

## Original Article

# Possible mechanism of PPAR $\alpha$ / $\gamma$ activator TZD18 in gastric cancer

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**Abstract:** The incidence and mortality rate of gastric cancer increased year by year. Current chemotherapy drug plays a key role in anti-tumor effect. PPAR is a ligand activated transcription factor that plays an important role in inflammation and tumor. We focused on investigating the mechanism of PPAR  $\alpha$ / $\gamma$  activator in gastric cancer cell growth. Different doses of TZD18 were used to treat gastric cell line MKN-45. MTT assay was used to test cell proliferation. Immunofluorescence was applied to test cell nuclear apoptosis. Flow cytometry was used to determine cell apoptosis rate. Western blot was performed to detect apoptosis related protein Bax, P27<sup>kip1</sup>, and Bcl-2 expression. Cell proliferation rate decreased significantly under different doses of TZD18 with time and dose gradient ( $P < 0.05$ ). Immunofluorescence revealed that the cells appeared karyopyknosis and chromatin deepening after TZD18 treatment. Flow cytometry showed that cell apoptosis rate increased after TZD18 treatment. Bax and P27<sup>kip1</sup> level elevated while Bcl-2 decreased after TZD18 treatment. PPAR  $\alpha$ / $\gamma$  activator TZD18 can effectively suppress cell proliferation and accelerate cell apoptosis, so as to slow down gastric cancer progression.

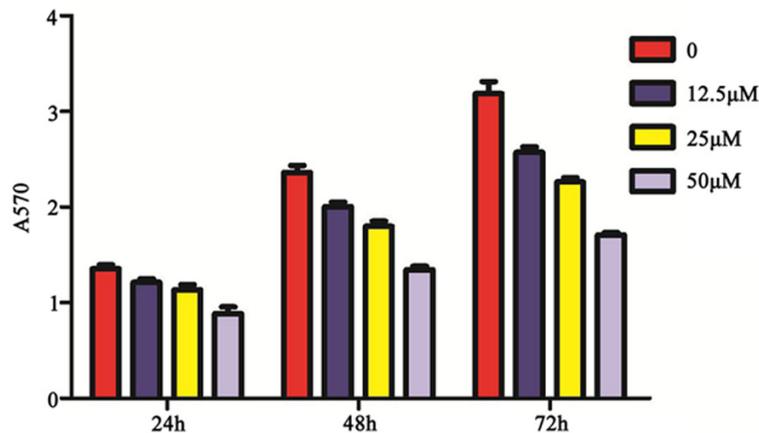
**Keywords:** PPAR  $\alpha$ / $\gamma$ , TZD18, gastric cancer

## Introduction

The mechanism of potential gastric cancer formation has not been clarified. Many gastric cancer patients' quick death is because of the rapid growth of tumor cells. Surgical resection is the best therapy method for patients with best prognosis. However, about 80% of the patients suffered from stomach inflammation, while only 10-15% of these patients can receive surgical resection [1, 2]. Though the morbidity and mortality of gastric cancer are on a declining curve in recent years, it is still a malignant tumor with high fatality rate. It still accounts for about 10% of all tumorigenesis [3]. Generally, chemotherapy drug is an important method for tumor treatment, especially targeting certain receptors, such as estrogen and retinoid receptor. For example, PGE receptor antagonist ONO-8711 can retard breast cancer by inducing it apoptosis. Moreover, it also can activate retinoic acid receptor to inhibit non-small cell lung cancer progression [4]. Recent studies have

shown that peroxisome proliferator activated receptors (PPARs) can change the way of tumor proliferation and apoptosis, and is considered to be a new kind of anti-tumor treatment method [5]. PPAR receptor includes PPAR $\alpha$ , PPAR $\beta$ , and PPAR $\gamma$ . PPAR is a type of ligand activated transcription factor that can regulate hormone balance and inhibit inflammation as an important regulatory factor. Oxidized fat sourced from LDL can effectively activate PPAR, and further impact inflammatory cytokine expression. Numerous studies revealed that PPAR $\gamma$  can affect tumor cells proliferation and differentiation as a regulatory factor of cell cycle [6, 7]. Furthermore, its ligand can obviously suppress colorectal cancer cells growth and carcinoembryonic antigen expression. Similarly, PPAR $\gamma$  activator TZD18 can lead to lipid accumulation in breast cancer. It also can inhibit cell growth by changing epithelial cells gene expression [8]. However, the role of PPAR $\gamma$  activator TZD18 in gastric cancer has not been fully investigated.

