Tropical Journal of Pharmaceutical Research July 2020; 19 (7): 1389-1395
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i7.8

Original Research Article

Down-regulation of F-actin and paxillin by N-(3-(1H-tetrazol-1-yl)phenyl) isonicotinamide derivative inhibits proliferation of prostate cancer cells

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Sent for review: 6 February 2020 *Revised accepted: 22 June 2020*

Abstract

Purpose: *To investigate the effect of N-(3-(1H-tetrazol-1-yl)phenyl) isonicotinamide derivative (TPIN) on prostate cancer cells, and the mechanism involved.*
Methods: *The cytotoxicity of TPIN in DU145 and PC3 cells was determined using Cell Counting Kit-8, while apoptosis induction was assayed by flow cytometry using Annexin V-fluorescein isothiocyanate dye. Changes in expressions of F-actin, RAC-α and paxillin were determined by western blot assay.*
Results: *Cell proliferation was effectively inhibited by TPIN in the concentration range of 0.75-15 μM. The values of half-minimum inhibitory concentration (IC_{50}) of TPIN for DU145 and PC3 cells at 48 h were 5.6 and 10.2 μM, respectively ($p < 0.05$). Treatment with 5.6 μM TPIN increased apoptosis to 59.64 % in DU145 cells, and 54.21% in PC3 cells. Cleaved caspase-3 and caspase-9 levels were increased by TPIN treatment in both cell lines ($p < 0.05$). Moreover, the levels of F-actin and paxillin were significantly downregulated by TPIN treatment in DU145 and PC3 cells ($p < 0.05$). In TPIN-treated DU145 and PC3 cells, cofilin-1expression was up-regulated, relative to control cells.*
Conclusion: *TPIN exhibits cytotoxic effect on prostate cancer cells via activation of apoptosis. It elevates cofilin-1 and the expressions of targets F-actin and paxillin in prostate cancer cells. Thus, TPIN is a potential chemotherapeutic agent for prostate cancer. However, further investigations, including clinical trials are required to authenticate these findings.*

Keywords: *Prostate cancer, F-actin, Paxillin, Apoptosis, Caspases*

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INTRODUCTION

Prostate cancer, one of the highly malignant tumors, is the fourth highest cause of cancer-related mortality in developed countries [1]. It has been shown that genetic variations determine susceptibility of people to prostate cancer [2]. Genomic studies have led to the identification of more than thirty sites susceptible to prostate cancer development [3]. It has been reported that prostate cancer gradually develops into androgen-independent carcinoma phenotype, at which stage its proliferation becomes independent of androgens. The androgen-independent stage of prostate cancer is generally resistant to chemotherapeutic

strategies and secondary endocrine treatments [4,5]. The prognosis of pancreatic carcinoma patients is very poor, with 5-year survival of only 8 %, according to current statistical data [1]. Advanced chemotherapeutic strategies have been ineffective for improvement of prognosis of pancreatic cancer [6]. Resection is the only effective curative strategy for pancreatic cancer. Unfortunately, pancreatic cancer is diagnosed late in 50 % of the patients, mostly at the stage of metastases. Moreover, the presently available chemotherapeutic agents are associated with adverse side effects, resulting in inadequate treatment and non-improvement in prognosis [6]. Thus, there is a dire need for identification of effective and novel therapeutic compounds for prostate cancer.

Apoptosis, a method for removal of unwanted cells, is stimulated either by endogenous cellular stimuli or exogenous stimuli [7]. Aggregation of unfolded proteins in endoplasmic reticulum, secretion of calcium ions and altered protein folding are the major pro-apoptotic stresses [7]. Apoptosis is the cause of continuous stress to the endoplasmic reticulum, leading to activation of pro-apoptotic proteins [8]. Caspase-3 which is located in endoplasmic reticulum, belongs to the family of caspase proteins, and it is activated by stress on the endoplasmic reticulum [9].

Cofilin, an actin-binding protein, has very low molecular weight, and its corresponding gene is present at chromosome 11q13 [10]. The reconstruction of schistose pseudopodia is regulated by F-actin, and it facilitates metastasis of cancer cells [11]. Over-expression of cofilin-1 has been reported in various kinds of cancer cells such as colon, esophageal and renal cancer cells [12]. The present study investigated the effect of TPIN (Figure 1) on prostate cancer cells, and the mechanism involved.

