A novel PPAR alpha/gamma dual agonist inhibits cell growth and induces apoptosis in human glioblastoma T98G cells[1]

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ABSTRACT

AIM: To examine the effect of a novel peroxisome proliferator-activated receptor (PPAR) α/γ dual agonist TZD18 on cell proliferation and apoptosis in human glioblastoma T98G cells and its possible mechanism. **METHODS:** RT-PCR, MTT, TUNEL, Flow cytometry, and Western blot analysis were employed. **RESULTS:** TZD18 inhibited the growth of T98G cells in a concentration-dependent manner, which was associated with a G1 to S cell cycle arrest. Besides, significant apoptosis was induced after treatment with a non-toxic dose of TZD18. During the process, the expression of Bcl-2 protein was down-regulated, while that of Bax and p27[kip] proteins was up-regulated, and the activity of caspase-3 was elevated. However, this effect appeared to be PPARα and PPARγ independent since their antagonists could not reverse this effect. **CONCLUSIONS:** TZD18, a novel PPARα/γ dual agonist, inhibited cell growth and induce apoptosis in human glioblastoma T98G cells *in vitro*, indicating a therapeutic potential for TZD18 in the treatment of glioblastoma.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor superfamily that includes receptors for steroids, thyroid hormone, vitamin D, and retinoic acid[1]. The PPAR α, β, and γ subtypes share a highly conserved DNA binding domain that matches with specific DNA sequences known as peroxisome proliferator response elements (PPREs). PPARs form a heterodimer with retinoid X receptor (RXR) and regulate the expression of target genes by binding to PPRE[2].

Recently, PPARγ was reported to be highly expressed in many types of cancer cells[3]. The thiazolidinedione (TZD) class of PPARγ ligands such as troglitazone (TGZ), pioglitazone (PGZ), ciglitazone (CGZ), exhibit growth inhibitory effects on colon cancer, breast cancer, prostate cancer, and gastric cancer by affecting differentiation and/or inducing apoptosis both *in vitro* and *in vivo*[4-9]. These findings suggest that TZD class of PPARγ agonists may represent a promising, novel therapeutic approach for certain human cancers.

Glioblastoma is the most common subtype of primary brain tumor in adults. These tumors are very aggressive with a highly invasive capacity and often infiltrate critical neurological areas within the brain[10]. Glioblastoma cells are notoriously resistant to apoptosis, a characteristic that contributes toward the failure of most standard clinical treatments[11]. Over the decades, no effective therapeutic approaches are available in spite

of advances in surgical techniques, radiotherapy, and chemotherapy[10]. Current research in this area is driven by the need to discover new agents that will be effective and have few side effects.

The new TZD class compound of 5-[3-(3-(4-phenoxy-2-propylphenoxy)propoxy)phenyl]-2,4-thiazolidinedione, designated TZD18 (Fig 1), is a novel PPAR α/γ dual agonist, newly synthesized by MERCK, NJ, USA [12,13]. The screening test showed TZD18 exhibited10-fold higher potency for human PPARγ than the common antidiabetic PPARγ agonist rosiglitazone, and it was more potent than human PPARα agonist fenofibrate[14]. We have previously demonstrated that the PPARγ ligand PGZ, induced apoptosis of glioblastoma cells[15]. In this study, we reported the first use of TZD18, a new and highly potent ligand for the nuclear receptor PPARα/γ, to inhibit cell growth and induce apoptosis in the human glioblastoma T98G cell line *in vitro*.
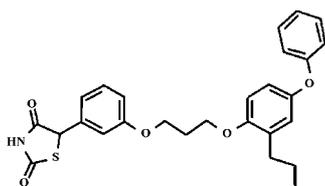

Fig 1. Structure of the dual PPARα/γ agonist TZD18.