## PPAR $\alpha/\gamma$ in gastric cancer



**Figure 1.** MTT detection of cell proliferation rate treated with TZD18 for different concentration and time.

In this study, we intended to explore the relationship between PPAR $\gamma$  activator TZD18 and gastric cancer. We analyzed the effect of PPAR $\gamma$  activator TZD18 on gastric cancer proliferation and apoptosis gene expression, aiming to clarify the mechanism of PPAR $\gamma$  activator TZD18 in gastric cancer.

### Materials and methods

Human gastric cancer cell line MKN-45 was purchased from Shanghai College of life science. The cells were maintained in RPMI-1640 medium containing 10% FBS and cultured in incubator with 5% CO<sub>2</sub> and 37°C. TZD18 was bought from Merck (USA).

#### MTT assay

Cell seeding: 1000-10000 cells in 200  $\mu\text{l}$  medium with 10% FBS were seeded in 96-well plate.

After MKN-45 adhering to the wall, 20  $\mu\text{l}$  MTT solution at 5 mg/ml (pH = 7.4) were added and culture for 4 h. And then 150  $\mu\text{l}$  DMSO solution was added and vibrated for 10 min.

The plate was read at 490 nm on microplate reader for growth curve analysis.

#### Western blot

100  $\mu\text{l}$  gastric cells were seeded in 96-well plate. The protein was extracted when density reached 70% using protein extraction kit (Beyotime) and quantified by BCA. Specially, fluid A and B were mixed at 50:1 to make BCA solution. 2  $\mu\text{l}$  cell supernatant together with 18  $\mu\text{l}$  PBS and 200  $\mu\text{l}$  AB mixture was blended for

measurement. The protein was separated by electrophoresis and transferred to PVDF membrane. After washed by TBST, the membrane was blocked in 5% milk for 1 h. Then the membrane incubated in primary antibody overnight. After that, the membrane was incubated in secondary antibody for 1 h at room temperature after three times' TBST washing. PVDF membrane was developed by ECL luminous fluid for analysis.

#### Immunofluorescent staining

The tissue was gradient dehydrated by sucrose after fixation. After frozen section (thickness = 6  $\mu\text{m}$ ), high temperature repair (5 min), washing (PBS, three times, 5 min), and blocking (10% BSA, 50 min), the slice was stained by primary antibody overnight. On the second day, the slice was washed by PBS for three times and stained with secondary antibody. All antibodies were purchased from Santa and used at 1:100. Secondary antibody was bought from ZSGB-Bio (Beijing, China).

#### Cell apoptosis detection

Cell apoptosis rate was detected by flow cytometry. The cells were collected and fixed in 70% ethanol at -20°C overnight. After stained at PBS for 30 min (PI, 50  $\mu\text{g}/\text{ml}$ , Sigma; RNase, 100  $\mu\text{g}/\text{ml}$ , Sigma),  $1 \times 10^6$  cells were tested by flow cytometry.

