and washed with PBS for 48 h. Proteins were extracted from the cells by treatment with lysis buffer used for western blotting, and IP (Beyotime Institute of Biotechnology). The protein samples in equal quantity (40 µg/lane) were loaded on SDS-polyacrylamide gel (10 %) for separation by electrophoresis. The proteins were subsequently

transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA, USA), and the membrane was blocked with 3 % (w/v) non-fat milk in TBST for 1 h at room temperature. The blots were incubated with primary antibodies at 4 ^{o}C overnight after dilution in TBST buffer. The primary antibodies used were rabbit monoclonal anti-Bcl-2, rabbit monoclonal anti-Bax, rabbit polyclonal anti-p-Akt, rabbit polyclonal anti-Akt, rabbit monoclonal anti-p-ERK-1/2, rabbit monoclonal anti-ERK1/2, rabbit polyclonal anti-MMP-2, rabbit polyclonal anti-MMP-9, and rabbit polyclonal anti-β-actin (Wuhan Boster Biological Technology, Ltd., Wuhan, China). The membranes were then washed and subsequently incubated for 1 h with horse radish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Wuhan Boster Biological Technology, Ltd.) at room temperature. The intensity of β-actin band was used as a loading control. Optical density was used as index of the relative expression of each target protein.

Statistical analysis

The data are presented as mean ± standard deviation, and were processed with Statistical Package for Social Sciences (SPSS for Windows, version 17.0; SPSS, Inc, Chicago, IL, USA). Statistical significance of differences were determined using Student's *t*-test or one-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Inhibition of HeLa and Caski cell viability by ITC

The cytotoxic effects of ICT on HeLa and Caski carcinoma cell lines and HCvEpC normal cervical epithelial cells following 48 h of exposure to ITC at doses of 2, 4, 6, 8, 10 and 12 µmol/L are shown in Figure 1. Exposure of HeLa and Caski cells to ITC at a dose of 2 µmol/L for 48 h produced very little change in their viabilities. However, reduction in viability of HeLa and Caski cells was evident with increase in the concentration of ITC from 4 - 12 µmol/L. At a concentration of 12 µmol/L, the viabilities of HeLa and Caski cells were reduced to 22.56 and 24.78 %, respectively. However, there was no evident change in the viability of HCvEpC cells at all the tested concentrations of ITC.

Exposure of HeLa cells to ITC caused induction of apoptosis

Following 48 h of exposure to ITC at concentrations of 4, 6, 8, 10 and 12 µmol/L, HeLa cells were assessed for apoptotic changes using flow cytometry **(**Figure 2). In HeLa cell cultures, ITC exposure enhanced the proportion of apoptotic cells, with increase in ITC concentration from 4 to 12 µmol/L. The degrees of apoptosis in HeLa cells were 7.76, 13.45, 21.98, 32.58 and 56.73 % on exposure to 4, 6, 8, 10 and 12 µmol/L ITC, respectively. In contrast, untreated HeLa cell cultures had apoptosis of only 1.98 %.

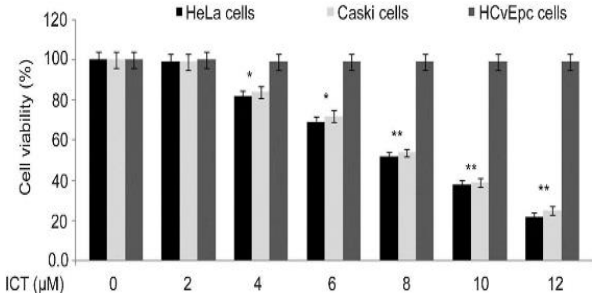

Figure 1: Effect of ITC on the viabilities of cervical cancer and normal epithelial cells. MTT assay was used for assessment of the viabilities of HeLa and Caski cancer cells, and HCvEpC normal epithelial cells following 48 h of exposure to ITC. The concentrations of ITC used were 2, 4, 6, 8, 10 and 12 µmol/L. The results are presented as mean ± standard deviation of triplicate experiments; *$p < 0.05$; **$p < 0.02$, versus untreated cells

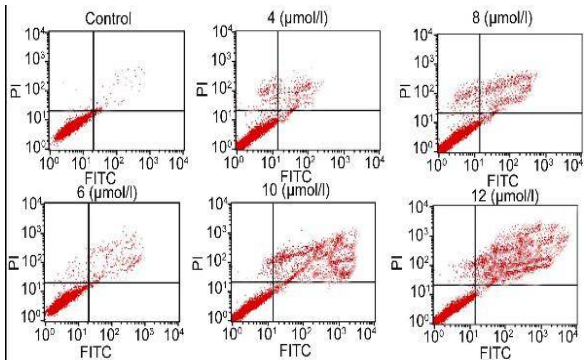

Figure 2: Effect of ITC exposure on apoptosis in HeLa cells. Cell cultures exposed to 4, 6, 8, 10 and 12 µmol/L ITC for 48 h were stained with Annexin V and propidium iodide. Flow cytometric assessment of stained cells was performed

