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Original Research Article

Topiramate inhibits the proliferation of bladder cancer cells via PI3K/AKTR signaling pathway

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Abstract

Purpose: *To explore new treatment options for bladder cancer (BC) based on topiramate (TPM).*
Methods: *The MTT assay and flow cytometry were used to determine the effect of topiramate on partial growth-related malignant phenotype of BC cells. Expression levels of apoptosis-related biomarkers and signaling pathway-related factors were analyzed using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting. In vivo experiments were conducted to investigate the role of TPM on tumor growth in mice with bladder cancer.*
Results: *The MTT results showed that topiramate blocked the growth of BC cells ($p < 0.05$). Growth inhibition was positively correlated with TPM concentration. Flow cytometry results revealed that bladder cancer cell apoptosis rose with increase in TPM concentration, while the mRNAs of apoptosis-associated factors Bcl-2 and Mcl-1 were down-regulated in a concentration-based manner by TPM ($p < 0.05$). Western blot assay indicated that Bax and Caspase-3 proteins were up-regulated, and the higher the concentration of TPM, the more significant the protein expression levels ($p < 0.05$).*
Conclusion: *Topiramate (TPM) slows down the rate of growth of BC cells and accelerates their rate of apoptosis through the regulation of P13K/AKT/mTOR signaling pathway. Thus, the compound has potentials for development as an anti-bladder cancer agent.*

Keywords: *Topiramate, Bladder cancer, Proliferation, Apoptosis, PI3K/AKT/mTOR*

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INTRODUCTION

The overall survival rate of patients with bladder cancer is relatively low [1]. There has been limited progress in the development of more precise therapies for improvement of patient survival [2]. Conventional drugs can only be used to maintain the lives of patients for as long as possible [3]. Thus, it is very necessary to use new methods for research on the treatment of bladder cancer.

Topiramate (TPM), a fructose pyran sulfamate drug, is known for its powerful antiepileptic effects [4]. Topiramate has a wide range of therapeutic effects on a variety of diseases [5]. Its range of treatment applications cover migraine, alcohol dependence, cocaine

addiction, bipolar disorder, neuroprotection, bulimia, post-traumatic stress disorder, obesity and IBD. Topiramate may also have therapeutic effects on tumors, since it inhibited the development and metastasis of ovarian cancer [6-8].

Phosphatidylinositol 3-kinase (PI3K) is known as an intracellular phosphatidylinositol kinase involved in many cancer processes. The PI3K/Akt signaling pathway is active in most tumor patients, while it is inhibited in normal cells [9]. The presence of neovascularity in bladder cancer may be due to an abnormal triggering of a link in the PI3K/AKT signaling pathway [10].

This research first studied the influence of TPM on some malignant phenotypes of bladder cancer in cells and rats, and initially investigated the specific impact of topiramate on bladder cancer. The research provides a different perspective for TPM application in the treatment of bladder cancer.

EXPERIMENTAL

Cell culture

All bladder cancer cell lines (5637, 253J-BV, T24, KU-19-19, and EJ) used in this study were purchased from American Type Culture Collection (ATCC). The reagents used in cell culture in this experiment were from Gibco, USA. The nutrition of the cells used in this study was provided via RPMI-1640 containing 20 % FBS and 1 % diabody (10000 µg/mL each). Cell culturing was done in a 5 % CO_2 incubator at a constant temperature (37 °C).

