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ORIGINAL RESEARCH

Platycodin D Enhances Glioma Sensitivity to Temozolomide by Inhibition of the Wnt/β-Catenin Pathway

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Background: Temozolomide (TMZ) is a first-line chemotherapeutic agent for gliomas. However, its efficacy is limited by drug resistance. Platycodin D (PD) exhibits notable anti-glioma activity The objective of this study was to investigate the potential of PD to augment glioma sensitivity to TMZ and the underlying mechanisms.

Methods: Cell viability and proliferation were assessed using CCK-8 and clonogenic assays, respectively, while flow cytometry was used to detect apoptosis. Cell migration and invasion were assessed using Transwell assays. Western blotting and immunohistochemistry analyses were performed to determine protein expression levels. A xenograft glioma model was established to investigate the in vivo effects of PD.

Results: PD augmented glioma cell sensitivity to TMZ, as evidenced by heightened inhibition of cell growth, colony formation, migration, and invasion, accompanied by elevated apoptosis. Treatment with PD or a combination of PD and TMZ robustly suppressed the expression of active β-catenin and c-Myc, which was reversed by the β-catenin activator, SKL2001. In vivo experiments demonstrated that PD amplified the anti-glioma efficacy of TMZ, resulting in diminished Ki67 expression and substantially reduced expression of active β-catenin and c-Myc in the tumor tissue.

Conclusion: PD augmented glioma cell sensitivity to TMZ by modulating Wnt/β-catenin pathway. Our findings demonstrate the potential of PD as an innovative therapeutic agent to enhance glioma treatment, especially in TMZ-resistant gliomas.

Keywords: platycodin D, TMZ, Wnt/β-catenin pathway, glioma

Introduction

Glioblastoma multiforme (GBM) is the predominant primary malignant brain tumor, and has a remarkably low 5-year survival rate of less than 5.5%.[1–3] In the conventional therapeutic paradigm for GBM, surgical resection is coupled with concurrent chemoradiotherapy featuring administration of temozolomide (TMZ). Notably, although promising, this approach is subject to a substantial hurdle: more than half of the patients exhibit resistance to TMZ, and an alarming 90% of recurrent gliomas are refractory to repeated cycles of TMZ therapy.[4]

Platycodon grandiflorum (PG) is a prominent traditional Chinese medication that is used to address different health conditions, including cough and chest pain.[5] A key bioactive component of PG is platycodin D (PD), a triterpenoid saponin found in high concentrations. Investigations have revealed the efficacy of PD against various tumors such as gastric cancer,[6] acute myeloid leukemia,[7] glioma,[8] and lung cancer.[9] Its anti-tumor properties have been attributed to the modulation of multiple signaling pathways. Research by Xu highlighted the antitumor efficacy of PD in gastric cancer, particularly through promotion of c-Myc protein ubiquitination and subsequent degradation.[6] In colorectal cancer cells treated with cetuximab, PD mitigated metastatic potential by suppressing β-catenin, particularly in cells containing wild-type KRAS.[10] Additionally,

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studies have underscored the role of PD in impeding glioblastoma progression through Skp2 regulation.[11] Furthermore, PD triggers apoptosis in cancer via the JNK1/AP-1/PUMA pathway[9] and heightens sensitivity to cetuximab by suppressing the PI3K/Akt pathway.[11] Ongoing investigations indicate the potential of PD as a versatile therapeutic agent with promise for treatment of diverse malignancies.

Aberrant activation of the Wnt/β-catenin pathway is a prevalent feature of many tumors, highlighting its potential as a therapeutic target.[12–15] This signaling cascade is notably heightened in GBM. Its active state is correlated with poor prognosis in GBM patients.[16–19] Hence, modulating the Wnt/β-catenin pathway represents a promising strategy with the potential to improve overall survival outcomes in individuals diagnosed with GBM. The Wnt/β-catenin pathway is crucial in promoting chemoresistance in diverse cancers by activating oncogenes such as c-Myc and survivin.[20]

To the best of our knowledge, this study is the first to demonstrate the potential of PD to enhance the responsiveness of glioma cells to TMZ. In this study, we probed the potential synergistic effects of the concurrent administration of PD and TMZ for the inhibition of glioblastoma. Our findings demonstrated that PD not only amplified the cytotoxicity of the chemotherapeutic agent TMZ but also exerted pronounced effects on apoptosis and inhibited cell invasion in vitro. Furthermore, through comprehensive in vivo and in vitro experiments, we substantiated the augmentation of glioblastoma cell sensitivity to temozolomide by PD and elucidated that the underlying mechanism involved the suppression of the Wnt/β-catenin pathway. These results contribute to a deeper understanding of the potential therapeutic avenues for glioblastoma treatment.