MATERIAL AND METHODS

Materials TZD18 was kindly provided by MERCK, NJ, USA. The PPARγ agonist GW7845 and antagonist GW9662 were gifts from GlaxoSmithKline (Hertfordshire, England). The PPARα agonist WY-14, 643 and antagonist MK-886 were purchased from Alexis Biochemicals (Germany). They were dissolved in ethanol, at 0.01 mol/L as stock solutions stored at -70 ℃ and were further diluted to appropriate concentrations with medium before use.

Cell culture Human glioblastoma T98G and breast cancer MCF-7 cell lines used in this study were purchased from American Type Culture Collection. It was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal calf serum and 1 % penicillin. Cells in logarithmic growth phase were used for further experiments. Cultures were maintained at 37 ℃, with an atmosphere of 5 % CO_2 and saturated humidity.

RNA extraction and cDNA preparation Total RNA from either untreated or treated cells was extracted by using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total RNA 1.6 µg from each sample was reverse transcribed in 40 µL reaction volume using an oligo d(T) primer and MMLV reverse transcriptase (Gibco Invitrogen Co, Germany) according to the protocol of the manufacturer. cDNA 1 µL was used for further quantitative analysis.

RT-PCR RT-PCR reactions were carried out with the Access RT-PCR System (MicroAmp®, PE Biosystems, Germany). Each reaction contained 0.5 mg RNA and 3 mmol/L Mg^{2+}. Primers for human PPARα (forward: 5´-ACTTATCCTGTGGTCCCCGG-3´, reverse: 5´-CCGACAGAAAGGCACTTGTGA-3´) and PPARγ (forward: 5´-CCCTCATG-GCAATTGA-ATGT-3´, reverse: 5´-GGCATTATGAGACATCCCCAC-3´) were designed to amplify 251 and 223 bp product. Following reverse transcription at 48 ℃ for 45 min with gene-specific primers, a stepdown PCR protocol was followed with the annealing temperature ranging from 65 ℃ to 49 ℃ for PPARα and from 60 ℃ to 45 ℃ for PPARγ[16]. For all reactions a human breast cancer cell line MCF-7 was used as a positive control[17], and the reactions run without template DNA was used as negative controls. RT-PCR products were analyzed by agarose gel electrophoresis, and bands were excised from the gel and sequenced to confirm identity.

Assessment of cell growth Cell proliferation was quantified using the cell proliferation MTT kit (Roche Diagnostics GmBH, Germany) according to the manufacturer's protocol. Briefly, cells at 5×10^4 per mL were treated with various concentrations of TZD18 in 96-well plates for 4 d. After incubation, MTT solution was added to each well and cells were incubated for 4 h. The water insoluble formazan was formed during incubation and it was solublized by adding solublization agent to each well. The formazan dye was quantitated using an Anthos HTII ELISA reader (Anthos Mikrosysteme GmBH, Germany).

Cell cycle analysis Cells were treated with TZD18 for 96 h, washed with PBS, and fixed with ice-cold methanol. These samples were then treated with *RN*ase, stained with propidium iodide (PI) and analysed with the FACSCalibur Flow Cytometer (Becton Dickinson, Germany).

Measurement of apoptosis by TUNEL Cells were cultured with TZD18 for 4 d, washed and cytospun onto glass slides. Apoptosis-induced nuclear DNA fragmentation was detected by terminal deoxynucleotidyl-

transferase-mediated UTP nick end labeling (TUNEL) technique, using the *in situ* cell-death detection kit, Fluo-rescein (Roche Diagnostics, Germany). Cells were fixed with a freshly prepared paraformaldehyde solu-tion (4 % in PBS, pH 7.4) at 15-25 ℃ for 1 h. The samples were washed three times with PBS and permeabilized by 0.2 % Triton X-100 in PBS for 2 min on ice. After being washed twice, cells were incubated in the presence of TUNEL reaction mixture at 37 ℃ for 60 min in the dark. The samples were washed three times with PBS and analyzed by fluorescence microscopy.

