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

Liang Wei¹, Ying Mu¹, Lina Ji²*, Xin Guo², Tongyi Li¹
¹Department of Urology, First Hospital of Shanxi Medical University, ²Department of Medical Oncology, Shanxi Bethune Hospital, Taiyuan, Shanxi 030032, China

*For correspondence: Email: JoyceBryanpm@yahoo.com; Tel: 0086-13277652399

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 (IC50) 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-1 expression 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

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](image)

**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 % CO2 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 x10⁶ 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 IC₅₀ 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.

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.

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 h, 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 paxillin expression was determined using western blotting assay
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 (mTORC1) 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.

**Open Access**

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0) and the Budapest Open Access Initiative (http://www.budapestopenaccessinitiative.org/resolve/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

**REFERENCES**

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics. CA Cancer J Clin 2017; 67: 7–30.
2. Yu EY, Massard C, Gross ME, Carducci MA, Culine S. Androgen receptor rearrangement and splicing variants in resistance to endocrine therapies in prostate cancer. Endocrinol 2017; 158: 1533-1542.
3. Houédé N, Pulido M, Mourey L, Joly F, Ferrero JM, Yu EY, Massard C, Gross ME, Carducci MA, Culine S, Hudes G, Posadas EM, Sternberg CN,Wilding G, Trudel GC, et al. Once-daily dasatinib: Expansion of phase II study evaluating safety and efficacy of dasatinib in patients with metastatic castration-resistant prostate cancer. Urol 2011; 77: 1166-1171.
4. Ho Y, Dehm SM. Androgen receptor rearrangement and splicing variants in resistance to endocrine therapies in prostate cancer. Endocrinol 2017; 158: 1533-1542.
5. Pedraza-Arevalo S, Hormaechea-Agulla D, Gomez-Gomez E, Requena MJ, Seith LA, Gahele MD, Castaño JP, Luque RM. Somatostatin receptor subtype 1 as a potential diagnostic marker and therapeutic target in prostate cancer. Prostate 2017; 77: 1499-1511.
6. Conroy T, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gengou-Gourdeau S, Fouchard C de la, Bennouna J, Bachet J-B, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 2011; 364: 1817-1825.
7. Obakan P, Arisam ED, Coker-Gurkan A, Palavan-Urnsal N. Epibrassinolide-induced apoptosis regardless of p53 expression via activating polyamine catabolic machinery, a common target for androgen sensitive and insensitive prostate cancer cells. Prostate 2014; 74: 1622-1633.
8. Reshma RS, Sreelatha KH, Somasundaram V, Satheesh Kumar S, Nadhan R, Nair RS, Srivinas P, Plumbagin, a napthaquinone derivative induces apoptosis in BRCA 1/2 defective castrate resistant prostate cancer cells as well as prostate cancer stem-like cells. Pharmacol Res 2016; 105: 134-145.
9. Kwegyir-Afful AK, Ramalingam S, Purushottamachar P, Ramamurthy VP, Njar VC. Gafetron and VNPT55 induce proteasomal degradation of AR/AR-V7, induce significant apoptosis via cytochrome c release and suppress growth of castration resistant prostate cancer xenografts in vivo. Oncotarget 2015; 6: 27440-27460.
10. Zhu B, Fukada K, Zhu H, Kypraiou N. Prohibitin and coflin are intracellular effectors of transforming growth factor beta signaling in human prostate cancer cells. Cancer Res 2006; 66: 8640-8647.
11. Lu L, Fu N, Luo XU, Li XY, Li XP. Overexpression of coflin 1 in prostate cancer and the corresponding clinical implications. Oncol Lett 2015; 9: 2757-2761.
12. Sundram V, Chauhan SC, Ebeling M, Jaggi M. Curcumin attenuates β-catenin signaling in prostate cancer cells through activation of protein kinase D1. PLoS One 2012; 7: e35368.
13. Wolpin BM, O'Reilly EM, Ko YJ, Blazekowsky LS, Ranick M, Rocha-Lima CM, Ritch P, Chan E, Spratlin J, Macarulla T, et al. Global, multicenter, randomized, phase II trial of gemcitabine and gemcitabine plus AGS-1C4D4 in patients with previously untreated, metastatic pancreatic cancer. Ann Oncol 2013; 24: 1792-1801.
14. Matsumoto K, Hagiwara M, Tanaka N, Hayakawa N, Ishida M, Ninomiya A, Nakajima Y, Nakamura S. Survival following primary androgen deprivation therapy for localized intermediate- or high-risk prostate cancer: Comparison with the life expectancy of the age-matched normal population. Med Oncol 2014; 31: 979.
15. Cella D, Ivanescu C, Holmstrom S, Bui CN, Spalding J, Fizazi K. Impact of enzalutamide on quality of life in men with metastatic castration-resistant prostate cancer after chemotherapy: Additional analyses from the AFFIRM randomized clinical trial. Ann Oncol 2015; 26: 179-185.
16. Kato M, Banuelos CA, Imamura Y, Leung JK, Caley DP, Wang J, Mawji NR, Sadar MD. Cotargeting androgen receptor splice variants and mTOR signaling pathway for the treatment of castration-resistant prostate cancer. Clin Cancer Res 2016; 22: 2744-2754.
17. Fang F, Wang L, Zhang S, Fang Q, Hao F, Sun Y, Zhao L, Chen S, Liao H, Wang L. CD147 modulates autophagy through PI3K/Akt/mTOR pathway in prostate cancer PC-3 cells. Oncol Lett 2015; 9: 1439-1443.
18. Chiou LY, Hu ME, Yang TY, Hsin IL, Ko JL, Tsai KJ, Sheu GT. Immunomodulatory protein from Ganoderma microsporum induces pro-death autophagy through Akt-mTOR-p70S6K pathway inhibition in multidrug resistant lung cancer cells. PLoS One 2015; 10: e0125774.

Trop J Pharm Res, July 2020; 19(7): 1394
19. Asahara S, Shibutani Y, Teruyama K, Inoue HY, Kawada Y, Etoh H, Matsuda T, Kimura-Koyanagi M, Hashimoto N, Sakahara M, et al. Ras-related C3 botulinum toxin substrate 1 (RAC1) regulates glucose-stimulated insulin secretion via modulation of F-actin. Diabetologia 2013; 56: 1088-1097.

20. Yu Y, Yang O, Fazli L, Rennie PS, Gleave ME, Dong X. Progesterone receptor expression during prostate cancer progression suggests a role of this receptor in stromal cell differentiation. Prostate 2015; 75: 1043-1050.

21. Luo Y, Cui X, Zhao J, Han Y, Li M, Lin Y, Jiang Y, Lan L. Cells susceptible to epithelial-mesenchymal transition are enriched in stem-like side population cells from prostate cancer. Oncol Rep 2014; 31: 874-884.

22. Wang F, Liu DZ, Xu H, Li Y, Wang W, Liu BL, Zhang LY. Thapsigargin induces apoptosis by impairing cytoskeleton dynamics in human lung adenocarcinoma cells. Scientific World Journal 2014; 2014: 619050.