ITC inhibited HeLa cell invasion

After exposure of HeLa cells to 2, 4, 6, 8, 10 and 12 µmol/L ICT, invasiveness was determined using Transwell assay. As shown in Figure 3, ITC exhibited inhibitory effect on the invasion of HeLa cells in concentration-dependent manner. Increasing the concentration of ITC from 4 to 12 µmol/L reduced invasiveness of HeLa cells. Exposure to 12 µmol/L ITC almost completely inhibited the invasive potential of HeLa cells.

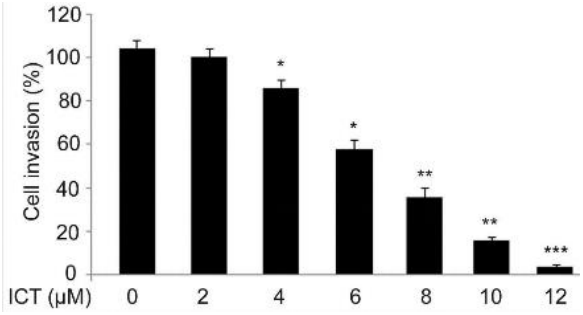

Figure 3: ITC inhibited HeLa cell invasion. Invasion of the cells exposed to ITC at 2, 4, 6, 8, 10 and 12 µmol/L was measured on Matrigel-coated membranes; *p < 0.05, **p < 0.02 and ***p < 0.01, versus untreated cells

ITC decreased metalloproteinase expressions in HeLa cells

Exposure of HeLa cells to ITC for 48 h caused reductions in protein expression levels of MMP-2 and MMP-9 (Figure 4). With increase in concentration of ITC from 4 to 12 µmol/L, marked decreases in the protein levels of MMP-2 and MMP-9 occurred in HeLa cells, as shown using western blotting.

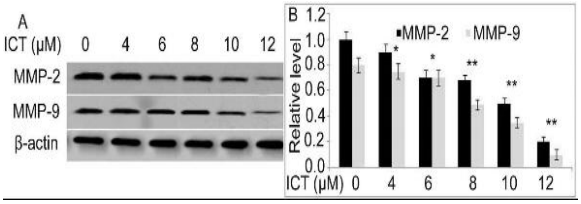

Figure 4: Effect of ITC exposure on expressions of matrix metalloproteinases. HeLa cells were exposed for 48 h to 4 - 12 µmol/L ITC. The protein expressions of MMP-2 and MMP-9 were determined using western blotting; *p < 0.05; **p < 0.02, versus untreated cells

ITC decreased expression of tumor malignancy protein

Exposure of HeLa cells to ITC for 48 h at concentrations of 4, 6, 8, 10 and 12 µmol/L was followed by western blotting to determine tumor malignancy protein expression (Figure 5). The ITC exposure markedly decreased the expressions of Bcl-2, p-ERK1/2 and p-Akt, when compared to untreated HeLa cells. On increasing the concentration of ITC from 4 to 12 µmol/L, the protein expressions of Bcl-2, p-ERK1/2 and p-Akt decreased. However, ITC exposure of HeLa cells did not change the expressions of t-ERK1/2 and t-Akt. The expression of Bax in HeLa cells was increased on exposure to ITC for 48 h, when compared to untreated cells. Moreover, the ITC exposure decreased Bcl-2/Bax ratio in HeLa cells.

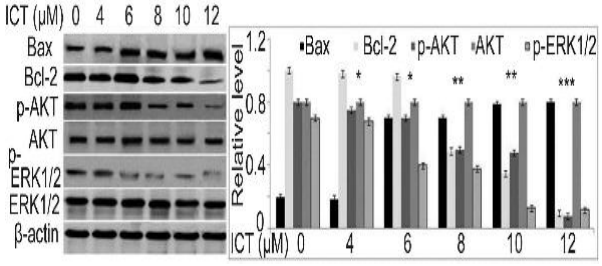

Figure 5: Effect of ITC on tumor-related protein expressions. HeLa cells were exposed to 4 - 12 µmol/L ICT for 48 h, and protein expression was assessed with western blotting. (A) Immunoblotting was used for determination of protein expressions related to tumor. (B) Expression of proteins are presented as mean ± standard deviation *p < 0.05, **p < 0.02, ***p < 0.01, versus untreated cells

DISCUSSION

The present study was designed to investigate the effect of ITC on cervical cancer and normal epithelial cell lines. Exposure of cervical cancer cells to ITC led to inhibition of carcinoma growth, and reductions in invasion and cell apoptosis. In the present study, the effect of ITC on HeLa, Caski carcinoma and HCvEpC normal cervical epithelial cells was assessed using MTT assay. The study showed that ITC exposure caused reduction in the proliferation of HeLa and Caski carcinoma cells without any evident change in HCvEpC normal epithelial cells. These findings suggest that ITC is a selective anti-proliferative agent against cancer cells, without any toxic effect on normal cells. To regulate cell division and put a check on uncontrolled cell proliferation, unwanted cells are eliminated from the body through apoptosis [11].

