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Original Research Article

Tiazofurin inhibits oral cancer growth *in vitro* and *in vivo* via upregulation of miR-204 expression

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Abstract

Purpose: *To investigate the effect of tiazofurin on proliferation and growth of oral cancer cells, and the associated mechanism(s) of action.*
Methods: *The effect of tiazofurin on the cytotoxicity of SCC-VII and SCC-25 oral cancer cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, while cell apoptosis was determined by flow cytometry. Western blotting was used for assaying protein expressions.*
Results: *Tiazofurin inhibited the viability of the oral cancer cells in a concentration-based manner ($p < 0.05$). Tiazofurin treatment at a dose of 2.0 μM reduced the proliferation of SCC-VII and SCC-25 cells to 25 and 22 %, respectively. Apoptosis was significantly increased in SCC-VII and SCC-25 cells by tiazofurin treatment, relative to untreated cells ($p < 0.05$). Tiazofurin also increased the activation levels of caspase-3 and caspase-9 and downregulated the expressions of p-Akt and p-mTOR in the two cancer cell lines. Moreover, miR-204 expression was significantly promoted in the tiazofurin-treated cells, when compared to control ($p < 0.05$). In SCC-VII cells, treatment with tiazofurin suppressed F-actin expression, relative to control.*
Conclusion: *These results demonstrate that tiazofurin inhibits the viability and proliferation of SCC-VII and SCC-25 cancer cells via induction of apoptosis and activation of caspase-3/caspase-9. Moreover, tiazofurin targets Akt/mTOR pathway, and upregulats the expressions of F-actin and miR-204 in the oral carcinoma cells. These findings suggest that tiazofurin has a potential for use as an effective treatment for oral cancer.*

Keywords: *Oral cancer, Tiazofurin, Apoptosis, Caspase, Cytotoxicity*

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INTRODUCTION

Oral cancer initially originates as small lesion from tissues in the mouth, and subsequently grows to a cancerous mass in the oral cavity [1]. In some cases, oral cancer develops from metastatic tumor cells invading oral tissues from distant organs [1]. However, approximately 90 % of oral cancer occur in the lining of the lips and mouth, resulting in what is called squamous cell cancer [2]. Presently, oral cancer is managed with chemotherapy, radiotherapy or resection, either alone or in combination [2]. The survival of oral carcinoma patients is less than 50%, despite

various treatment techniques [2]. Thus, there is need for effective and novel treatment methods for squamous cell cancers to improve patient survival.

Apoptosis is triggered either by exogenous or endogenous signals, and it regulates cell death [3]. In multicellular organisms, the signals are generated due to aggregated misfolded proteins, irregularity in Ca^{2+} release, and protein processing in non-specific manner in endoplasmic reticulum [4]. The activation of these stresses for prolonged durations serves as signal for induction of apoptosis [5]. Caspase-3 activation by apoptotic signals in endoplasmic reticulum has crucial role in cellular death and removal of surplus cells [6]. Eukaryotic cells harbor a protein known as cofilin which binds and induces reconstruction of structural framework in actin [7]. The binding of cofilin to actin promotes cellular movement by facilitating pseudopodia-like structures [8]. Elevated F-actin has been reported in colon, kidney, prostate and esophageal carcinoma cells [9]. F-Actin also enhances cellular adhesion and migration of localized carcinoma cells in different tissues [10].

Natural products serve as invaluable resources for drug candidates due to their low toxicities in living systems, relative to synthetic drugs. Diverse plant-derived secondary metabolites are included in clinical drug list because of their potential pharmacological properties [11]. Flavonoids exert beneficial influence on human health by reducing risk of disorders via targeting tumor cell proliferative potential and inhibiting inflammation [12]. The current study investigated the anti-proliferative effect of tiazofurin (Figure 1) in oral cancer cells and determined the associated mechanism(s).

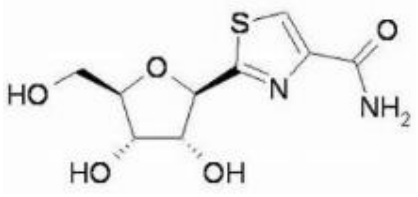

Figure 1: Molecular structure of tiazofurin

EXPERIMENTAL

Cell culture

Oral cancer cell lines (SCC-VII and SCC-25) were provided by Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM mixed containing 10 % fetal bovine serum, penicillin (100 µg/mL) and streptomycin (100 µg/mL) in an incubator in a 5 % CO_2 atmosphere at 37 ˚C.