MTT assay

The MTT assay was carried out as described previously [11]. The first step was to dilute 5637 and T24 cells into cell suspension at a density of 1×10^5 cells/mL, and then inoculate on a 96-well plate. After culturing in an incubator for 24 h, serum media with different TPM (Sigma, Germany) concentrations were added, while DMSO (Sigma, Germany) was added to the control wells. After culturing in an incubator for 24, 48, and 72 h, 20 µL of MTT (5 mg/mL) (Abcam, USA) was added to each well and placed in an incubator for 4 h. Thereafter, each well medium was replaced with 150 µL of DMSO. The 96-well plate was then placed on a shaker for 20 min, after which the absorbance value (A) was read at 490 nm. The % inhibition of bladder cancer was obtained from Eqn 1.

$$\text{Inhibition (\%)} = (1 - A_{test} / A_{control}) \times 100 \quad \ldots\ldots\ldots (1)$$

Apoptosis assay

The Annexin V-FITC/PI apoptosis kit (ThermoFisher Scientific, USA) was used to determine the apoptotic effect of graded concentrations of TPM on human bladder cancer 5637 cells/T24 cells. The main steps were as follows: 5637 cells/T24 cells were inoculated in 6-well plates. Different doses of TPM in 10 % serum medium were added and cultured for 24 h. The cells were rinsed 3 times with cold PBS at 4 ℃, followed by staining using Annexin V and propyl iodide in the dark for 10 min. Then, apoptosis was analyzed using flow cytometry.

Colony formation assay

Bladder cancer cells were exposed to varying TPM doses. The cell concentration was adjusted, and the cells were inoculated in 6-well plates, with 3 multiple wells per TPM concentration in each group. The cells were incubated for 3 weeks, and culturing was terminated when the 6-well plate had visible clone set. The supernatant was gently removed, and cells were washed twice with PBS and allowed to dry. Then, 2 mL of methanol and 1 mL of Giemsa dye solution (Sigma-Aldrich, USA) were put into every well. After fixation for 20 min, the cells were placed at laboratory temperature for 30 min. The dye was gently washed off with running water, and dried. The number of cloned colonies was counted visually.

Quantitative real time-polymerase chain reaction (qRT-PCR)

This was done according to general procedure reported earlier [12]. The brief procedure used was as follows: first, the bladder cancer cells were treated with TPM at concentrations of 0, 1, 100 and 1500 microns. Then, RNA was obtained from bladder cancer cells with RNA extraction kit (ThermoFisher Scientific, USA). Under the guidance of the Takara reverse transcription kit (RR047A, PrimeScript™ RT reagent Kit), the RNA was reverse-transcribed into cDNA. All operations were performed on ice, and RT fluorescence qPCR was performed using the Takara kit (RR820A, SYBR® Premix Ex Taq™ II). The reaction was carried out with ABI StepOnePlus™ Real-Time fluorescence quantitative PCR (ABI, USA). The primer sequences involved in this experiment are: Bcl-2 forward: 5'-TGCACCTGACGCCCTTCAC-3′, reverse: 5′: AGACAGCCAGGAGAAATCAAACA G-3′; Mcl-1 forward; 5'-CGGTAATCGGACT CAACCTC-3', reverse: 5'-CCTCCTTCTCCGTA GCCAA-3'.

Western blot analysis

The two different types of bladder cancer cell lines (5637 and T24 cells) were cultured in a constant-temperature incubator for 24 h with different concentrations of TPM in 10 % serum medium. After washing twice with pre-cooled PBS at 4 ℃, 200 μL RIPA lysis buffer was used lyse the cells. After centrifugation, the proteins were quantified with BCA procedure. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done on equal amounts of samples, and transferred to polyvinylidene fluoride (PVDF) membrane using semi-dry transfer method, incubated with 3 % fetal bovine serum, followed by overnight incubation with corresponding primary antibodies at 4 ºC, and washing thrice with PBST. This was followed by incubation with 2º antibody at laboratory temperature for one hour. Thereafter, ECL reagent was added, and the target bands were developed and photographed. The primary antibody concentration was diluted 1:1000. The reagents involved in western blot analysis were purchased from ThermoFisher Scientific Company (USA) and the antibodies were supplied by Abcam.