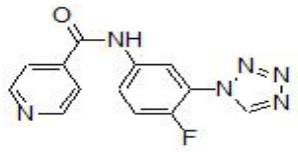

Figure 1: Molecular structure of *N-(3-(1H-tetrazol-1-yl)phenyl) isonicotinamide derivative*

EXPERIMENTAL

Cell lines

The DU145 and PC3 cell lines were provided by Biochemistry and Cell Biology Institute of Chinese Academy of Sciences, China. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) mixed with 10 % FBS at 37˚C under humidified atmosphere in an incubator with 5 % CO_2 and 95 % air.

Cell viability assay

Changes in proliferation of DU145 and PC3 cell lines were determined with Cell Counting Kit-8. The cells were plated at 2 x10^5 cells/well and maintained for 24 h, followed by incubation with TPIN at doses of 0.75, 1.5, 3.0, 6.0, 12 and 15 μM at 37˚C for 48 h. Then, commercial CCK-8 solution (12μL) was added to each of the plates, and incubation was performed for 5 h. Cellular proliferation was indirectly measured in a microplate reader at 455 nm.

Apoptosis analysis

The cells were seeded in well-plates at a density of 2 x10^5 cells per well, and treated with 5.6 μM TPIN, followed by incubation at 37˚C for 48 h, and washing in cold PBS. The cells were re-suspended in 450 μl of binding buffer. Thereafter, they were treated with Annexin V-FITC (5 μL) and PI (10 μL) for 40 min in the dark at 4˚C. Cellular apoptotic changes were detected using FACSCalibur flow cytometer. The data were analyzed with Flowjo software (version 7.6.1, USA).

Determination of activation of caspase-3 and caspase-9

The cells were plated at a density of 2 x10^5 cells per well, and treated with 5.6 μM TPIN at 37˚C for 48 h. Then, they were incubated with reagents for caspase-3 and caspase-9 (100 μL) for 3 h at room temperature. The levels of caspase-3 and caspase-9 in the cells were determined through measurement of absorbance at 487 in a microplate.

Cell cycle analysis

The cells were seeded in well-plates at a density of 2 x10^5 cells per well, and treated with 5.6 μM TPIN at 37˚C for 48 h. Then, the cells were harvested and subjected to washing with PBS, followed by fixing in 70 % ethyl alcohol for 150 min. This was followed by centrifugation for 5 min at 80 x g, washing in PBS, and re-suspending in 450 μL buffer mixed with RNase (12 μL) and PI (20 μL). Then, the cells were incubated in the dark for 20 min, and subjected to flow cytometric analysis.

Western blot assay

The cells were seeded in well-plates at a density

of 2 x10^6 cells per well in 6-well plates and treated with 5.6 µM TPIN for 48 h. After harvesting, the cells were lysed by treatment with RIPA buffer, and the insoluble protein lysate was centrifuged at 11,000 g for 35 min at 4˚C. Nano-Drop 1000 spectrophotometer was used for estimation of protein concentration of the lysates. Proteins in lysates were separated using 10 % SDS-PAGE, and the resolved proteins were transferred to PVDF membranes. The membranes were blocked by incubation with 5 % non-fat milk for 150 min, and the proteins were probed by incubation with primary antibodies overnight at 4˚C. After washing twice in PBS, the membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody at room temperature for 150 min. The detection and quantification of the signals were carried out using ECL in line with instructions on chemiluminescence kits. The antibodies used were anti-Akt, anti-F-actin, anti-p-Akt, anti-p-mTOR, anti-cofilin-1, anti-paxillin and anti-β-actin.

Statistical analysis

Data are presented as mean ± standard deviation (SD, n = 3)). Analysis of the data was performed using SPSS software (version 17.0, Inc, Chicago, IL, USA). Differences amongst groups were determined with one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Differences were considered significant at $p < 0.05$.