Assessment of caspase-3 activity The activity of caspase-3 proteases was measured using a caspase-3 colorimetric activity assay kit (Chemicon International, Gemany). Briefly, after being cultured with 20 μmol/L TZD18 for varying periods of time, approximately 2×10^6 T98G cells were lysed and incubated for 1 h at 37 ℃ with the caspase specific substrate in the provided reaction buffer. Cleavage of Ac-DEVD-pNA by active caspase resulted in the liberation of pNA (p-nitroanilide) into solution. The release of pNA was quantitated spec-trophotometrically by measuring absorbance at 405 nm using an Anthos HTII ELISA reader (Anthos Mikro-systeme GmBH, Germany). All experiments were re-peated in triplicate.

Western blot analysis Protein concentrations of whole cell lysates were measured using the BCA pro-tein assay kit (Perbio Science Deutschland GmbH, Germany). Total 50 μg of protein was separated by 4 %-12 % gradient sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to PVDF membranes (BIO-RAD, Germany), blocked in PBS-Tween (0.1 %) with 5 % non-fat dry milk overnight at 4 ℃ and subsequently incubated with primary antibodies for 2 h at room temperature. After thorough washing, the membranes were incubated with peroxidase-conjugated secondary antibodies for 90 min. Signal was detected by chemiluminescence using the ECL detection system (Amersham Pharmacia Biotech, Germany). The following antibodies were used in this study: anti-p27 [kip1] (sc-528), anti-p21 [cip1] (sc-397), anti-Bax (sc-493) (all from Santa Cruz, Germany), anti-bcl-2 (Clone 124, DAKO Diagnostika GmbH, Germany). As an internal control, β-actin was detected with an anti-β-actin antibody (sc-1616, Santa Cruz, Germany).

Statistical analysis Data were presented as mean±SD of at least three experiments. The Student's *t*-test for paired samples was used to evaluate the sta-tistical significance of differences between parameters.

RESULTS

Expression of PPARα and PPARγ in T98G cells The expression of PPARα and PPARγ in T98G cells was examined using RT-PCR. The results showed T98G cells expressed both PPARα and PPARγ (Fig 2).
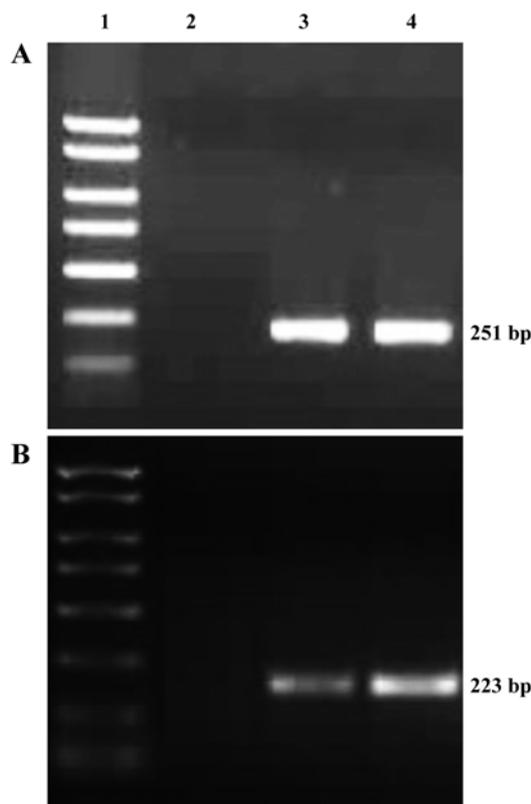

Fig 2. RT-PCR amplification of a 251-bp PPARα (A) and 223-bp PPARγ (B) fragment in T98G cells. Lane1: marker; lane2: negative control (without cDNA); lane3: positive con-trol (MCF-7); lane 4: T98G cells.