Animal experiments

Experiments on animals were approved by the Animal Ethics Committee of Nanjing Drum Tower Hospital Group, Suqian Hospital (approval no. 202003AA018) and followed WHO guidelines for the animal studies [13]. All mice used in the animal experiments were BALB/C57 nude mice aged 3 - 4 weeks. The mice were obtained from WTLH Co. Ltd, China. Bladder cancer cells were injected into mice. All mice were kept in separate cages for 10 - 15 days for tumors to be palpable. Three animals were injected with 1.5 mM TPM (10 mg/kg; the TPM was dissolved in ethanol and then diluted with PBS). The mice were treated 10 times every three days. Four mice which were assigned to the control group were injected with equivalent dose of ethanol solution in place of TPM. Body weight and tumor volume were recorded. After the injection, the mice were euthanized. The tumor volume and tumor weight were determined after tumor excision.

Statistical analysis

The data are expressed as mean ± standard deviation (SD), and were analyzed by two-tailed *t*-test. The data are presented as mean ± standard deviation (SD). Significance of difference was assumed at $p < 0.05$.

RESULTS

TPM suppressed bladder cancer cell multiplication

The results of the MTT indicated that the anti-proliferative influence of TPM on BC cells was more obvious with increase in TPM concentration. TPM blocked BC cell proliferation in a concentration-based fashion. Two important forms of population dependence and proliferation ability can be analyzed using clonal formation rate. After 14 days of exposure to 1500 μM TPM, the colony-forming potential of 5637 and T24 cells were reduced (Figure 1b). From these results, it can be inferred that TPM blocked BC cell multiplication in a dose-based fashion. Thus, as TPM concentration increased, it played a more significant role in inhibiting the growth of bladder cancer cells.

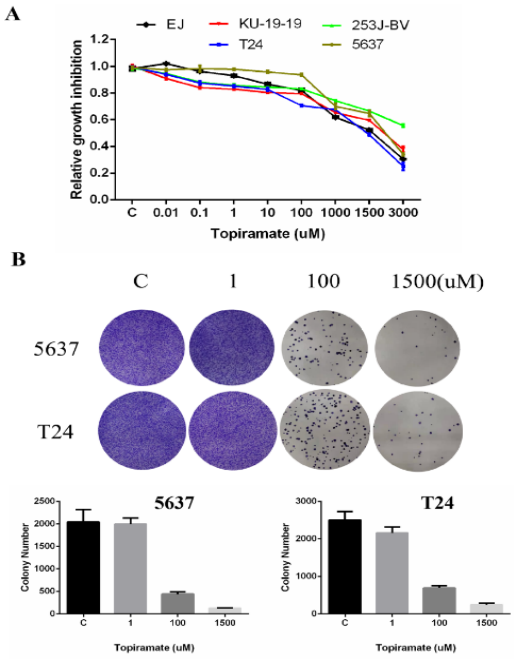

Figure 1: Inhibition of TPM on cell proliferation in bladder cancer cells. Five different types of bladder cancer cells were cultured in different concentrations of TPM for 72 h. (A) Cell proliferation, as determined using MTT assay. Topiramate blocked the growth process bladder cancer cells. (B) The higher the concentration of TPM used to treat bladder cancer cells, the fewer the colonies formed in bladder 5637 cells and T24 cells. The quantitative analysis of data is shown on the right

Induction of apoptosis in bladder cells by topiramate treatment

In this study, flow cytometry was used to determine whether the growth inhibition by TPM was associated with apoptosis. The result

revealed that apoptosis of 5637 cells and T24 cells were markedly increased after TPM treatment (Figure 2a and Figure 2b). Furthermore, it was found that the degree of TPM-induced apoptosis was positively correlated with concentration. Apoptosis induction in bladder cancer cells by TPM was validated by carrying out qPCR and immunoblot assays for levels of apoptosis-related mRNAs and proteins. After 24 h of TPM administration, the mRNA and protein expressions of Bcl-2 and Mcl-1 were determined, as well as protein expressions of Caspase-3, Survivin, Bax and Bcl-2.