Cytotoxicity assay

Cytotoxic changes induced by tiazofurin in the oral cancer cells were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were separately seeded in 96-well plates, each at a density of 1 x 10^6 cells/well in DMEM and incubated for 24 h. Tiazofurin was added to the medium at separate doses at 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 µM, and incubated with the cells for 48 h. Cells treated with only 30 mM HEPES for 48 h served as negative control. Following incubation, MTT solution (5 µg/mL) was added to each plate, followed by further incubation of the cells for 3 h at 37 ˚C. Thereafter, the medium was replaced with 120 µl of DMSO to solubilize the formazan crystals formed, and the optical density of each solution was read in an EL800-Microplate Reader at 557 nm. Cell viabilities were estimated from the readings.

Determination of cell apoptosis

The cells were seeded in 6-well plates, each at a density of 1 x 10^5 cells/well in DMEM, and were cultured for 24 h at 37 ˚C, followed by incubation for 48 h with 2.0 µM tiazofurin. Then, the cells were rinsed twice in PBS, and re-suspended in 300 µL of binding buffer. Annexin V-fluorescein isothiocyanate (5 µL) was added to each well, and the wells were incubated in the dark at 4 ˚C for 40 min. Then, still in the dark, incubation was carried out for 20 min with 10 µLPI at room temperature. Cellular apoptosis was analyzed using FACSCalibur flow cytometer equipped with Flowjo software.

Determination of caspase activities

The cells were seeded at density of 1 x 10^5 cells/well in a96-well plate and treated with tiazofurin at a dose of 2.0 µM. Then, the cells were incubated for 48 h at 37 ˚C, followed by addition of 130 µL commercially-available caspase-3 and caspase-9 reagents (Beyotime Institute of Biotechnology). Thereafter, incubation was carried out for 3 h at room temperature, and absorbance was read at 488 nm in a microplate reader (Bio-Rad Laboratories, Inc.). The readings were used for estimation of caspase levels.

Western blotting analysis

The cells were seeded at a density of 1 x 10^6 cells/well in 6-well plates and treated for 48 h with 2.0 µM tiazofurin. Following rinsing with

PBS, the cells were lysed in RIPA buffer, and the protein content of the lysate was determined using BCA protein assay kit (Thermo Fisher Scientific Inc.). Then 30-µg protein samples were resolved on 10-12 % SDS-PAGE and transferred to PVDF membranes. The membranes were treated with 5 % non-fat milk in TBST at 37 ˚C, to block non-specific binding of the blot. Then, the membrane was incubated overnight with primary antibodies i.e. anti-p-Akt, anti-Akt, anti-p-mTOR, anti-mTOR and anti-F-actin at 4˚C, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 2.5 h at room temperature. The blots were detected using enhanced chemiluminescence (ECL) and analyzed with Image Lab software.

Statistical analysis

Results are expressed as mean ± standard deviation (SD) of three experiments performed independently. Statistical analysis was done using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). Differences were determined using Students *t*-test, one-way analysis of variance (ANOVA) and Tukey's post-hoc test. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

Tiazofurin inhibited SCC-VII and SCC-25 cell proliferation

Tiazofurin exhibited cytotoxic effect on viabilities of SCC-VII and SCC-25 cells in a concentration-based manner (Figure 2). Tiazofurin treatment at doses of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 µM reduced SCC-VII cell proliferation to 93, 85, 75, 60, 48, 41, 34 and 25 %, respectively. The levels of viability of SCC-25 cells were 91, 82, 70, 59, 44, 39, 31 and 22 %, on treatment with tiazofurin at doses of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 µM, respectively. Thus, tiazofurin suppressed the proliferative potential of the oral carcinoma cells in a concentration-dependent manner.

Tiazofurin induced apoptosis in SCC-VII and SCC-25 cells

Values of percentage apoptosis were significantly increased in SCC-VII and SCC-25 cells by tiazofurin treatment, relative to untreated control ($p < 0.05$; Figure 3). Apoptosis in tiazofurin treated SCC-VII cells increased to 68.71 % on treatment with 2.0 µM dose, relative to 1.98 % in control cells. Treatment with tiazofurin at 2.0 µM increased apoptotic in SCC-25 cells to 70.46 % relative to 2.56 % in control cells. Thus, tiazofurin exhibited cytotoxic effect on the oral carcinoma cells by activating apoptosis.

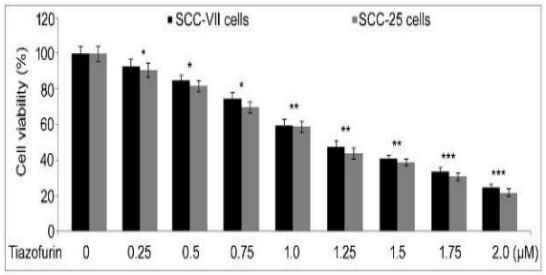

Figure 2: Effect of tiazofurin on the viability of oral carcinoma cells. The SCC-VII and SCC-25 cells were treated with tiazofurin at indicated doses, and viability measurements were made using MTT assay; *p < 0.05; **p < 0.02; ***p <0.01, vs. untreated treated cells

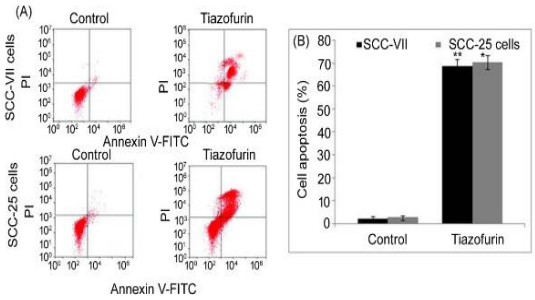

Figure 3: Effect of tiazofurin on apoptosis in oral carcinoma cells. The SCC-VII and SCC-25 cells were treated with 2.0 µM tiazofurin and apoptosis was assayed using flow cytometry. *P < 0.02; **p < 0.01, vs untreated cells

Tiazofurin activated caspase-3 and caspase-9 activities in SCC-VII and SCC-25 cells

The effect of tiazofurin on the activities of caspase-3 and caspase-9 in SCC-VII and SCC-25 cells was assayed with western blot assay (Figure 4). Tiazofurin at a dose of 2.0 µM markedly activated caspase-3activities in SCC-VII and SCC-25 cells, relative to control cells. Moreover, it significantly activated caspase-9 activities in SCC-VII and SCC-25 cells, when compared to untreated cells.

Tiazofurin suppressed Akt activation in SCC-VII and SCC-25 cells

Changes in p-Akt and Akt levels in SCC-VII and SCC-25 cells due to tiazofurin treatment were assayed using western blotting cells (Figure 5). The expressions of p-Akt in 2.0 µM tiazofurin-treated SCC-VII and SCC-25 cells were suppressed markedly, relative to control cells. However, tiazofurin treatment caused no

changes in expressions of total Akt protein in SCC-VII and SCC-25 cells.

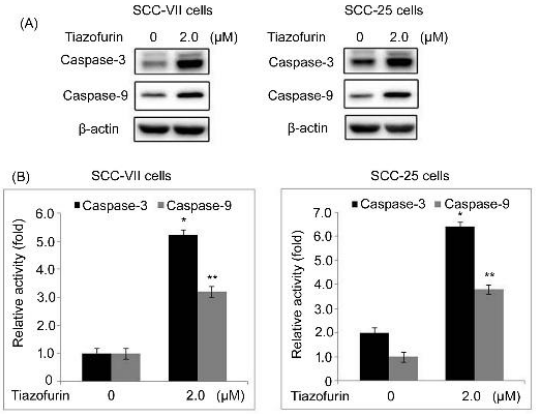

Figure 4: Effect of tiazofurin on caspase-3 and caspase-9 activities in SCC-VII and SCC-25 cells, as assayed using western blotting. *P < 0.02; **p < 0.01, vs. untreated cells

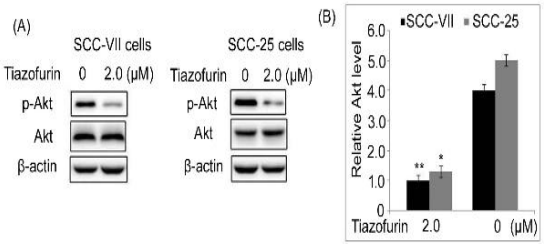

Figure 5: Effect of tiazofurin on Akt and p-Akt expressions. (A) SCC-VII and SCC-25 cells were treated with tiazofurin at a dose of 2.0 μM, and the expressions of p-Akt and Akt were determined with western blot assay. (B) Densitometric data for p-Akt and Akt expressions. *P < 0.02; **p < 0.01, vs untreated cells

Tiazofurin suppressed p-mTOR expressions in SCC-VII and SCC-25 cells

Changes in p-mTOR expression due to tiazofurin treatment were assayed with western blotting in SCC-VII and SCC-25 cells (Figure 6). The expression of p-mTOR in 2.0 μM tiazofurin-treated SCC-VII and SCC-25 cells were suppressed markedly, relative to untreated cells. However, tiazofurin treatment caused no changes in expressions of total mTOR protein in SCC-VII and SCC-25 cells.

Tiazofurin inhibited F-actin expressions in SCC-VII and SCC-25 cells

The expressions of F-actinin tiazofurin-treated SCC-VII and SCC-25 cells were assayed with western blotting technique. In SCC-VII cells, treatment with 2.0 μM tiazofurin markedly suppressed F-actin expression, relative to control. The F-actin expression in SCC-25 cells was also reduced by treatment with 2.0 μM tiazofurin. These results are shown in Figure 7.

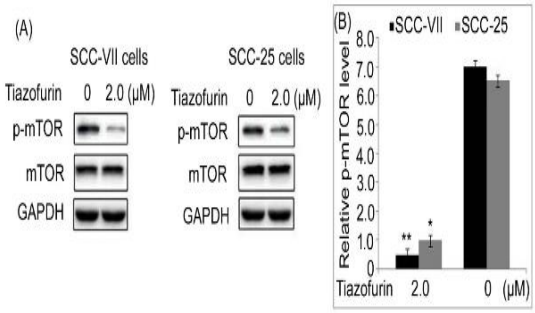

Figure 6: Effect of tiazofurin on p-mTOR expression. (A) SCC-VII and SCC-25 cells were treated with tiazofurin at a dose of 2.0 μM, and p-mTOR expression was measured with western blot assay. (B) Densitometric data for p-Akt and Akt expression. *P < 0.02; **p < 0.01, vs. untreated cells

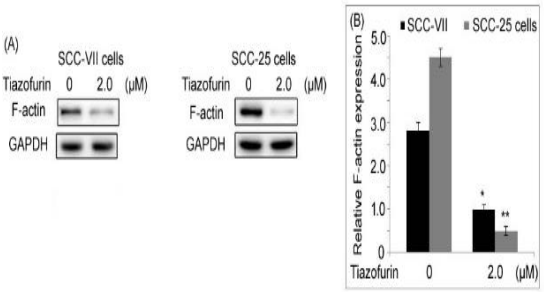

Figure 7: Effect of tiazofurin on F-actin expression. (A) The SCC-VII and SCC-25 cells were treated with tiazofurin at a dose of 2.0 μM, and F-actin expression was measured using western blotting assay. (B) Densitometric data for F-actin expression; *p <0.02 and **p <0.01 vs. 0 μM tiazofurin-treated cells

DISCUSSION

Oral cancer belongs to the category of head and neck carcinoma and is the 6th most commonly diagnosed malignancy throughout the world [13]. It is the fourth highest cause of death related to cancer in males, and it ranks highest in mortality among people of young age in Taiwan [14]. Although diagnostic techniques and screening processes have improved significantly over the years, morbidity due to oral cancer has increased significantly in recent years in Asian countries and China [15]. In the present study, tiazofurin significantly suppressed the viabilities of SCC-VII and SCC-25 cells in dose-based manner, and significantly induced apoptosis in the cancer cells, relative to untreated control cells. Stress-mediated caspase activation in endoplasmic reticulum initially enhances mitochondrial

permeability, with subsequent cytochrome c efflux [16]. This is immediately followed by apoptotic complex formation, cleavage of effector caspases, and cellular apoptosis [6]. The current study showed that tiazofurin markedly elevated caspase-3 and caspase -9 activities in SCC-VII and SCC-25 cells, relative to untreated cells.

Protein synthesis for multiple functions (including cell survival) is regulated by the mTOR pathway which serves as an important target for treatment of various cancers [17]. The PI3K/Akt pathway which regulates cellular proliferation and viability uses mTOR as a downstream effector [17]. Autophagic death has been reported in multidrug-resistant lung cancer cells via inhibition of the Akt/mTOR pathway [18]. In the current study, tiazofurin reduced phosphorylation of Akt (p-Akt) in SCC-VII and SCC-25 cells without influencing total Akt expressions. Tiazofurin treated SCC-VII and SCC-25 cells also showed lower level of p-mTOR, relative to untreated cells. This suggests that tiazofurin inhibited the growth of SCC-VII and SCC-25 cells by targeting the Akt/mTOR pathway.

The internal proteins in cells like microfilaments and canaliculi together comprise the cytoskeleton framework [19]. Among the structural constituents of the cytoskeleton, microfilaments are the smallest units, and they consist of globular-form actin protein called F-actin [19]. The data from the present study have shown that tiazofurin treatment suppressed expression of F-actin in the two oral cancer cells. MicroRNAs (miRNAs) contribute to the development of various cancers, including gastric carcinoma [20]. These microRNAs are involved in regulation of target genes via suppression of translation or degradation of mRNAs [21]. Studies have shown that miR-204 expression is markedly down-regulated in carcinoma cells [22]. Therefore, elevation of miR-204 expression has therapeutic role in treatment of cancers. The present study demonstrated that tiazofurin induced up-regulation of miR-204 expression in SCC-VII and SCC-25oral cancer cells.

CONCLUSION

The findings in this study demonstrate the inhibitory effect of tiazofurin on oral cancer cells via induction of apoptosis and activation of caspase-3 and caspase-9. Furthermore, tiazofurin targets the Akt/mTOR pathway, and elevates the expressions of F-actin and miR-204 in oral carcinoma cells. These results suggest that tiazofurin may be an effective therapeutic agent for oral 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 author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Aimin Zhao - conceived and designed the study; Xiaoying Tang, Yanhuan Hong - collected and analyzed the data; Xiaoying Tang, Aimin Zhao -wrote the manuscript. All authors read and approved the manuscript for publication.

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