RESULTS

TPIN inhibited cellular proliferation

Treatment with TPIN produced inhibitory effect on cellular proliferation in time- and concentration- based manners (Figure 2). The proliferations of DU145 and PC3 cells were inhibited by TPIN in the concentration range of 0.75-15 µM. Treatment with TPIN at doses of 0.75, 1.5, 3.0, 6.0, 12 and 15 µM suppressed DU145 cell proliferation to 88, 73, 61, 48, 30 and 18 %, respectively, after 48 h. The PC3 cell proliferation was decreased to 90, 76, 65, 52, 45 and 23 %, respectively, on treatment with 0.75, 1.5, 3.0, 6.0, 12 and 15 µM TPIN. The IC_{50} values of TPIN for DU145 and PC3 cells after 48 h were 5.9 and 10.2 µM, respectively. The proliferation of DU145 cells was decreased to 43 % on treatment with 15 µM TPIN at 24 h, while treatment with 15 µM TPIN suppressed PC3 cell proliferation to 51 % after 24 h.

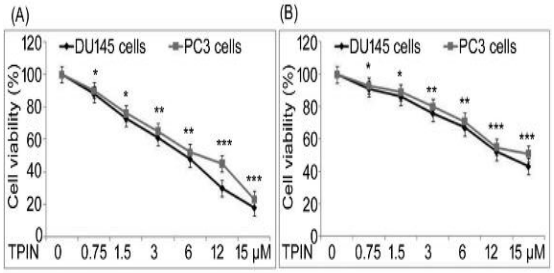

Figure 2: Effect of TPIN on cell proliferation. Treatment of cells with 0.75, 1.5, 3.0, 6.0, 12 and 15 µM TPIN for (A) 48 and (B) 24 h was followed by measurement of proliferation using Cell Counting Kit-8; *$p < 0.05$; **$p < 0.02$; ***$p < 0.01$, vs untreated cells

TPIN induced apoptosis

Treatment of the cells with 5.6 µM TPIN led to a significant enhancement in % apoptosis, relative to control cells (Figure 3). Apoptotic cell count was 59.64 in DU145 cells treated with 5.6 µM TPIN for 48 h, relative to 2.34 % in untreated cells. Treatment at doses of 5.6 µM for 48 h enhanced PC3 cell apoptotic count to 54.21 %, when compared to 2.97 % in control.

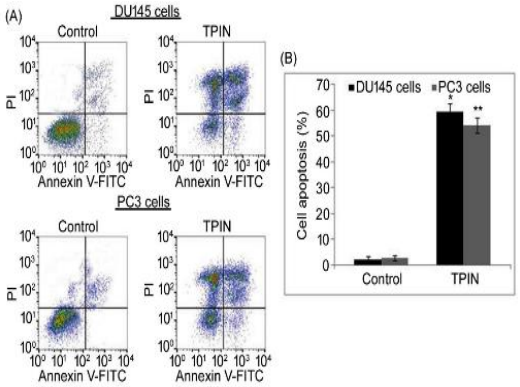

Figure 3: Effect of TPIN on cellular apoptosis. Apoptosis was determined in cells treated with 5.6 µM TPIN for 48 h, and in untreated cells using flow cytometry following Annexin-V staining; *$p < 0.02$; **$p < 0.01$, vs. untreated cells

TPIN arrested cell cycle

In DU145 cells, treatment with 5.6 µM TPIN significantly increased the fraction of cells in G1/G0 phase, relative to control cells (Figure 4). There was also an increase in G1/G0 phase fraction in PC3 cells on treatment with 5.6 µM TPIN. Cell counts in S and G2/M phases were suppressed in both cells after treatment with 5.6 µM TPIN for 48 h.

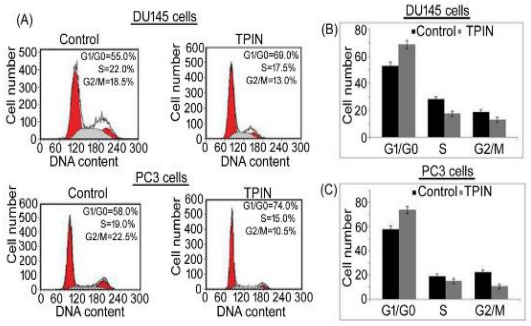

Figure 4: Effect of TPIN on cell cycle. A: DNA content, as determined in cells treated with 5.6 µM TPIN for 48 h and in untreated cells, using flow cytometry PI staining. B & C: Quantification of data. *P < 0.02; **p < 0.01, vs untreated cells

TPIN enhanced activities of caspase-3 and caspase-9

As shown in Figure 5, marked increases in caspase-3 and caspase-9 activities were seen in TPIN-treated DU145 cells after 48 h, relative to control cells. The PC3 cells also showed marked elevations in caspase-3 and caspase-9 activities after 48 h, relative to control cells. These findings confirmed the inhibition of prostate carcinoma cell proliferation by TPIN via the apoptotic pathway.