Effect of TZD18 on the T98G cell proliferation T98G cells were incubated with various doses of TZD18 for 4 d. MTT test showed that TZD18 significantly inhibited cell proliferation in a concentration-dependent manner (Fig 3).

Growth inhibitory effect of TZD18 was inde-pendent of PPARα and PPARγ To make clear whether the observed growth inhibition was mediated by activation of PPARα and/or PPARγ, first the effect of PPARα antagonist MK-886 and/or PPARγ antago-nists GW9662 were evaluated. They were unable to reverse the inhibitory effect of TZD18 when used alone
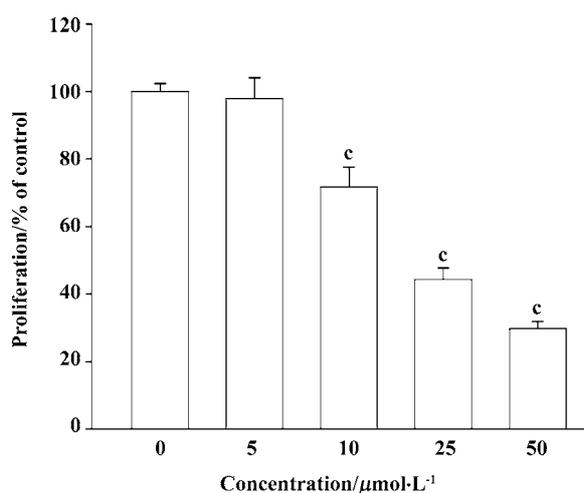
Fig 3. Effect of TZD18 on proliferation of T98G cells. *n*=6. Mean±SD. ᶜ*P*<0.01 *vs* untreated control.

or together (Fig 4). Furthermore, the effect of PPARα agonist WY-14, 643 and/or a high-affinity PPARγ ligand GW7845 had no significant influence on the proliferation of T98G cells (Fig 4).
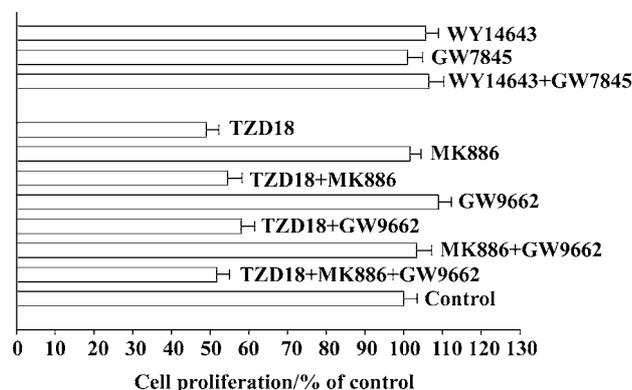

Fig 4. Growth inhibitory effect of TZD 18 was independent of PPARα and PPARγ. Cells 5×10⁴ cells/mL were incubated in the presence or absence of TZD18 (25 μmol/L), WY-14, 643 (20 μmol/L), MK-886 (10 μmol/L), GW7845 (10 μmol/L), GW9662 (5 μmol/L), or their combinations for 4 d. *n*=6. Mean±SD.

Effect of TZD18 on the T98G cell cycle After being treated with TZD18 10 μmol/L and 20 μmol/L for 4 d, the cells exhibited an increase in the G0/G1 phase associated with a decrease of the cells in S phase (Fig 5). These data were quite parallel to the MTT results, indicating that cell cycle arrest, at least in part, may contribute to the inhibitory effects of TZD18 on the growth of T98G cells.