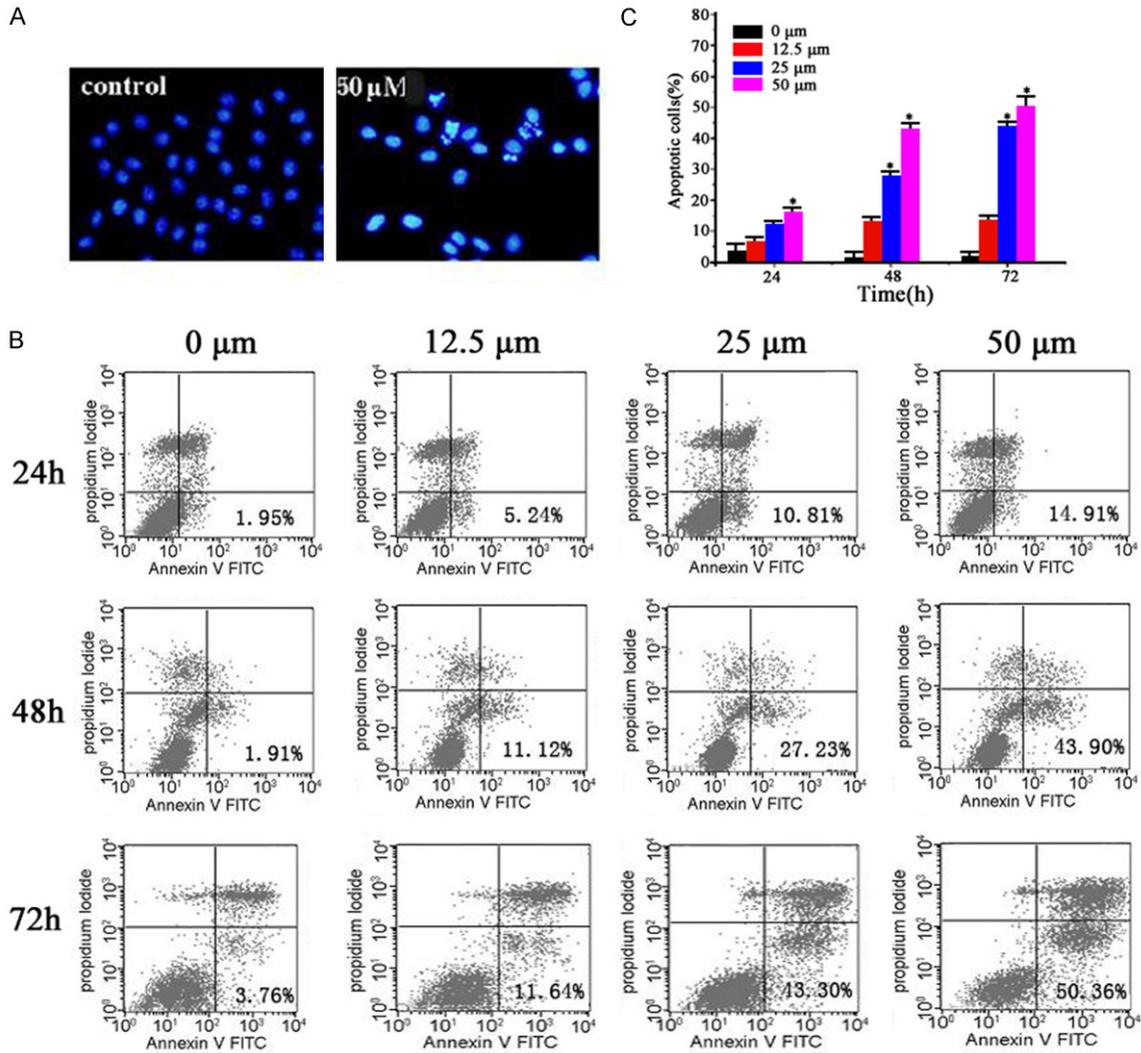
#### Statistical analysis

All statistical analyses were performed using SPSS11.0 software. All data was presented as mean  $\pm$  standard deviation. T test, Pearson correlation analysis, and chi-square test were applied for data analysis.  $P < 0.05$  was considered as significant difference. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Western blot analysis was treated by the ratio of target protein and  $\beta$ -actin.

### Results

#### Cell proliferation rate detection

The effect of TZD18 on gastric cancer cell proliferation was detected by MTT (**Figure 1**). The



**Figure 2.** TZD18 induced cell apoptosis. A. Immunofluorescence showed karyopyknosis; B. Flow cytometry detected cell apoptosis; C. Apoptosis statistical result,  $P < 0.05$ .

proliferation rate in control group was 91.3%. In comparison, TZD18 treatment group showed lower proliferation rate than the control. In specific, 10  $\mu\text{M}$  TZD18 for 12 h treatment decreased proliferation rate from 91.3% to 84.2% and for 72 h to 69.8%. 50  $\mu\text{M}$  TZD18 for 12 h can decline the proliferation rate to 75.3% and for 72 h to 45.3%.