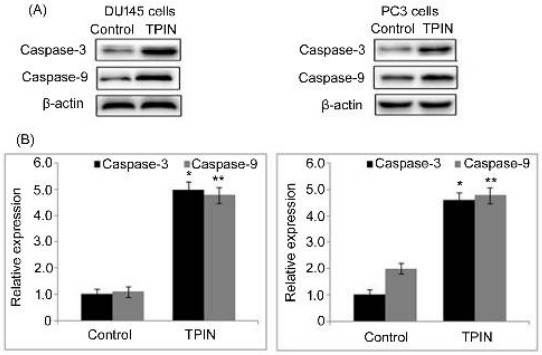

Figure 5: Effect of TPIN on activities of caspase-3 and caspase-9. The activities of caspase-3 and caspase-9 were assayed in cells treated with 5.6 µM TPIN for 48, and also in control cells, using western blot assay. *P < 0.02; **p < 0.01, vs untreated cells

TPIN inhibited phosphorylation of Akt

Changes in Akt activation and mTOR expression caused by TPIN in prostate carcinoma cells were assayed with western blotting assay. As shown in Figure 6, TPIN treatment at a dose of 5.6 µM markedly downregulated phosphorylation of Akt in DU145 cells. The phosphorylation of Akt was also suppressed in TPIN-treated PC3 cells. However, no change in total Akt expression was observed in TPIN-treated cells after 48 h. The activation of mTOR was also suppressed in cells on treatment with TPIN. Similar to Akt, TPIN treatment did not alter the expression of total mTOR in the tested cells.

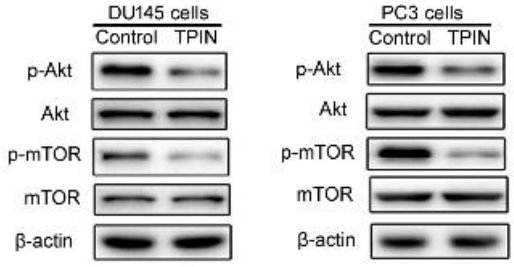

Figure 6: Effect of TPIN on expressions of PI3K and Akt. Cells treated with 5.6 µM TPIN for 48 h and untreated cells were subjected to determination of expressions of PI3K/Akt using western blot assay

TPIN suppressed F-actin and promoted cofilin-1 expression

In DU145 cells, treatment with 5.6 µM TPIN for 48 h markedly reduced the protein expression of F-actin (Figure 7). The level of F-actin in TPIN-treated DU145 cells was markedly lower than that in untreated cells. Treatment with 5.6 µM TPIN also reduced F-actin expression in PC3 cells, relative to control. Changes in cofilin-1 expression in prostate carcinoma cells treated with TPIN were assayed using western blotting. The results showed markedly elevated cofilin-1 expression in the cells treated with 5.6 µM TPIN for 48 h.

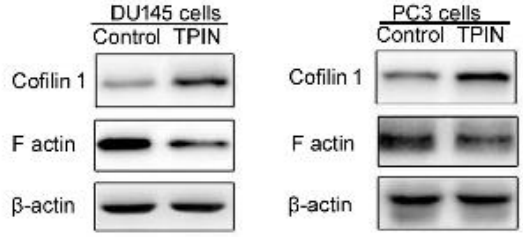

Figure 7: Effect of TPIN on F-actin and cofilin-1 expressions. The cells were treated with 5.6 µM TPIN for 48 h, and the expressions of F-actin and cofilin-1 were determined using western blotting assay

TPIN suppressed paxillin expression

As shown in Figure 8, treatment of DU145 and PC3 cells with 5.6 µM TPIN for 48 h caused a marked reduction in paxillin expression. Compared to untreated DU145 and PC3 cells, the paxillin expression was markedly downregulated in TPIN-treated cells.

