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Original Research Article

Indole-thiazolidinone conjugate inhibits nasopharyngeal carcinoma cell migration and invasion by targeting NF κB pathway

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Abstract

Purpose: *To investigate the effect of indole-thiazolidinone on metastasis in HK1 nasopharyngeal carcinoma cells.*
Methods: *HK1 cell proliferation was determined colorimetrically using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Invasion and migration of HK1 cells were assessed using Matrigel™ chamber coated invasion and wound healing assays, respectively.*
Results: *Indole-thiazolidinone suppressed proliferation of HK1 and NPC 039 NPC cell lines at 72 h. The degree of proliferation of HK1 cells on treatment with 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 μM indole-thiazolidinone was 99, 87, 71, 64, 49, 38 and 31 %, respectively. In HK1 cell cultures, migration potential was reduced to 58.32, 47.54, 28.91 and 17.65 %, on exposure to 1.5, 2.0, 2.5 and 3.0 μM indole-thiazolidinone, respectively. Incubation with 1.5, 2.0, 2.5 and 3.0 μM indole-thiazolidinone resulted in cell invasion values of 63.41, 49.37, 35.12 and 19.67 %, respectively. There was a marked decrease in the expressions of matrix metalloproteinase 2 and matrix metalloproteinase 9 in HK1 cells on treatment with indole-thiazolidinone ($p < 0.05$). In addition, indole-thiazolidinone treatment resulted in decrease in p65 and p50 in nuclear fraction. Treatment of HK1 and NPC 039 cells with indole-thiazolidinone and henenalin synergistically decreased cell proliferation. Indole-thiazolidinone treatment caused significant decrease in tumor growth in mice ($p < 0.05$).*
Conclusion: *Indole-thiazolidinone inhibits proliferation and metastasis in nasopharyngeal carcinoma cells. Therefore, it has potential for development as a therapeutic management of nasopharyngeal carcinoma in humans.*

Keywords: *Indole-thiazolidinone, Nasopharyngeal carcinoma, Translocation, Radiotherapy, Proteinase, Apoptosis*

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is common in the southern parts of China and Southeast Asia [1,2]. According WHO, more than 80 % of cases of NPC are diagnosed in China alone. Nasopharyngeal carcinoma (NPC) affects the head and neck, and it is different from other types of cancers [3]. In most cases, NPC cell invasion is seen in lymph, blood and

subsequently to hepatic tissues, bone and liver [4]. The high rate of NPC metastasis is a big hurdle in its treatment [5]. At the early stage of NPC, radiotherapy is the main therapeutic strategy used, although the relapse rate is very high, and the 5-year survival is low [6]. Therefore, the evolvement of newer treatments for NPC is of great significance for increasing the survival period of patients.

Metastasis of cancer cells involves reduction in cell adhesion, extracellular matrix (ECM) decomposition, and high rate of cell mobility [7]. One of the main factors involved in the cancer cell metastasis is ECM decomposition and basement membrane breakdown [7,8]. The ECM decomposition is catalysed by proteinase known as matrix metalloproteinases [9, 10]. Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) are overexpressed in carcinoma cells. Thus, inhibition of these proteinases plays an important role in suppression of tumor growth by preventing cancer cell metastasis and invasion [9,10].

Regulation of cancer growth by apoptosis is often used in the treatment of several types of carcinomas [11,12]. Apoptosis onset is controlled in cancer cells by the activation of nuclear factor-kappa B (NF-κB) [13-18]. Activated NF-κB is translocated from the cytosol to the nucleus where it induces the expressions of genes linked to apoptosis [13-18]. The present study investigated the effect of indole-thiazolidinone on proliferation and metastasis of NPC cancer cells. The anti-metastatic activity of indole-thiazolidinone was investigated *in vivo* in mice model of NPC. In addition, the mechanism of the inhibitory effect of indole-thiazolidinone on NPC was studied.

EXPERIMENTAL

Cell culture

The cells (HK1 and NPC-039) were supplied by American Type Culture Collection (ATCC; Rockville, MD, USA). The medium used for cell culture was DMEM medium containing 10 % FBS, glutamine (2 mM) and antibiotics [penicillin (100 U/mL) and streptomycin (100 µg/mL)]. The cell culture was performed in an incubator under humidified atmosphere containing 5 % carbon dioxide at a temperature of 37 ˚C.

Cell proliferation assay

Changes in proliferation of HK1 and NPC-039 cells due to indole-thiazolidinone treatment was determined colorimetrically using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay. The cells were seeded in 6-well plates (Nunc A/S Plastfabrikation, Roskilde, Denmark) at a density of 2 x 10^5 cells/well. Incubation of the cells for 12 h was followed by replacement of medium with new medium containing 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µM indole-thiazolidinone. The cells were incubated for 72 h, following which 50 µL of MTT (5 µg/mL) solution was put into each well of the plate. After incubation for 2 h, DMSO (150 µL) was added to the plates to dissolve the resultant formazan crystals. After incubation for 10 min, cell proliferation was measured by reading the absorbance at of the solutions at 587 nm using Universal Microplate Reader (EL800Bio; Tek Instruments, Inc., Winooski, VT, USA).

Evaluation of cell invasion

First, HK1 cells were subjected to 72 h pre-treatment with 1.5, 2.0, 2.5 and 3.0 indole-thiazolidinone or DMSO (control). The cells were then distributed into the upper chambers of Matrigel™ (Becton Dickinson, Bedford, MA, USA) invasion chamber (Corning Inc, Tewksbury, MA, USA) containing serum-free DMEM medium. After incubation for 72 h, cotton swabs were used to remove the cells from the upper membrane surface. On the bottom filter side, the cells were fixed with 90 % ethanol and subsequently subjected to Crystal Violet (Sigma-Aldrich) staining. Then, cell invasion was determined using Olympus-CX31 microscope (Olympus Corp., Tokyo, Japan).

Determination of HK1 cell migration

The HK1 cells were seeded into 6-well culture plates. The cells were allowed to attain 100 % confluency, and then a sterile P200 pipette tip was used to scratch the monolayer. The detached cells were removed by washing the monolayers with PBS twice. Then, the cells were incubated for 72 h with 1.5, 2.0, 2.5 and 3.0 µM indole-thiazolidinone or DMSO (control). Images of the cell monolayers treated with different doses of indole-thiazolidinone were captured. Olympus-CX31 microscope (Olympus Corp.) was used for the measurement of distances through which the cells migrated.

Western blot analysis

The HK1 cells were treated with 1.5, 2.0, 2.5 and 3.0 µM indole-thiazolidinone or DMSO (control) for 72 h. Then, the cells were seeded at a density of 2 x 10^5 cells into a lysis buffer [Tris-hydrochloride (40 mmol/L), ethylene

diamine tetraacetate (1 mmol/L), potassium chloride (150 mmol/L), sodium vanadate (100 mmol/L), Triton X-100 (1 %) and PMSF (1 mmol/L)] at pH 7.5. Commercially available NucBuster™ Protein Extraction kit (Novagen®; Merck KGaA, Darmstadt, Germany) was used for the preparation lysates of the cell nuclei as per the manual procedure. Proteins in the lysates were separated on 10 % SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore Corp, Billerica, MA, USA).

Non-specific sites in the membranes were blocked by incubation with non-fat milk for 1 h at 37 ˚C. Incubation of the membranes with primary antibodies was performed overnight at a temperature of 4 ˚C with. The antibodies used were against: rabbit polyclonal MMP-2 (catalogue number #4002), rabbit polyclonal MMP-9 (catalogue number #2270; both from Cell Signaling Technologies; Danvers, MA, USA), NF-κB p50 (catalogue number #sc-271908), NF-κB p65 (catalogue number #sc-71676) and β-actin (catalogue number #sc-376421; Santa Cruz Biotechnology, Inc.; Dallas, TX, USA). The membranes were washed with PBS and then incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse (catalogue number #EK010) at room temperature. The bands were visualized using Enhanced Chemiluminescence kit (ECL Plus; GE Healthcare Europe GmbH, Freiburg, Germany).

Experimental animals

Male BALB/c mice (5 weeks old, 18 ± 2 g) were purchased from Shanghai Laboratory Animal Co. (SLAC, Shanghai, China). The mice were acclimatized under an atmosphere of 57 % humidity, temperature of 25 ± 2 ˚C and alternating 12-h light and 12-h dark cycles. The animals were provided free access to standard laboratory feed and water. The experimental procedures involving mice were carried out according to the guidelines issued by the National Institute of Health [19]. The animal studies were also approved by the Animal Care and Use Committee of Shanghai Tongji University (approval no. STU/16/1002).