Materials and Methods

Reagents

PD (purity > 99%) was obtained from Tianjin Shilan Technology Co. Ltd. (China). Antibodies against Bcl-2 (26593-1-AP), Bax (50599-2-Ig), c-Myc (10828-1-AP), β-catenin (51067-2-AP), and MMP9 (10375-2-AP) were purchased from Proteintech. Antibodies against caspase-3 (#9664), Ki67 (#12202), and MMP2 (#40994) were obtained from CST. Temozolomide (TMZ) was procured from Sigma–Aldrich. Antibodies specific for active β-catenin (AB305261) and c-Myc (ab32072) were purchased from Abcam. SKL2001 (HY-101085) was obtained from MedChem Express. U87 and U251 cells were purchased from the Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College.

CCK-8 Assay

The cells were then treated with different concentrations of PD and TMZ. After 24 h, the CCK-8 solution was added to each well and incubated for 1 h. Absorbance was quantified as previously described.[21]

Colony Formation Assay

Individual wells of a 6-well plate were inoculated with 500 cells and incubated overnight. The cells were cultured for 2 weeks after the administration of TMZ and PD until the emergence of discernible colonies. The culture medium was renewed every 48 h. Subsequently, the colonies were fixed in ethanol and stained with crystal violet. Finally, the colonies were assessed microscopically.

Apoptosis Assay

Apoptosis was evaluated using an Annexin V Apoptosis Detection Kit (BD Pharmingen). Treated cells were harvested and suspended in 500 μL of buffer. Fluorescein isothiocyanate (FITC) and propidium iodide (PI) solutions (5 μL each) were added and incubated for 15 min. The samples were then analyzed using flow cytometry.

Transwell Assay

To evaluate the potential of PD to amplify the effect of TMZ on glioma cell motility, we used a Transwell chamber (Corning, USA). For the migration assay, cells were placed in the upper chamber with 200 μL Dulbecco's modified Eagle's medium (DMEM). Simultaneously, the lower chamber was loaded with 600 μL of DMEM supplemented with

20% fetal bovine serum (FBS) and the designated concentrations of either PD or TMZ. After 24 h, the cells that migrated across the membrane were stained with 0.2% crystal violet and imaged randomly. Invasion experiments were conducted in a Matrigel-coated chamber (10 μL).

Western Blotting

Following lysis in radioimmunoprecipitation assay buffer, cells or tumor tissues underwent centrifugation at 12,000 × g for 15 min to obtain the protein supernatant. Proteins (30 μg) were isolated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membrane was blocked with 5% bovine serum albumin (BSA) for 1 hour and then incubated with primary antibodies overnight at 4 ◦C. Following incubation with secondary antibodies, protein expression was assessed through enhanced chemiluminescence (ECL) (Millipore). Western blot analyses were conducted as described previously.[22]

In vivo Experiments

Male BALB/c mice (5-weeks-old) were obtained from the Beijing Charles River Laboratory Animal Technology Company. Ethical clearance for experimentation involving animals was obtained from the Ethics Committee of the Peking University People's Hospital (No. 2021- PHE072). This study adheres to the principles outlined in the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health (NIH). The mice were implanted with 5×10^6 U87 cells, and when the tumor diameter reached 50 mm^3, were randomly assigned to four groups: control, PD, TMZ, and PD + TMZ groups. Mice were treated intraperitoneally with PD or TMZ every other day at a fixed dose of 20 mg/kg.

Immunohistochemistry (IHC) Analysis

The tumor tissues were embedded in paraffin and subjected to deparaffinization and dehydration after sectioning. Subsequently, the samples were boiled in sodium citrate buffer (pH 6) for 20 min, treated with 3% H_2O_2 for 20 min, and incubated overnight with primary antibodies against Ki67 (1:500), β-catenin (1:500), and c-Myc (1:200; Abcam) at 4°C. After three washes with phosphate-buffered saline (PBS), the slides were incubated for 1 h with secondary antibodies and then subjected to a peroxidase reaction with diaminobenzidine (DAB). Finally, the samples were photographed under a microscope.

Statistical Analysis

Each experiment was performed independently with at least three biological replicates. Data are presented as mean ± standard deviation (SD). Group differences were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for comparisons among multiple groups. For comparisons between two groups, an independent *t*-test was used. The assumption of normality was verified using the Shapiro–Wilk test, and homogeneity of variance was confirmed using Levene's test. Statistical significance was set at $P < 0.05$.