The mRNA expressions of Bcl-2 and Mcl-1 showed completely inverse relationship with the concentration of TPM (Figure 2 c). The Bax and Caspase-3 proteins were increased, and the higher the concentration of TPM, the more significant the protein expressions, while Survivin and Bcl-2 proteins were down-regulated in bladder cancer cells. There were negative correlations between TPM concentration and the expressions of Survivin and Bcl-2 (Figure 2 d). The above results were verified in both 5637 cells and T24 cells, thereby further indicating that TPM induced bladder cancer cell apoptosis.

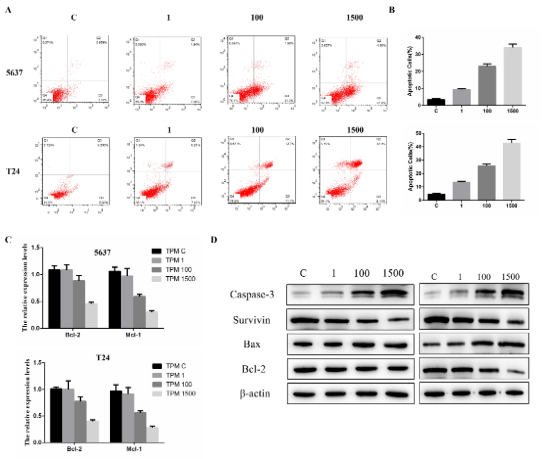

Figure 2: Topiramate stimulated apoptosis in BC cells. (A) Apoptosis level of 5637 cells, as determined using flow cytometry. The percentage apoptosis was statistically treated. (B) Percentage apoptosis of T24 cells, as measured using flow cytometry. Treatment with TPM enhanced apoptosis of T24 cells, as shown in the quantitative analysis of results. (C) The mRNAs of Bcl-2 and Mcl-1, as determined using qPCR assay. The expressions of Bcl-2 and Mcl-1 decreased with the increase in TPM concentration. (D) Levels of Caspase-3, Survivin, Bax and Bcl-2 proteins in 5637 and T24 cells, as assayed using Western blotting, with β-actin employed as a standard

Topiramate inhibited the proliferation of bladder cancer cells via PI3K/AKT/mTOR signaling pathway

Cell proliferation is a part of malignant phenotype of tumors, and it is the basis of tumor growth. One currently accepted theory is that Akt is triggered by activation of the lipid kinase PI3K, leading to the production of PIP3 in the plasma membrane of the cell. When Akt binds to PIP3 through the homologous PH domain, it translocates itself to the cell membrane. When Akt is phosphorylated, it activates downstream signaling pathways and regulates the processes involved in cell growth. In order to unravel how TPM affects the multiplication of BC cells, the phosphorylation levels of Tyr458 in PI3K and Thr308 of Akt were assayed through western blot analysis (Figures 3a and 3b). Taking the blank group as a reference, the phosphorylation of PI3K Tyr458 in 5637 and T24 cells was most significantly reduced at TPM concentration of 1500 μM. In terms of p-Akt and AKT protein expression levels, there were only little changes in total AKT when TPM was used at levels of 100 and 1500 μM.

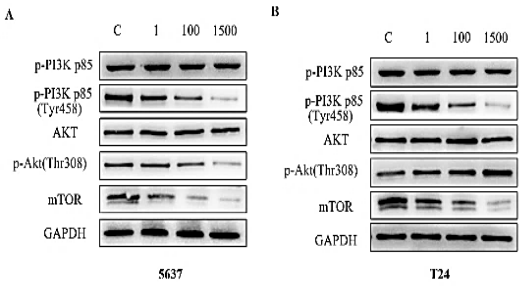

Figure 3: Effect of Topiramate on PI3K/AKT/mTOR signal route in 5637 and T24 cells. (A & B) 5637 and T24 cells were treated with three different concentrations of TPM for follow-up experiments, and immunoblotting was used to assay proteins associated with signaling route, with GAPDH as internal control

TPM suppressed bladder cancer cell proliferation *in vivo*

Figure 4a is a representative image of tumor formation in mice, while an image of regenerated tumor tissue in mice is shown in Figure 4b. It can be seen that the volume of tumor formed in the mice injected with TPM was markedly small, relative to control injected with ethanol solution (Figure 4c). Moreover, it is evident that there was delayed tumor growth in mice injected with TPM (Figure 4d). The weight of experimental group increased much slower than that of the control mice (Figure 4e).