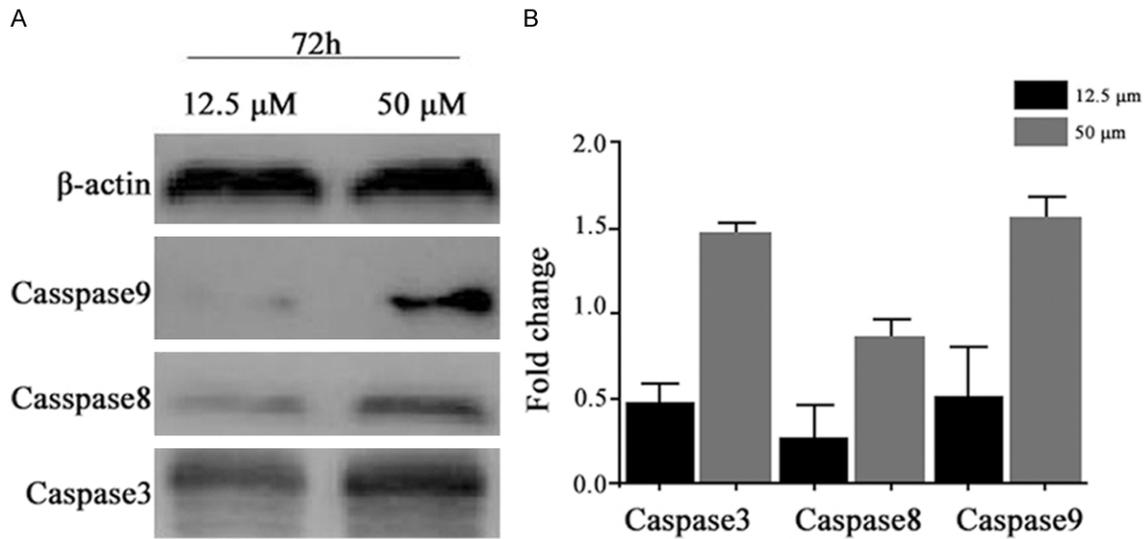
*TZD18 induced cell apoptosis*

To investigate the mechanism of TZD18 inducing cell death, we speculated whether TZD18 lead to cell apoptosis. Immunofluorescence revealed that the apoptotic cells appeared karyopyknosis and chromatin deepening (**Figure 2A**) with 6.1% apoptosis rate in control

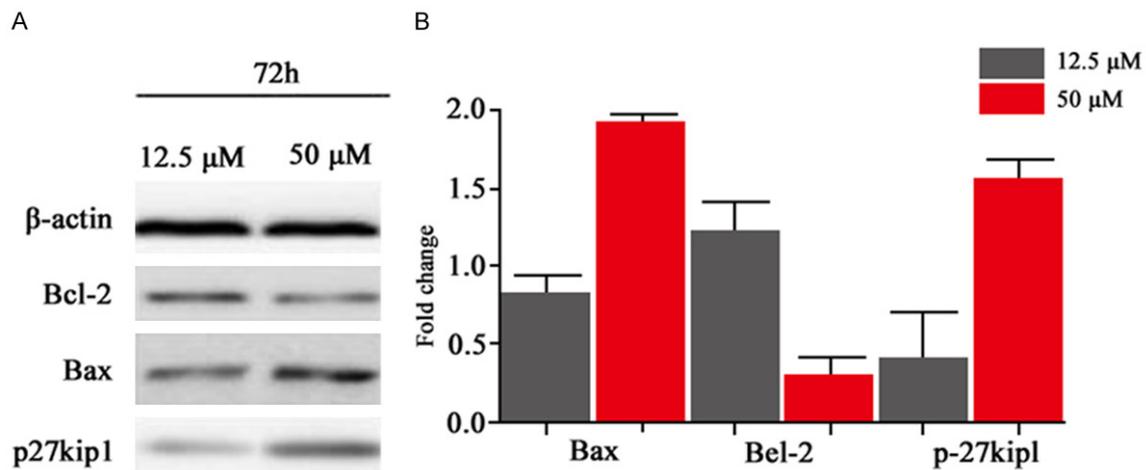
group. As shown in **Figure 2B** and **2C**, TZD treatment at 12.5  $\mu\text{M}$  increased apoptosis rate from 8.2% at 24 h to 18.5% at 72 h, while at 50  $\mu\text{M}$  elevated to 47.1% at 72 h ( $P < 0.05$ ).