Results

PD Heightened the Responsiveness of Glioma Cells to TMZ

To elucidate the effect of PD on glioma cell sensitivity to TMZ, U87 and U251 cells were treated with different concentrations of TMZ (5, 10, 20, 30, and 50 μM) in combination with PD (1 μM) for 24 h. CCK8 assay showed that both PD and TMZ treatment reduced the viability of glioma cells. Co-treatment with PD and TMZ further inhibited the cell viability (Figure 1A). Colony formation assay was performed to examine the prolonged repercussions of PD on glioma cell proliferation. We observed that both PD and TMZ treatment markedly suppressed colony formation by U87 and U251 cells. Moreover, concurrent exposure to PD and TMZ synergistically suppressed glioma cell colony formation (Figure 1B and C).

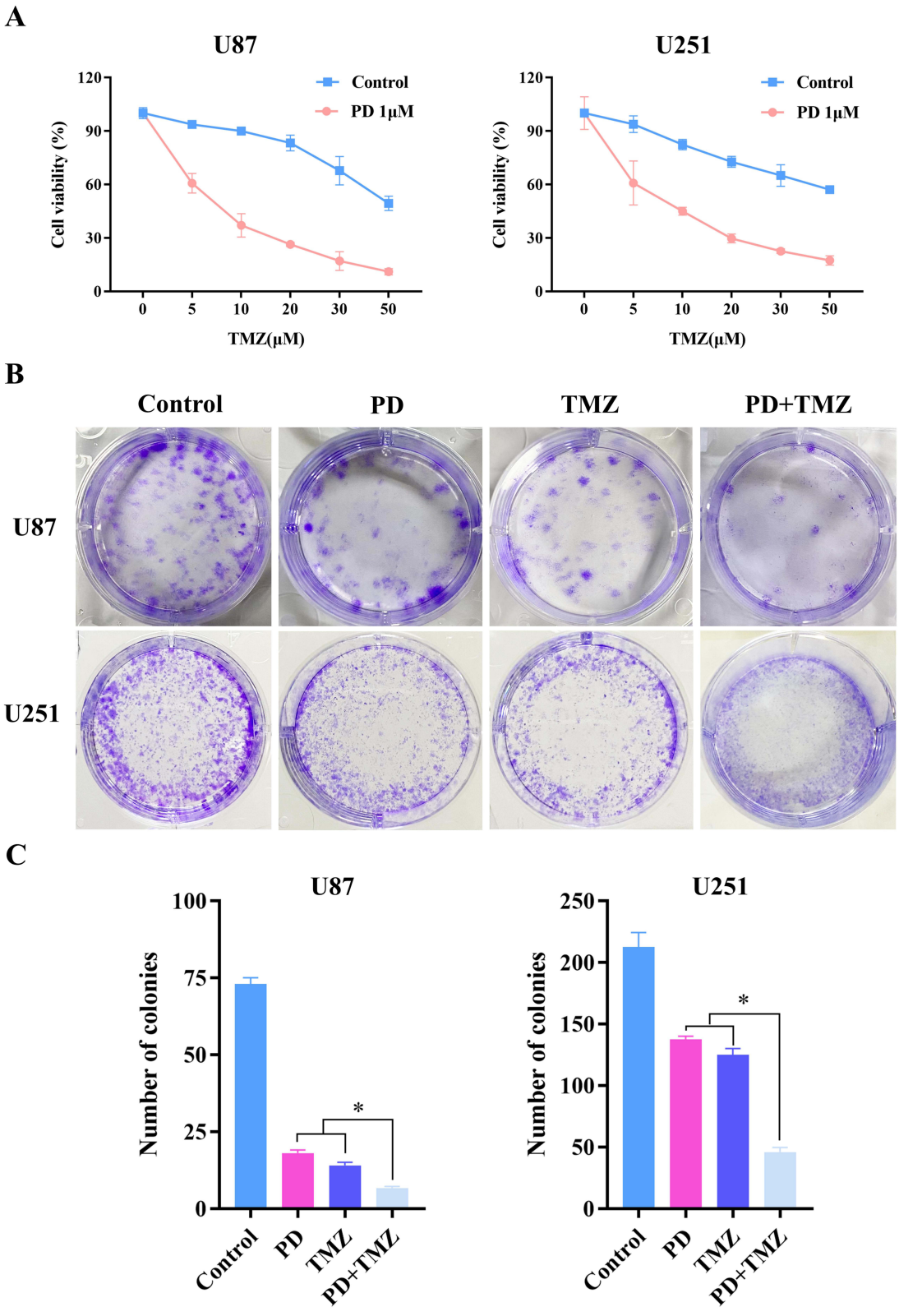

Figure 1 PD heightens the susceptibility of glioma cells to TMZ. (**A**) Effects of PD in combination with TMZ on the proliferation of glioma cells. (**B** and **C**) Impact of PD in combination with TMZ on colony formation of U87 and U251 cells. Data are represented as mean ± SD (n= 5). *P < 0.05.

PD Augments the Suppressive Impact of TMZ on the Migratory and Invasive Properties of U87 and U251 Cells

To investigate the potential of PD to augment the effects of TMZ on the migration and invasion of glioma cells, U87 and U251 cells were treated with PD, TMZ, or PD + TMZ. Both PD and TMZ independently suppressed glioma cell migration, and combined PD and TMZ treatment exerted a more pronounced inhibitory effect on migration than administration of each drug alone (Figure 2A and B). A similar behavior was observed in the invasion experiments, in which concurrent administration of PD and TMZ more potently inhibited invasion than the administration of each drug individually (Figure 2C and D).

To further validate the molecular-level impact of PD on enhancing the inhibitory effects of TMZ on glioma cell motility, Western blotting was used to evaluate MMP2 and MMP9 expression in response to individual interventions with PD, TMZ, and combined PD and TMZ treatment. Treatment with PD or TMZ decreases the expression of MMP2 and MMP9 in glioma cells. Moreover, the combined treatment with PD and TMZ exhibited a stronger inhibitory effect on the expression of MMP2 and MMP9 than each drug alone (Figure 2E).

PD Promotes Apoptosis in Glioma Cells and Enhances Glioma Cell Sensitivity to TMZ

Flow cytometry was used to assess the apoptotic status of glioma cells following treatment with PD (5 or 10 μM) for 24 h. PD treatment significantly promoted apoptosis of U87 and U251 cells (Figure 3A and B). To further confirm the involvement of PD in glioma cell apoptosis, Western blotting was performed to determine the expression levels of apoptosis-related proteins including Bax, Bcl2, and cleaved caspase-3. Following treatment with PD, there was substantial upregulation of Bax and cleaved caspase-3 expression, accompanied by a pronounced decrease in Bcl2 expression (Figure 3C and D).

The combined application of PD and TMZ yielded the highest apoptotic rate in glioma cells as determined by flow cytometry. This implies that PD has the potential to augment the responsiveness of U87 and U251 cells to TMZ (Figure 4A and B). To further validate the enhancement of glioma cell sensitivity to TMZ induced by PD, we assessed the expression of apoptosis-associated proteins. A notable increase in Bax and cleaved caspase-3 expression, along with a prominent reduction in Bcl2 expression, was observed in cells treated with the PD + TMZ combination compared to those treated with either drug alone (Figure 4C and D).

The β-Catenin Activator SKL2001 Can Reverse the Effects of PD on Expression of Active β-Catenin and c-Myc in Glioblastoma Cells

Following treatment with PD (5 or 10 μM), decreased expression of active β-catenin and c-Myc was detected in glioma cells (Figure 5A and B). We posited that the augmentation of glioma cell sensitivity to TMZ by PD could be attributed to the reduced expression of active β-catenin and c-Myc in the glioblastoma cells. Subsequently, we determined the active β-catenin and c-Myc expression in glioma cells after individual treatment with PD or TMZ, as well as their combination. In contrast to the individual interventions, the concurrent application of PD and TMZ resulted in notably decreased active β-catenin and c-Myc expression in both U87 and U251 cells (Figure 5C and D).

To further substantiate whether PD augmented the sensitivity of glioma cells to TMZ by reducing the expression of active β-catenin and c-Myc, we treated glioma cells with the β-catenin activator SKL2001 to determine its ability to counteract the PD-induced downregulation of β-catenin. We found that SKL2001 effectively reversed PD-induced downregulation of both β-catenin and c-Myc (Figure 6A and B). Additionally, we found that SKL2001 reversed the downregulation of β-catenin and c-Myc caused by combined treatment with PD and TMZ (Figure 6C and D).

PD Augments the Responsiveness of Glioma Cells to TMZ in an in vivo Tumor Xenograft Model

To validate the ability of PD to augment glioma cell responsiveness to TMZ, we conducted rigorous in vivo experiments in a subcutaneous glioma model. