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Original Research Article

Arylsulfonyl indoline-enzamide exhibits inhibitory effect on nasopharyngeal carcinoma

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Abstract

Purpose: *To investigate the effect of arylsulfonyl indoline-benzamide (ASIB) on the viability of nasopharyngeal carcinoma (NPC) cells, and the underlying mechanism of action.*
Methods: *The viability of C666 and NPC 039 cells was determined using 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) assay. Cell migration was analysed by wound healing assay, while protein expression levels of matrix metalloproteinases (MMPs), p50, p65 and NF κB were assayed using western blotting.*
Results: *MTT assay results showed that ASIB treatment led to significant and dose-dependent reductions in the viability of C666 and NPC 039 ($p < 0.05$). The migration and invasive potential of C666 cells were decreased on incubation with ASIB for 48 h. Western blotting data showed significant decrease in MMP 2/9 expressions in C666 cells on treatment with ASIB ($p < 0.05$). The levels of p65 and p50 in the nuclear fraction of C666 cells were markedly lower than those in the negative control group. Arylsulfonyl indoline-benzamide (ASIB) treatment for 48 h decreased the level of NF κB expression in C666 cells ($p < 0.05$). The volume of tumor excised from ASIB-treated NPC mice was lower than that of the untreated group.*
Conclusion: *Arylsulfonyl indoline-benzamide (ASIB) exhibits inhibitory effects on the viability and metastasis potential of NPC cells. Thus, it may be beneficial in the treatment of nasopharyngeal carcinoma but this has to be further investigated.*

Keywords: *Metastasis, extracellular matrix, nuclear fraction, pathogenesis*

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is one of the fatal human diseases diagnosed regularly in the southern parts of China and Southeast Asia [1]. China dominates the list of NPC cases, and alone accounts for approximately 80 % of the patients as per WHO data [1]. It has been reported that about 95 % of clinically diagnosed NPC are very poorly differentiated and are sensitive to X-ray treatments [2]. The cancer metastasizes to the lymph nodes in more than 70 % NPC patients, leading to worsening of the disease [3]. In addition, NPC undergoes metastasis to distant organs such as lungs, liver and bone [4]. The currently available treatment for NPC at the early stage is radiotherapy,

although the 5-year survival of patients is very low [5]. In most NPC patients, cancer relapse has been detected after radiotherapy [5]. Thus, the development of new and efficient treatment for NPC which can inhibit the cancer and improve the survival of patients is of great medical significance.

Tumor metastasis involves several steps such as loss of adhesion between the cells, extracellular matrix degradation, up-regulation of migration and increased neo-vascularization [6]. Metastasis is facilitated by the degradation of extracellular matrix and various components of the basement membrane [6,7]. The extracellular matrix degradation and basement membrane break down is catalysed by matrix metalloproteinases [8,9]. Two most important matrix metalloproteinases involved in the extracellular matrix degradation and basement membrane break down are MMP-2 and MMP-9 [8,9]. Downregulation of MMP-2 and MMP-9 is important in the suppression of NPC invasion and metastasis [8,9].

In various biologically-active natural compounds and drug candidates, indoline is present as the main pharmacophore [10-12]. In medicinal chemistry, indoline is considered an important scaffold for the development of drug molecules [10-12]. It has been reported that 7-aroyl-aminoindoline-sulfonamide is a potential anti-proliferative compound due to its antimitotic and vascular disrupting effects [12]. These findings attracted chemists and clinicians to develop indoline-based structures for investigation of anti-cancer effects. Benzamides act as potential anti-proliferative compounds and have been found to exhibit their effects through diverse mechanisms [13-15]. Recently, *N*-aryl-4-[(1-arylsulfonyl)-indolin-7-yl)amino methyl]benzamides were shown to be promising anti-proliferative agents in cancer cells [16]. In the present study, the effect of arylsulfonyl indoline-benzamide (ASIB) on NPC cell viability, and the underlying mechanism were investigated.

EXPERIMENTAL

Cell culture

The C666 and NPC-039 cells were supplied by the Institute of Virology, Chinese Academy of Preventive Medicine (Beijing, China). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing 10 % FCS. The medium was also mixed with the antibiotics penicillin (100 U/mL) and streptomycin (100 µg/mL). Cell culture was carried out at 37 ˚C under a humidified atmosphere of 5 % CO_2.