Effect of TZD18 on the induction of T98G cell

apoptosis In order to know whether the cell cycle arrest is associated with apoptosis, we performed TUNEL assay. Cells were treated with TZD18 10 μmol/L and 20 μmol/L for 4 d. TZD18 significantly increased the number of TUNEL positive cells in a concentration-dependent manner (Fig 6A, 6B).
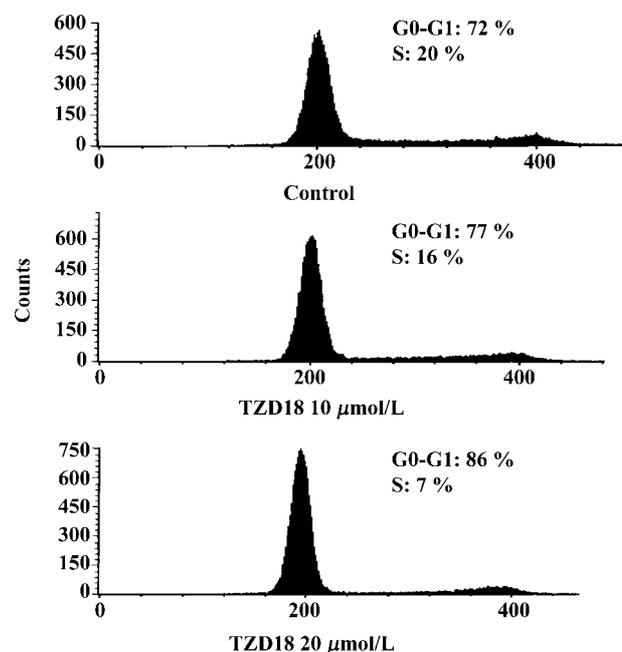

Fig 5. Effect of TZD18 on the T98G cell cycle.

Caspase-3 activity In consideration of frequent involvement of caspases activation in apoptosis, caspase-3 activity was assessed in T98G cells after treatment with TZD18 20 μmol/L. The caspase-3 activity increased as early as 12 h after treatment and reached a maximal level at 24 h (Fig 7).

Effect of TZD18 on the expression of Bcl-2, Bax, p21ᶜⁱᵖ¹, and p27ᵏⁱᵖ¹ To investigate the mechanism by which TZD18 induced apoptosis and caused cycle arrest of T98G cells, the expression level of Bcl-2, Bax, p21ᶜⁱᵖ¹, and p27ᵏⁱᵖ¹ proteins correlated with apoptosis and cell cycle arrest were detected by Western blot (Fig 8). The expression of cell cycle regulator p27ᵏⁱᵖ¹ was upregulated by TZD18, while p21ᶜⁱᵖ¹ had no change, suggesting p27ᵏⁱᵖ¹ may be involved in this process. Also, up-regulation of pro-apoptotic proteins Bax and downregulation of anti-apoptotic protein Bcl-2 were noted, indicating that alternation of these protein levels may account for the observed apoptosis by TZD18.

DISCUSSION

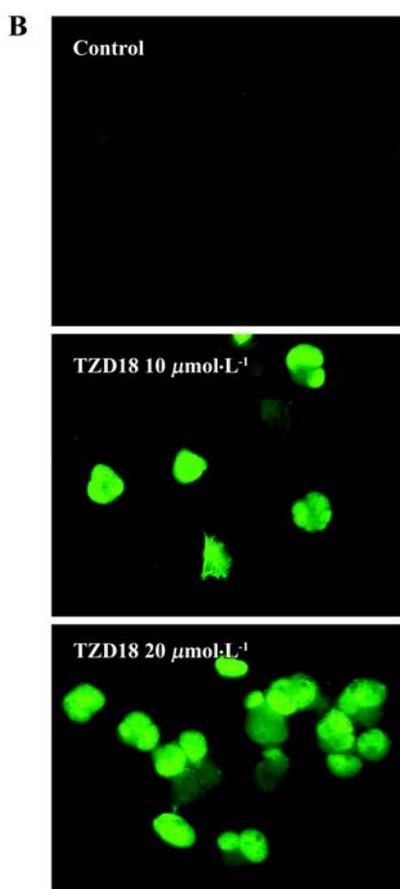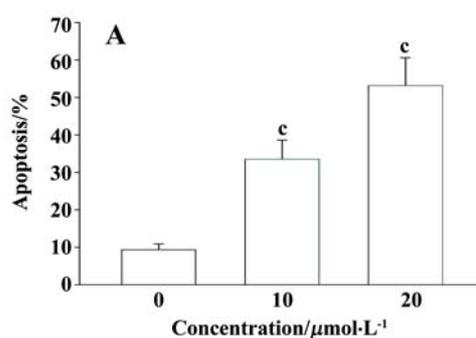We have previously demonstrated that PPARγ