Establishment of NPC tumor xenograft mice model

In order to study the role of indole-thiazolidinone in *in vivo* tumor growth inhibition, HK1 cells were xenografted into nude mice using the procedure reported earlier [18]. In essence, HK1 tumor cells were implanted subcutaneously at a concentration of 2 x 10^5 cells per animal, into the right flank under anaesthesia. The mice were then randomly assigned to two groups of five mice each. Mice in the treatment group were intragastrically administered daily doses of indole-thiazolidinone (10 mg/kg) for 30 days, while those in the untreated and normal control groups received equivalent doses of normal saline. Tumor development in the mice was recorded by measuring the tumor volumes on alternate days using callipers. The mice were sacrificed on day 31 by cervical dislocation, and the weight of tumor tissue in each animal was measured.

Statistical analysis

Data are presented as mean ± standard deviation (n = 3) of three experiments performed independently. Data analysis was performed using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA). Comparisons were made using Student's *t*-test and one-way analysis of variance. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Indole-thiazolidinone inhibited proliferation of HK1 and NPC-039 NPC cells

Results from MTT assay showed that indole-thiazolidinone suppressed proliferation of HK1 and NPC-039 NPC cell lines at 72 h. Treatment of HKH1cells with indole-thiazolidinone at doses of 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µM resulted in 99, 87, 71, 64, 49, 38 and 31 % inhibitions in proliferation, respectively (Figure 1). Treatment of NPC-039 cells with 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µM indole-thiazolidinone decreased proliferation to 97, 84, 69, 58, 44, 35 and 28 %, respectively. Thus, indole-thiazolidinone exhibits anti-proliferative effect on NPC cells in a concentration-dependent manner.

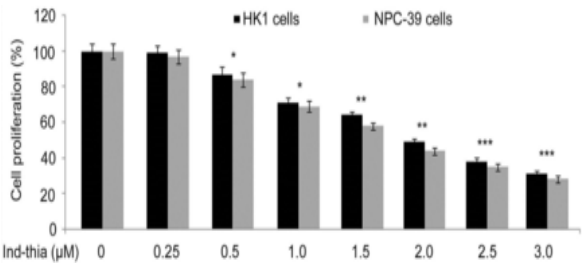

Figure 1: Anti-proliferative effect of indole-thiazolidinone on NPC and NPC-39 cells. HK1 and NPC-039 cells were treated with 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µM indole-thiazolidinone for 72 h. Cell proliferation was determined by MTT assay. Values are expressed as mean of triplicate measurements performed independently; *$p < 0.05$, **$p < 0.02$, ***$p < 0.01$, relative to untreated control cells

Migration and invasion of HK1 NPC cells was inhibited by indole-thiazolidinone

Treatment of HK1 cells with indole-thiazolidinone for 72 h decreased cell migration significantly ($p < 0.05$). In HK1 cell cultures, migration potential was reduced to 58.32, 47.54, 28.91 and 17.65 % on treatment with 1.5, 2.0, 2.5 and 3.0 µM indole-thiazolidinone, respectively (Figure 2 A and C). Indole-thiazolidinone also significantly inhibited the invasiveness of HK1 cells at 72 h ($p < 0.05$). Quantification of cell invasion showed 63.41, 49.37, 35.12 and 19.67 % reductions on treatment with 1.5, 2.0, 2.5 and 3.0 µM indole-thiazolidinone, respectively (Figures 2 B and C).

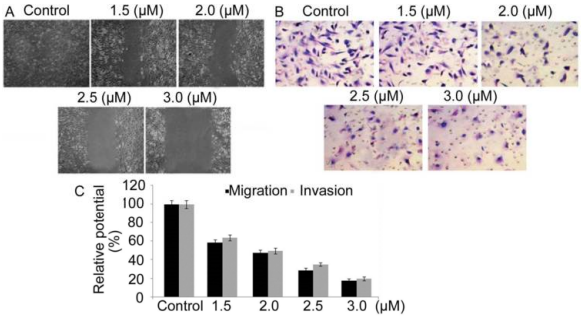

Figure 2: Inhibition of migration and invasion of HK 1 cells by indole-thiazolidinone. A: The cells were cultured in DMEM medium containing 1.5, 2.0, 2.5 and 3.0 µM indole-thiazolidinone and subsequently analysed for migration using wound healing assay. B: The cells were incubated with 1.5, 2.0, 2.5 and 3.0 µM indole-thiazolidinone for 72 h and invasion was determined using an Olympus-CX31 microscope. Cell migration in DMSO was taken as control (magnification, x150). Data are presented as mean ± standard deviation of three independent experiments; *$p < 0.05$, **$p < 0.02$, ***$p < 0.01$, compared to untreated control cells

Indole-thiazolidinone reduced the expression of matrix metalloproteinases

Western blot assay was used to determine the effect of indole-thiazolidinone on expressions of MMP-2 and MMP-9. Marked decreases in the expressions of MMP-2 and MMP-9 in HK1 cells were induced by indole-thiazolidinone at 72 h (Figure 3). The decreases in MMP-2 and MMP-9 expressions were maximum at 3.0 µM indole-thiazolidinone.