*TZD18 regulated caspase activity*

Caspase activity, including Caspase 3, Caspase 8, and Caspase 9, is considered to be important factors in the process of apoptosis. Caspase 3 activity increased in 72 h under 12.5  $\mu\text{M}$  TZD treatment, and reached top under 50  $\mu\text{M}$  by increasing 3.1 times. Caspase 8 activity elevated significantly under 12.5  $\mu\text{M}$  and reached top at 50  $\mu\text{M}$  by increasing 4.3 times. Caspase 9 increased for 3.8 times (**Figure 3A** and **3B**,  $P < 0.05$ ).



**Figure 3.** A. Western blot detected Caspase activity. B. Statistical result,  $P < 0.05$ . TZD18 impact on apoptotic protein P27<sup>kip1</sup>, Bcl-2, and Bax.



**Figure 4.** A. Western blot detected apoptotic related protein P27<sup>kip1</sup>, Bcl-2, and Bax. B. statistical result,  $P < 0.05$ .

To explore the effect of TZD18 on apoptotic related protein expression, the cells were treated by 12.5  $\mu\text{M}$  or 50  $\mu\text{M}$  TZD18 for 72 h. As shown in **Figure 4A**, P27<sup>kip1</sup> level increased significantly after TZD18 treatment. Bax expression showed elevation trend with dose dependent. On the contrary, Bcl-2 declined after treatment. It indicated that TZD18 suppress tumor by affecting cell apoptosis (**Figure 4B**,  $P < 0.05$ ).

**Discussion**

Gastric cancer is a type of malignant tumor with complicated pathogenesis. Gastric cancer

occurrence and development is a typical multiple factors and multi-step process involving abnormal cell proliferation, apoptosis, invasion, and metastasis [9, 10]. PPAR $\gamma$  is a ligand dependent transcription factor that can regulate a variety of physiological processes, including fat cells occurrence and maintaining glucose stability [11]. We found that PPAR $\gamma$  plays an important role in anti-tumor process by promoting apoptosis. It can increase P27<sup>kip1</sup> expression in gastric cancer through P53 dependent mechanism. Furthermore, its activator troglitazone can effectively inhibit colorectal cancer cell growth [12, 13]. TZD18 is a newly synthesized PPAR $\alpha/\gamma$  activator. It presents anti-

tumor activity through PPAR $\alpha$  and PPAR $\gamma$  [14]. Studies have reported that TZD18 can effectively inhibit T98G cell proliferation and promoting cell apoptosis [15]. However, the role of TZD18 in gastric cancer has not been reported. In this study, we found that MKN-45 cell proliferation decreased with dose dependent under TZD18 treatment, suggesting that TZD18 has the effect of inhibiting tumor growth.

Studies have shown that TZD18 can restrain cell in G1 phase by elevating P21 and E-cadherin expression in colon cancer, pancreatic cancer, and breast cancer [16, 17]. Generally, TZD can block cell cycle and associated with the cell cycle proteins, such as P27<sup>kip1</sup>. In this study, TZD18 treatment can increase P27<sup>kip1</sup> expression in gastric cancer cells. Previous research revealed that P27<sup>kip1</sup> level upregulated in T98G cells, indicating that TZD18 may block gastric cell in G1 phase [18].

Cell cycle arrest, apoptosis is the precondition of cell apoptosis. To investigate the role of TZD18 in cell proliferation reduction, we tested cell apoptosis and found that TZD18 can increase cell apoptosis with dose dependent. Caspase plays an important role in cell apoptosis. Caspase 3 activated by caspase 9 is the two critical genes in cell function and apoptotic body formation [19, 20]. In this article, we found that Caspase 3 and Caspase 9 elevated with the increase of TZD18 concentration, indicating that TZD18 may mediate apoptosis through regulating caspase activity. Bcl-2 plays an important role in apoptosis by inhibiting the programmed cell death, while Bax is the composition of proapoptotic protein that can regulate cell apoptosis through p53 [21-23]. Previous report revealed that TZD18 can increase cell apoptosis through elevating Bax expression in leukemia cells. Following the increase of Bax, Bcl-2 level reduced. This phenomenon has been confirmed in glioma cell line [24, 25]. In this study, we found that TZD18 can effectively inhibit Bcl-2 expression and upregulate Bax level, indicating that it induced apoptosis through increasing Bax and reducing Bcl-2.

Our study explained the mechanism of TZD18 in gastric cancer and confirmed that TZD18 affected gastric cell growth by apoptosis. It has guiding significance for the following gastric cancer investigation.

#### Disclosure of conflict of interest

None.

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