One of the adverse effects of impaired apoptotic pathway is tumor formation. In the present study, exposure of HeLa cell cultures to ITC increased significantly the apoptotic cell proportion, relative to untreated cultures. With increase in the concentration of ITC, the population of apoptotic cells in the cultures was enhanced. Apoptosis is regulated by a balance between the expression of pro-apoptotic and anti-apoptotic proteins [12]. These proteins control cell apoptosis through the regulation of permeability of mitochondrial membrane and the subsequent caspase activation [12]. The present study investigated changes in expressions of proteins of Bcl-2 family in HeLa cells on exposure to ITC. Western blotting revealed that exposure of HeLa cells to ITC promoted the expression of Bax and downregulated the expression of Bcl-2 protein. A marked difference in the expressions of Bax and Bcl-2 proteins was recorded in ITC-treated and

untreated HeLa cell cultures. The initiation of apoptotic signalling in cancer cells is strongly associated with the Bcl-2/Bax ratio [13]. The results of the present study indicate that exposure of HeLa cells to ITC caused a significant decrease in Bcl-2/Bax ratio. This finding provides a clear indication that ITC exposure inhibited HeLa cell proliferation through promotion of Bax and suppression of Bcl-2 protein expressions. The advanced and fatal stage of cancer is characterized by higher metastasis which is associated with invasion of carcinoma cells.

In this study, ITC exhibited inhibitory effect on the invasive potential of HeLa cells. Carcinoma cell metastasis requires breakdown of extracellular matrix which is catalysed by increased expressions of MMPs [14]. Dissociation of the extracellular matrix components by higher expression of MMPs also promotes carcinoma cell migration to distant organs [15]. Higher expressions of MMPs also cause degradation of type IV collagen membrane thereby enhancing carcinoma cell metastasis [16]. The levels of MMP-2 and MMP-9 expressions in carcinoma tissues are markedly higher than those of the corresponding normal samples [17]. In the present study, exposure of HeLa cells to ITC markedly reduced the protein expression levels of MMP-2 and mMP-9 at 48 h. This suggests that ITC inhibited HeLa cervical cancer cell invasion through the suppression of MMP-2 and MMP-9 expressions.

The network of EGFR signalling responsible for the regulation of cell proliferation, metastasis and apoptosis via transfer of signals and activation of corresponding genes is mediated by two pathways: PI3K/Akt and MAPK/ERK [18]. Any change in the EGFR network leads to the transfer of abnormal signals and development of various types of cancers [19]. An important member of PI3K pathway which is linked to enhancement of cell proliferation and prevention of cells from undergoing apoptosis is Akt [20]. In various types of cancer cells such as bladder carcinoma, the expression of activated Akt is markedly increased [21]. The results obtained in this study also showed higher level of activated Akt in the HeLa cervical cancer cells, which is consistent with an earlier report [21].

However, exposure of HeLa cells to ITC caused a significant decrease in the level of activated Akt in a concentration-dependent manner. Since Akt is a member of PI3K pathway, inhibition of Akt activation indicates that ITC exposure inhibits PI3K pathway in HeLa cells. It has been shown that phosphorylation of ERK activates MAPK pathway which subsequently enhances proliferation and suppresses onset of apoptosis [22]. In the present study, ITC exposure caused significant suppression of ERK phosphorylation in HeLa cells, when compared to the untreated cells. This suggests that ICT treatment inhibits activation of MAPK pathway in HeLa cells, since ERK is a target in the MAPK pathway.

CONCLUSION

The results obtained in this study reveal that ITC inhibits the growth of cervical cancer cells, suppresses their invasion, and induces their apoptosis. These effects occur via the suppression of Akt and ERK phosphorylation, thereby down-regulating PI3K and MAPK pathways. Therefore, ITC may be of great benefit for the treatment of cervical cancer. However, more studies are required to fully investigate the role of ITC in cervical carcinoma in animal models.

DECLARATIONS

Acknowledgement

The authors are thankful to Head of Department of Pharmacy, Ankang Hospital of Traditional Chinese Medicine, Ankang, Shaanxi, China for his support for this study.

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. Xia Xu and Hongbing Ma performed the experimental work. Fujun Wang, carried out literature review and compiled the data. Jiamiao Yan designed the study and wrote the manuscript. All the authors approved the final version of the manuscript.

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