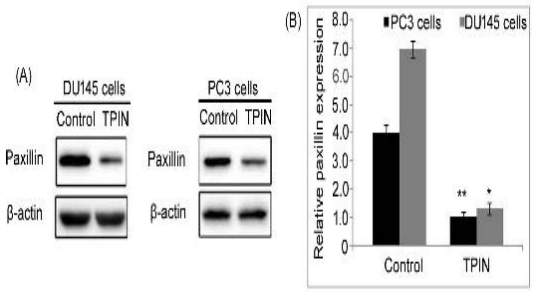

Figure 8: Effect of TPIN on paxillin expression. The cells were treated with 5.6 µM TPIN for 48 h, and the expression of paxillin was determined with western blot assay. B: Densitometric analysis of data. *P < 0.02; **p < 0.01, vs untreated cells

DISCUSSION

Prostate cancer is a malignant tumor which ranks 6th highest in mortality worldwide [13]. It is diagnosed in approximately 10,00,000 cases every year, and it is responsible for about 2,60,000 deaths globally [13]. Although diagnostic techniques and therapeutic strategies have been tremendously improved, mortality from prostate cancer is on the increase, especially in Asian countries, including China [14]. The current study investigated the effect of TPIN on prostate cancer cells, and the mechanism involved. Stress in endoplasmic reticulum during initial stage of apoptosis is associated with enhancement of mitochondrial membrane permeability, and subsequent activation of caspases [15].

Cytochrome C efflux into the cytoplasm leads to development of apoptotic complex, followed by effector caspase activation, and ultimately induction of apoptosis [9]. The present study investigated the effect of TPIN on the viability of prostate carcinoma cells, DNA content distribution, and apoptotic changes. The results showed that TPIN treatment led to inhibitory effects on the proliferation of DU145 and PC3 cells via activation of apoptosis and upregulation of caspase-9 and caspase-3. The mTOR pathway plays a vital role in the synthesis of various proteins involved in cellular apoptosis [16,17]. Therefore, the mTOR pathway serves as a target for development of prostate cancer treatment strategies [21]. It is known that mTOR is a member of phosphoinositide-3-kinase (PI3K) family, and a major downstream executor molecule of the PI3K/Akt pathway [17]. The expression of mTOR is widely found in cells where it regulates various cellular processes such as proliferation and survival [17].

Mammalian target of rapamycin complex-1 (m-TORC1) is associated with the translocation of mRNAs e.g. 5' terminal oligopyrimidine tract mRNAs [16]. Autophagic cell death is induced in lung carcinoma cells resistant to multiple drugs via inhibition of the Akt-mTOR pathway [18]. The present study has shown that TPIN treatment suppressed the phosphorylation of mTOR and Akt in prostate carcinoma cells. However, TPIN treatment did not change the levels of total mTOR protein and total Akt protein in these cells. These results suggest that TPIN-mediated suppression of cell proliferative potential is associated with targeting of the mTOR/Akt pathway.

The cell skeleton is comprised of protein network which consists of microfilaments, various intermediate filaments and canaliculi [19]. The smallest of these are microfilaments which consist of actin protein, either in free form or globular form (G-actin or F-actin) [19]. It has been found that the pattern of polymerization in actin regulates the phenotypic behavior of different types of malignant cells [20]. Alteration in reconstruction of F-actin interferes with the cellular skeleton, and it is considered an important target for anticancer drugs [21]. The expression of cofilin-1 is associated with development of schistose pseudopodia which play a key role in metastasis of cancer cells [10]. The level of cofilin-1 is markedly elevated in various cancers e.g. breast cancer cells [12]. The regulation of cofilin-1 and paxillin by therapeutic agents has been shown to inhibit pulmonary adenocarcinoma cell viability via the apoptotic pathway [22]. In the current study, TPIN reduced F-actin level and elevated cofilin-1 protein expression in prostate carcinoma cells. In these cells, treatment with TPIN caused a marked reduction in paxillin expression.

CONCLUSION

The present study has demonstrated that TPIN suppresses the proliferative potential of prostate cancer cells via apoptosis induction and arrest of cell cycle. Moreover, TPIN down-regulates Akt-mTOR pathway and regulates the expressions of cytoskeleton proteins in prostate cancer cells. Thus, TPIN may be useful in the development of a therapeutic strategy for prostate 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. Liang Wei and Ying Mu contributed to this work equally. Lina Ji conceived and designed the study; Liang Wei, Ying Mu, Xin Guo, Tongyi Li - collected and analyzed the data; Liang Wei, Ying Mu, Xin Guo -wrote the manuscript. Lina Ji -Approved final version of the manuscript. All authors read and approved the manuscript for publication.

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