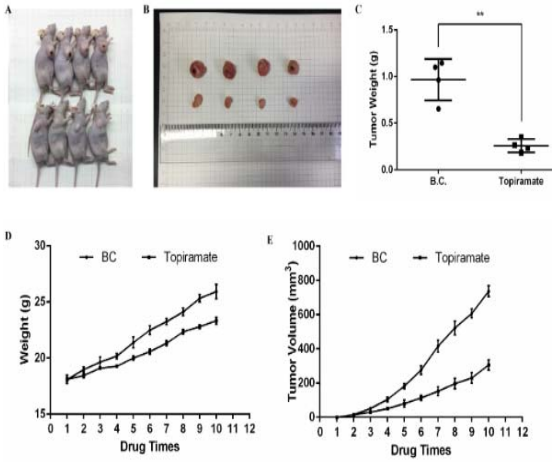

Figure 4: Topiramate inhibited tumor growth in rats. (A) Image of tumor-bearing mouse. (B) image of tumor tissues. (C) Quantitative analysis of tumor weights in the control group and TPM treatment group. (D) Growth curve showing changes in animal body weight during the administration period. (E) Growth curve showing changes in tumor volume. $^{**}P < 0.01$

DISCUSSION

Due to continuous advancements in modern medicine, bladder cancer has been identified as one of the most common malignant tumors in urology. The pathogenesis bladder cancer is now considered a very complicated process involving multiple steps and factors related to patients' living habits, occupations and genetics. It has been found that the incidence of bladder cancer is on the increase all over the world. Majority of bladder tumors are epithelial cell carcinomas (UCC), and about 75 % are non-muscle-invasive. In general, the prognosis of bladder cancer patients whose pathological classification is non-muscular invasiveness is good. However, the remaining 25 % are muscular invasive bladder cancer which usually has long-term metastasis, and the degree of patient's survival is low. Although transurethral resection of bladder tumors is an effective treatment for bladder cancer, about half of patients experience relapse within 1 year, and may develop a more malignant form with a poor prognosis. Bladder perfusion therapy is often used after surgery in combination with antitumor drugs such as mitomycin and anthracyclines [14]. At present, bladder cancer has become an extremely important public health problem. Thus, there is a particular need to identify novel therapeutic drugs for bladder cancer. Research on development of new drugs for bladder cancer is on-going.

Topiramate is an antiepileptic drug with a wide range of clinical applications, including analgesia, emotional stability, and management of cocaine addiction and other neuropsychiatric diseases. In addition, topiramate exerts therapeutic effect on obesity. The new use of an old medicine has become a new direction in modern medicine. Studies have reported a potential usefulness of TPM in inflammatory bowel disease, leading to the idea that TPM may have a similar effect on bladder cancer. The effect of TPM on cancer has also been reported: it markedly suppressed the expression of malignant phenotypes of ovarian cancer. Traditional perceptions have limited the use of TPM in cancer. However, new use of an old drug is in line with contemporary research trends. Given the wide range of pharmacological mechanisms of TPM, it was hypothesized that topiramate might play a role in bladder cancer. Studies have shown that promoting apoptosis through chemotherapy is an effective strategy for killing cancer cells, but the sensitivity of tumor cells to drugs is also a part of the effect of treatment outcome [15]. In this study, topiramate was used to induce cell apoptosis, and its inhibitory effect on BC cell multiplication was determined. Topiramate blocked the growth of BC cells dose-dependently. Results from flow cytometry showed that TPM-induced apoptosis varied with concentration of the drug: the higher the TPM concentration, the higher the % apoptosis of bladder cancer cells. In contrast, mRNA expressions of Bcl-2 and Mcl-1 showed completely inverse relationship with TPM concentration. The Bax and Caspase-3 proteins were increased, and the higher the concentration of TPM, the more significant the protein expression levels. However, survivin and Bcl-2 proteins were down-regulated. There were negative correlations between TMP concentration and the expressions of Survivin and Bcl-2.