Indole-thiazolidinone inhibited p50 and p65 nuclear translocation

Western blotting was used to determine the levels of p65 and p50 in nuclear fraction of HK1 cells after treatment with 1.5, 2.0, 2.5 and 3.0 µM indole-thiazolidinone. Marked decreases in p65 and p50 levels in the nuclear fraction were observed on treatment with indole-thiazolidinone, when compared to untreated cells (Figure 4).

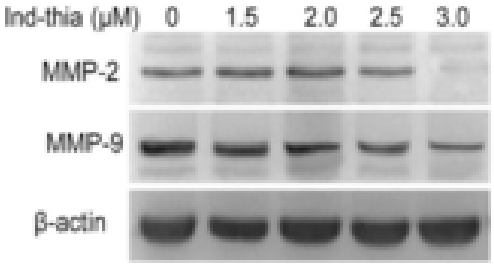

Figure 3: Effect of indole-thiazolidinone on the expressions of MMP-2 and MMP-9. A: HK1 cells were incubated with 1.5, 2.0, 2.5 and 3.0 µM indole-thiazolidinone, after which the expressions of MMP-2 and MMP-9 were determined using western blotting. B: The expression of MMP-2 and MMP-9 were quantified. β-Actin was used as loading control. Values are presented as mean of triplicate measurement; *$p < 0.05$, **$p < 0.02$, ***$p < 0.01$, relative to untreated control cells

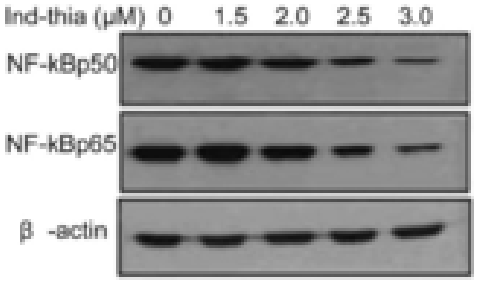

Figure 4: Indole-thiazolidinone suppresses p50 and p65 nuclear translocation. The cells incubated with indole-thiazolidinone (1.5, 2.0, 2.5 and 3.0 µM) were subjected to western blotting to determine the expressions of p50 and p65 in the nuclear fraction. β-Actin served as control

Combined effect of indole-thiazolidinone and NF-κB inhibitor, henenalin, on NPC cell proliferation

To confirm the involvement of NF-κB in indole-thiazolidinone-mediated inhibition of proliferation, HK1 cells were treated with a combination of indole-thiazolidinone and henenalin for 72 h (Figure 5). The results showed that treatment of HK1 and NPC-039 cells with indole-thiazolidinone and henenalin combination synergistically decreased their proliferation. The proliferations of HK1 cells on treatment with indole-thiazolidinone (3.0 µM), henenalin and indole-thiazolidinone-henenalin combination were 31, 43 and 12 %, respectively, while the proliferation of untreated culture was 99 % (Figure 5). The degrees of proliferation of NPC-039 cells on treatment with indole-thiazolidinone (3.0 µM), henenalin and indole-thiazolidinone-henenalin combination were 28,

39 and 11 %, respectively, while the proliferation of untreated culture was 99 % (Figure 5).

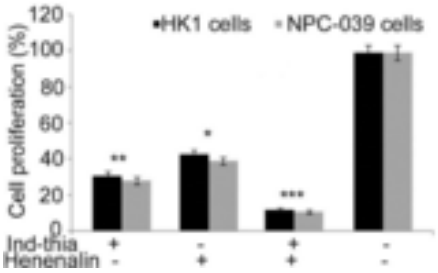

Figure 5: Effect of indole-thiazolidinone and henenalin on NPC cell proliferation. Following exposure to indole-thiazolidinone (3.0 µM), henenalin, combination of indole-thiazolidinone and henenalin or DMSO alone, HK1 cells were subjected to MTT assay. Values are expressed as mean of triplicate measurements performed independently; *$p < 0.05$, **$p < 0.02$, ***$p < 0.01$, compared to untreated control cells

Indole-thiazolidinone inhibited NPC growth *in vivo*

Tumor volume was measured on alternate days over a period of 30 days after treatment with indole-thiazolidinone at a dose of 10 mg/kg (Figure 6). Treatment of mice with indole-thiazolidinone caused a significant ($p < 0.05$) decrease in tumor growth. On day 31, tumor weight in animals treated with indole-thiazolidinone was significantly lower than that in untreated mice ($p < 0.05$). The mean tumor weights in indole-thiazolidinone-treated and untreated mice on day 31 were 0.23 and 4.8 g, respectively.

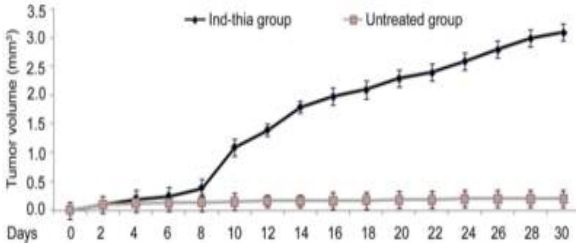

Figure 6: Effect of indole-thiazolidinone on tumor growth in mice. HK1 cells were subcutaneously implanted in mice to establish NPC mice model. The mice were then intraperitoneally injected indole-thiazolidinone (10 mg/kg), or normal saline. Tumor volume was measured on alternate days over 30 days, while tumor weight was measured on day 31 of treatment; *$p < 0.05$, **$p < 0.01$, relative to normal control mice. Ind-thia: indole-thiazolidinone

DISCUSSION

Nasopharyngeal carcinoma is one of the fatal diseases in southern part of China and Southeast Asia [1]. The present study was designed to investigate the role of indole-thiazolidinone in the treatment of nasopharyngeal carcinoma. The failure of current treatment strategies for nasopharyngeal carcinoma, and low survival rate of the patients are associated with the high metastasis potential of NPC. Carcinoma cell migration and invasion are facilitated by the weakening and decomposition of extracellular matrix [7]. The movement of cancer cells into vascular systems like lymph, blood, and subsequently to distant organs is possible only after extracellular matrix degradation [7].

In the present study, indole-thiazolidinone treatment markedly decreased migration and invasion tendency of HK1 cells. The wound healing assay showed inhibition of migration potential in HK1 cells by indole-thiazolidinone treatment. In Matrigel™ coated chamber assay, the invasion tendency of HK1 cells was markedly decreased on incubation with indole-thiazolidinone. These observations proved that indole-thiazolidinone exhibits anti-metastatic activity on nasopharyngeal carcinoma cells. Overexpression of matrix metalloproteinase plays a vital role in the process of extracellular matrix decomposition [20-22]. Down-regulation of matrix metalloproteinase-2 and metalloproteinase-9 expressions by drug candidates has been shown to suppress carcinoma growth [23-26].

In view of this, the present study investigated the effect of indole-thiazolidinone on levels of matrix metalloproteinases in HK1 cells. Western blotting assay showed that indole-thiazolidinone treatment caused marked decreases in matrix metalloproteinase expressions in HK1 cells. Thus, indole-thiazolidinone inhibits HK1 cell metastasis by inhibition of extracellular matrix decomposition and down-regulation of matrix metalloproteinase levels. Matrix metalloproteinase levels are enhanced in cancer cells by the up-regulation of NF-κB [23-25]. It has been demonstrated that targeting NF-κB expression in carcinoma cells regulates tumor growth [27-30]. Thus, NF-κB level in HK1 cells was determined after indole-thiazolidinone treatment.

The results showed that the levels of p65 and p50 levels were markedly increased in nuclear fractions of indole-thiazolidinone-treated HK1 cells. To confirm whether NF-κB was involved in inhibition of HK1 cell proliferation by indole-thiazolidinone, the cells were treated with a combination of indole-thiazolidinone and henenalin, an NF-κB inhibitor. The results revealed that treatment of HK1 cells with a

combination of indole-thiazolidinone and henenalin decreased HK1 cell proliferation in a synergistic manner. In addition, indole-thiazolidinone treatment inhibited tumor growth and development in nasopharyngeal carcinoma mice model *in vivo*.

CONCLUSION

Indole-thiazolidinone inhibits nasopharyngeal carcinoma cell proliferation and metastasis via regulation of matrix metalloproteinase and NF-κB expressions. Therefore, indole-thiazolidinone is a potential therapeutic agent for the management of nasopharyngeal carcinoma.

DECLARATIONS

Acknowledgement

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Conflict of interest

The authors declare that 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. Guoping Zhang designed the study, compiled the data, performed literature survey and wrote the paper. Sheng Zhang performed the experimental work. Both the authors read the paper thoroughly before communicating it for publication.

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