Fig 6. (A) The effect of TZD18 on the induction of apoptosis of T98G cells. *n*=3. Mean±SD. [c]*P*<0.01 *vs* untreated control. (B) Apoptotic T98G cell after treatment with TZD18 (TUNEL staining, ×400).

Fig 7. Effect of TZD18 on the activity of caspase-3. *n*=3. Mean±SD.

Fig 8. Effect of TZD18 on the expression of Bcl-2, Bax, p21[cip1], and p27[kip1]. Lane 1: untreated control; lane 2: TZD18, 10 µmol/L; lane 3: TZD18, 20 µmol/L.

ligand PGZ treatment inhibited cell growth and induced apoptosis of glioblastoma cells[15]. In this study, the newly synthesized α/γ dual agonist TZD18 showed a more potent anti-proliferative effect on T98G cells. Our results showed that the growth of T98G cells was inhibited by TZD18 in a dose-dependent manner. The dose response was found to be comparable to those of colon cancer cells[18] and gastric-cancer cells[19].

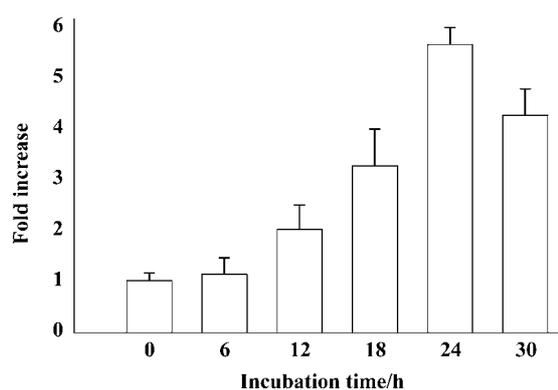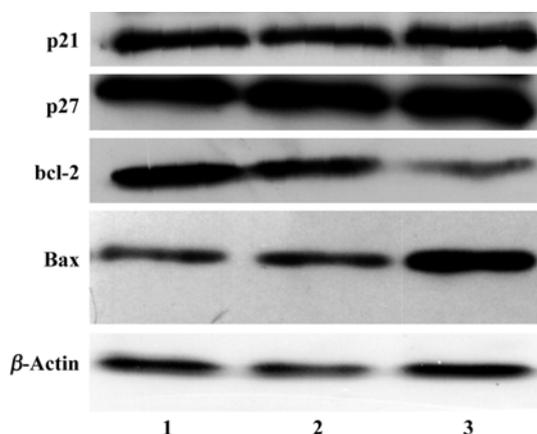Because TZD18 is a dual agonist for PPARα/γ, its anti-prolierative effect may be through the activation of PPARα and/or PPARγ. However, a PPARα-specific antagonist MK-886 could not block the anti-proliferative effect of TZD18. To further confirm this result, we examined the effect of the PPARα agonist WY-14,643 on the proliferation of T98G cells. The result showed that WY-14,643 had no significant effect on the proliferation of T98G cells. These data indicated that the anti-proliferative effect of TZD18 was independent of PPARα.

Recent reports suggested that the anti-proliferative effects of PPARγ agonists such as CGZ, TGZ, PGZ, and 15-deoxy-delta12,14-prostaglandin J2 were independent of PPARγ activation in many cell types[20-26]. To determine whether the inhibitory effect of TZD18 is mediated by PPARγ, GW7845, a high-affinity PPARγ agonist, was incubated with T98G cells for 96 h and showed no inhibition of proliferation of T98G cells. In addition, TZD18 induced proliferation inhibition was not

reversed by PPARγ antagonists GW9662. These data suggested that the inhibitory effect of TZD18 was also independent of PPARγ.

To make clear whether simultaneous activation of PPARα and PPARγ were required for the inhibitory effect of TZD18, we used PPARα and PPARγ antagonist at same time. However, the inhibitory effect of TZD18 could not be reversed. Furthermore, the combination of PPARα and PPARγ agonist had no effect on the proliferation of T98G cells. Taken together, these results indicated that the inhibitory effect of TZD18 was PPARα and PPARγ co-independent.

A large body of evidence demonstrated that an alteration of the cell cycle accounts for the growth inhibition of cancer cells by agonists of synthetic TZD class[27-29]. We also observed that TZD18 increased T98G cells accumulating in the G1 phase and decreased in the S phase in dose-dependent manner. The cell cycle withdrawal in cancer cells by TZDs may be related to the increased level of CDK inhibitors such as $p21^{cip1}$, $p27^{kip1}$[28,30]. In our experiment, we only found expression of $p27^{kip1}$ increased, while $p21^{cip1}$ did not have obvious changes. Thus, T98G cell cycle withdrawn may be associated with upregulation of $p27^{kip1}$. However, further studies are required to elucidate the exact mechanism of induced G1 arrest in T98G cells.

It is likely that cell growth arrest is a prerequisite for apoptosis and one consequence of cell cycle arrest can be apoptosis[31]. To determine the underlying mechanisms of the growth inhibitory effect of TZD18, we also investigated whether it acted by inducing apoptosis of T98G cells. For this purpose, we performed DNA fragmentation assay (TUNEL) and found that TZD18 induced apoptosis of T98G in a dose-dependent manner.

As the main executor of apoptosis, caspase-3 activation by PPARγ agonists has been reported recently[32-35]. Therefore, we measured the activity of caspase-3 using Ac-DEVD-pNA as substrate. In our experiment, we found that the increase of caspase-3 activity was time-dependent and reached the highest at 24 h, which further confirm TZD18 inducted apoptosis of T98G cells, indicating that DEDV-dependent protease activity may be responsible for apoptotic characteristics induced by TZD18.

We then investigated how TZD18 induced apoptosis in T98G cells. Among dozens of apoptotic related molecules, we concentrated on Bcl-2 since we have previously found that downregulation of Bcl-2 by either PGZ and/or ATRA were closely associated with induction of apoptosis in MCF-7 cells[8]. Also, we focused on Bax because it was also associated with TZD-induced apoptosis in human glioblastomas[15,36,37]. We found in this study that TZD18 inhibited the expression of Bcl-2 at the protein level. Since Bcl-2 was one of the key determinants in TZD-induced apoptosis in both breast cancer[8] and colon cancer[38], we assume that Bcl-2 may also play an important role in apoptosis of T98G cells. Further functional analysis of Bcl-2 in human glioblastoma is required to clarify this issue. In this study we also observed an upregulation of Bax in T98 cells after exposure to TZD18. Western-blot analysis of TZD18-treated T98G cell protein extracts revealed reduced bcl-2, and increased $p27^{kip1}$ and Bax expression, suggesting a mechanism for the TZD18 mediated cell-cycle arrest and apoptosis.

In conclusion, the current study showed that, as a novel agent, TZD18 inhibited cell growth and induced apoptosis in human glioblastoma T98G cells, which was associated with activation of caspase-3 and down-regulation of Bcl-2 expression, and up-regulation of Bax and $p27^{kip}$ expression, suggesting that TZD18 might have therapeutic role in the treatment of human glioblastoma. Further studies using other cells, as well as potential synergy with other agents should now be pursued.

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