Many pathways related to cell proliferation and apoptosis have been discovered. The PI3K/Akt pathway is a classic anti-apoptotic and pro-survival signal route that regulates a variety of processes in tumors, including resistance to chemotherapy, proliferation, invasion, angiogenesis, and metastasis. Phosphorylated PI3K (p-PI3K) and AKT (p -Akt) are in their activation states. Up-regulation of this pathway leads to a variety of tumors [16]. The PI3K is a member of the phospholipid kinase family. It has lipid kinase and protein kinase activities. It is composed of a regulatory subunit P85 and a catalytic subunit P110, and it plays a signal transduction function in the cell. Protein kinase B, also known as Akt, is highly homologous to protein kinases A and C. It (Akt) is considered an effector molecule of PI3K signaling pathway. In this pathway, activated PI3K phosphorylates Akt, thereby activating it (Akt). The PI3K/AKT

signaling pathway has a wide range of functions: it participates in regulating cell proliferation, cell survival, cell metabolism, angiogenesis, tumor radiation resistance and chemotherapy resistance. The regulation of apoptosis is particularly important. Abnormal stimulation of this signaling route usually occurs in malignant tumors. In the normal population, the negative regulation of PI3K/AKT signal route which inhibits the occurrence of tumors, in some aspects, depends on the activity of PTEN protein. Consistent with the results obtained in this study, some studies have reported that PTEN is significantly down-regulated in invasive BC, when compared with superficial bladder cancer. Indeed, PTEN dysfunction usually occurs in patients with invasive bladder cancer. As a result, it loses its inhibitory effect on PI3K, leading to excessive activation of the PI3K/AKT/mTOR signaling pathway. The degree of activation is reflected in the extent of BC malignancy [10,17]. The use of Akt inhibitors to block PI3K/AKT signaling route in bladder cancer cells enhanced the killing potential of paclitaxel on bladder cancer cells [18]. Therefore, any marker in this signal route is likely to be an essential target in tumor therapy. This research found that TPM intervention changed p-PI3K and p-Akt levels: as the concentration of TPM increased, the phosphorylation of these two factors decreased. These results suggest that TPM slowed down the rate of multiplication of BC cells and accelerated their rate of apoptosis. This was achieved through the regulation of P13K/AKT/mTOR signaling pathway.

Limitations of the study

This research did not cover cell invasion and metastasis. Invasion and metastasis are also prominent features of malignant tumors. Thus, there is need to supplement the results with other experimental evidence in subsequent studies. Moreover, this study was conducted only at the human bladder cancer cell and nude mouse levels.

CONCLUSION

This study has shown that TPM slows down the rate of growth of bladder cancer cells and accelerates their rate of apoptosis via the regulation of P13K/AKT/mTOR signaling route. Furthermore, TPM inhibits the growth and proliferation of bladder cancer cells. Thus, TPM has a potential for development as an anti-bladder cancer drug. However, more in-depth mechanisms of action of TPM needs to be studied.

DECLARATIONS

Conflict of Interest

No conflict of interest 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. Liu Chao and Weiqi Cai conceived and designed the study, and drafted the manuscript. Liu Chao, Shaoqi Zhang, Jianjun Zhang, Longjun Cai, Xiangyu Wang and Fanlai Meng, collected, analyzed and interpreted the experimental data. Shaoqi Zhang and Weiqi Cai revised the manuscript for important intellectual content. All authors read and approved the final manuscript for publication.

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