MTT assay

Changes in viabilities of C666 and NPC-039 cells on exposure to ASIB were determined using 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded at a density of 2 x 10^6 cells/well in 96-well culture plates and grown for 24 h. The medium was then replaced with fresh medium containing 10, 20, 30, 40, 50 or 60 µM ASIB. After 48 h of incubation, 20 µL of MTT solution (5 mg/mL; Sigma-Aldrich) was added to each well of the plate and incubation was continued for 4 h. Then, the medium was discarded and 150 µL DMSO was added to each well to dissolve the blue formazan crystals formed. The absorbance of the supernatant collected from each well was measured at 450 nm, using an MK3 micro-plate reader (Thermo).

Analysis of cell migration

The migration potential of C666 cells after 48 h of incubation with ASIB was assessed using wound healing assay. The cells were seeded into the six-well plate in DMEM medium containing 10 FCS and cultured to 70 % confluency. The plastic tip of a 100 µL pipette was used to scratch the cell monolayer by drawing a straight line through the centre of the plate. The cells were then separately exposed to 10, 20, 30, 40, 50 and 60 µM ASIB or DMSO alone (control) for 48 h. Thereafter, cell migration was measured using an Olympus-CX31 microscope (Olympus Corp.).

Analysis of cell invasion

The 24-well invasion chamber for cell culture (Corning Inc., Tewksbury, MA, USA) was coated with 8.0 µm Matrigel™ (Becton Dickinson, Bedford, MA, USA) before the seeding of cells. The C666 cells were separately pre-treated with 10, 20, 30, 40, 50 and 60 µM ASIB or DMSO alone (control) for 48 h. Then, cells at a density of 2 x 10^6/200 µl of serum-free medium were seeded into the upper compartment of the chamber. Into the lower chamber was placed DMEM medium containing 10 % FCS. After 48 h of incubation, the cells on the upper chamber were cleaned using cotton swab and the cells that invaded lower chamber were fixed with 90 % ethanol. The cells were then subjected to crystal violet staining and counting was performed using an Olympus-CX31 (Olympus Corp., Tokyo, Japan) microscope.

Western blot analysis

The C666 cells were exposed to 10, 20, 30, 40, 50 and 60 µM ASIB or DMSO alone (control) for 48 h. Thereafter, the cells were treated with 200 µL of the lysis buffer (containing 50 mmol/l Tris-hydrochloride, 2 mmoL ethylene diamine tetraacetate, 300 mmol potassium chloride, 200 mmoL sodium vanadate, 2 mmoL PMSF and 2 % Triton X-100 at a pH of 7.4). The lysates of C666 cell nuclei were collected using the NucBuster™ extraction kit for proteins (Novagen®; Merck KGaA, Darmstadt, Germany) in accordance with the manual protocol. The protein separation was performed by subjecting 30-µg samples to 10 % SDS-polyacrylamide gel electrophoresis. The proteins were subsequently transferred onto polyvinylidene fluoride membrane which was incubated for 1 h at 37˚C with non-fat milk [containing Tris-buffered saline and TBST Tween®-20] to block non-specific binding sites. Thereafter, the membrane was incubated at 4˚C overnight with anti-p50, anti-p65, anti-MMP-2, anti-MMP-9 and anti-β-actin primary antibodies, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody for 1 h at room temperature. An Enhanced Chemiluminescence kit (ECL Plus; GE Healthcare Europe GmbH, Freiburg, Germany) was used for the band visualization.

Experimental animals

Forty (40) male BALB/c nude mice (5-week old) were supplied by the Chinese Academy of Sciences, Shanghai Institute of Cell Biology (Shanghai, China). The mice were housed under 12 h light and dark cycles and provided access to laboratory feed and water. The temperature in the laboratory was maintained at 22 - 23 ˚C, and humidity was 55 - 60 %. All experimental procedures on mice were performed in accordance with the guidelines for the Care and Use of Laboratory Animals, National Institute of Health, China. The study was approved by the Ethics Committee of The Second Affiliated Hospital of Xi'an Jiaotong University (Xibei Hospital) (approval no. TXY20160621), and animal study was followed by the guidelines of the Chinese Academy of Sciences [17].

Establishment of NPC tumor model and treatment

Forty mice were assigned randomly to four groups of 10 each. Nasopharyngeal carcinoma was induced in 30 mice using standard protocol. The C666 cells were administered into in the right flank regions of mice at a dose of 2 x 10^6 *cells* per mouse. Mice in two treatment groups were injected ASIB at separate doses of 30 and 50 µM intragastrically on the same day of cancer cell administration. The third group of mice was not given ASIB, and served as untreated control. The mice in untreated control and normal control groups received normal saline alone. The tumor volume in each mouse under study was measured using callipers on alternate days over a period of one month after tumor administration. On the day 31st of tumor cell administration, the mice were sacrificed using cervical dislocation method. The tumor from each mice was excised and weighed to determine the effect of ASIB on tumor growth.

Statistical analysis

Data presented are the mean of ± SD of three assays carried out independently. The data were analysed statistically using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). Differences were determined using Student's *t*-test and a one-way analysis of variance. The differences were taken as statistically significant at $p < 0.05$.

RESULTS

Arylsulfonyl indoline-benzamide inhibited proliferation of C666 and NPC-039 NPC cells

Arylsulfonyl indoline-benzamide (ASIB) reduced C666 and NPC-039 cell viabilities in concentration-based manner (Figure 1). Increasing the concentration of ASIB from 10 to 50 µM caused decreases in viability in both the cell lines. No further reduction in cell viability was observed on increasing the concentration of ASIB above 50 µM. The viability of C666 cells was reduced to 92.34, 81.67, 69.09, 53.86, 38.32 and 38.31 %, on exposure to 10, 20, 30, 40, 50 and 60 µM ASIB, respectively. Similarly, in NPC-039 cell cultures exposed to 10, 20, 30, 40, 50 and 60 µM ASIB, viability was reduced to 91.75, 78.13, 65.48, 49.56, 34.86 and 34.81 %, respectively.

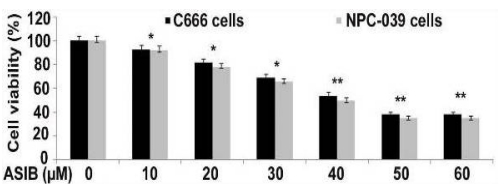

Figure 1: Effect of ASIB on viabilities of C666 and NPC-039 cells. The cell lines were exposed for 48 h to 10, 20, 30, 40, 50 and 60 µM ASIB. Changes in the cell viability were determined using MTT assay. The values are mean of ± SD of three experiments performed independently; *$p < 0.05$ and **$p < 0.02$, vs. control

Arylsulfonyl indoline-benzamide inhibited C666 cell migration

The cells were exposed to ASIB at concentrations of 10, 20, 30, 40, 50 and 60 µM for 48 h to determine changes in migration potential (Figure 2). Arylsulfonyl indoline-benzamide (ASIB) reduced migration of C666 cells by 15.22, 34.78, 47.91, 68.84 and 95.57 %, at concentrations of 10, 20, 30, 40 and 50 µM, respectively, when compared to 100 % in the control.

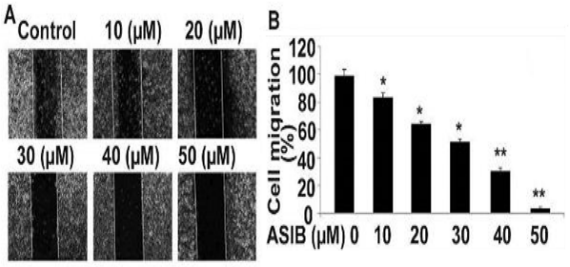

Figure 2: Effect of ASIB on C666 cell migration. Changes in C666 cell migration due to ASIB treatment were determined using wound healing assay. Arylsulfonyl indoline-benzamide was added to the cultures of C666 cells at indicated concentrations and images were taken at x200 magnification; *p < 0.05 and **p < 0.02 vs. control

Arylsulfonyl indoline-benzamide inhibited C666 cell invasion

Matrigel™ TransWell assay was used for assessment of the effect of ASIB on C666 cell invasion (Figure 3). Treatment of C666 cells with ASIB for 48 h caused a significant decrease in cell invasion. Arylsulfonyl indoline-benzamide (ASIB) at 10, 20, 30, 40 and 50 µM reduced invasion of C666 cells by 11.32, 26.77, 37.46, 72.62 and 93.85 %, respectively, when compared to 100 % in the control.

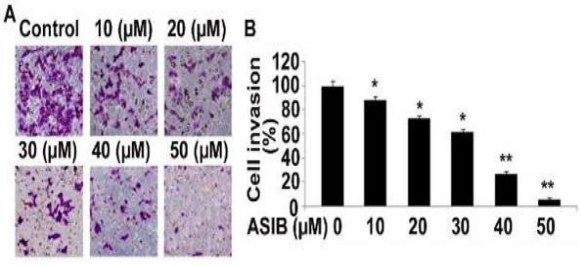

Figure 3: Effect of ASIB on C666 cell invasion. Cell exposure to 10, 20, 30, 40 and 5 µM ASIB for 48 h was followed by assessment of invasion using TransWell assay. The images were captured at x200 magnification; *p < 0.05 and **p < 0.02, vs. control cell viability

ASIB Inhibited MMP expression in C666 cells

The C666 cells were exposed for 48 h to 10, 20, 30, 40, 50 and 60 µM ASIB (Figure 4). Marked decreases in MMP-2 and MMP-9 protein expressions were seen in C666 cells on exposure to ASIB. The reductions in MMP-2 and MMP-9 protein levels by ASIB treatment in C666 cells was concentration-dependent. The inhibition of protein expressions of MMP-2 and MMP-9 was maximum at 50 µM ASIB.

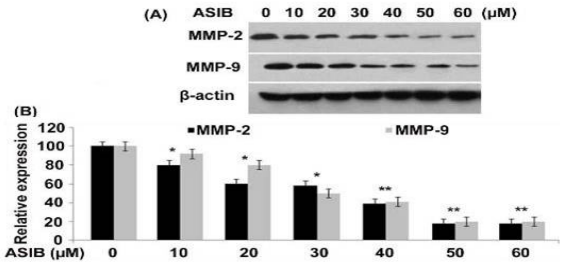

Figure 4: Effect of ASIB on MMP-2 and MMP-9 expressions. (A) Western blotting was used for determination of ASIB-induced changes in MMP-2 and MMP-9 protein expressions in C666 cells. (B) The protein expressions were quantified and the values presented as average of ± SD of three experiments. *p < 0.05 and **p < 0.02, vs. control C666 cells

Inhibition of p50 and p65 nuclear translocation in C666 cells by ASIB

The effect of ASIB on protein expressions of p65 and p50 in C666 cells was determined with western blotting (Figure 5). In C666 cell cultures, exposure to ASIB significantly reduced the expression of p65 and p50 proteins in the nuclei. The down-regulation of p65 and p50 protein expressions in C666 cells by ASIB was concentration-dependent. The inhibitions of p65 and p50 proteins by ASIB were significant from 10 µM, and the effects were maximum at 50 µM in 48 h.

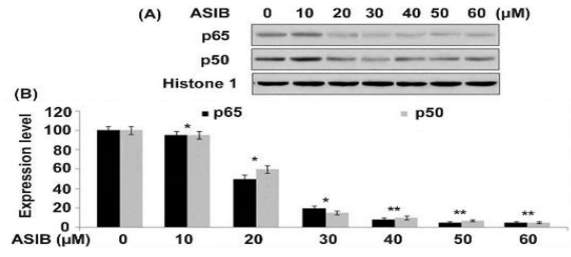

Figure 5: Effect of ASIB on NF-κB expression in C666 cells. (A) Protein expressions of p65 and p50 in C666 cell nuclei after 48 h of ASIB treatment were determined with western blotting. (B) The protein level was quantified and values are presented as mean ± SD of three experiments; *p < 0.05 and **p < 0.02, versus control C666 cells

Arylsulfonyl indoline-benzamide suppressed NPC development *in vivo*

On day 31st of tumor implantation, the mice were sacrificed and the tumors were excised and weighed (Figure 6). Tumor growth in mice was significantly suppressed by ASIB treatment, when compared to the untreated group.

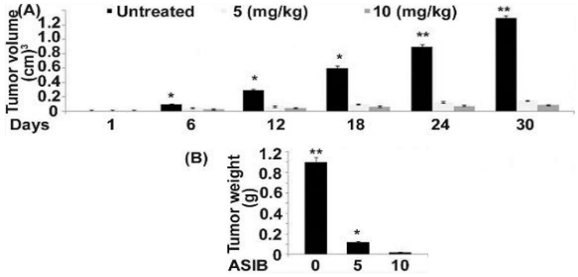

Figure 6: Effect of ASIB on tumor growth *in vivo*. Mice implanted with C666 cell xenograft subcutaneously were treated with ASIB at doses of 30 and 50 mg/kg. (A) Tumor volume in the mice was recorded on alternate days over a period of one month. (B) Tumors were excised on day 31st of ASIB treatment and their weights were obtained. *$p < 0.05$ and **$p < 0.02$, vs. normal control

DISCUSSION

Benzamides act as potential anti-proliferative compounds and exhibit their effects through diverse mechanisms [13-15]. Aryl-4-[(1-(arylsulfonyl)-indolin-7-yl)aminomethyl)benzamides constitute an important class of benzamide family which possesses significant anti-proliferative properties [16]. The present study investigated the effect of arylsulfonyl indoline-benzamide on the migration and invasion potential of NPC cells. The study demonstrated reduction of viability, and suppression of invasive and migratory potential of NPC cells by arylsulfonyl indoline-benzamide through down-regulation of NF-κB expression. In the present study, the effect of arylsulfonyl indoline-benzamide on viability of C666 and NPC-039 cells was determined. Arylsulfonyl indoline-benzamide treatment reduced the viability of both cell lines at 48 h in a concentration-based manner.

These findings suggest that arylsulfonyl indoline-benzamide acts as an anti-proliferative agent against the NPC cells. The major challenge to the treatment of NPC is its metastasis to the lymph nodes and distant organs such as lungs, liver and bones [3,4]. The present study investigated the effect of arylsulfonyl indoline-benzamide on migration and invasion potential of C666 NPC cells. Wound healing assay showed that exposure of C666 cells to arylsulfonyl indoline-benzamide significantly reduced their migration potential. The invasive potential of NPC cells was also reduced significantly on exposure to arylsulfonyl indoline-benzamide. These results showed that arylsulfonyl indoline-benzamide significantly reduced viability and suppressed metastasis of NPC cells.

The metastasis of tumor cells is associated with the extracellular matrix degradation, breakdown of basement membrane components and increased neo-vascularization [6]. Degradation of the extracellular matrix and basement membrane is induced by the expression of matrix metalloproteinase [18-20]. In the present study, the effect of arylsulfonyl indoline-benzamide on expression of matrix metalloproteinase in C666 NPC cells was investigated. The study showed that arylsulfonyl indoline-benzamide exposure caused a marked decrease in the expression of matrix metalloproteinases in C666 NPC cells. These observations suggest that arylsulfonyl indoline-benzamide suppresses NPC cell metastasis through down-regulation of protein expressions of MMP-2 and -9.

Protein expressions of MMP-2 and MMP-9 in carcinoma cells is up-regulated by nuclear factor (NF)-κB-related proteins [21, 22]. Therefore, the effect of arylsulfonyl indoline-benzamide on the expression of NF-κB in C666 cells was investigated. The results revealed that exposure of C666 cells to arylsulfonyl indoline-benzamide significantly decreased the expressions of p65 and p50 in the nuclei of the cells. The effect of arylsulfonyl indoline-benzamide on tumor growth was also studied *in vivo* in mice model of NPC. The study revealed that arylsulfonyl indoline-benzamide treatment significantly decreased volume and weight of tumor in the mice model of NPC.

CONCLUSION

The findings of the study show that arylsulfonyl indoline-benzamide reduces NPC cell viability and metastasis via down-regulation of NF κB pathway. Therefore, arylsulfonyl indoline-benzamide can potentially be developed into a drug for the treatment of nasopharyngeal carcinoma.

DECLARATIONS

Acknowledgement

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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. Yang Jing, Wen Zhang and Hui Liu performed the experimental work. Yujuan Wang carried out the literature study and compiled the data. Guoxi Zheng designed the study and wrote the paper. All the authors wrote the paper before communication.

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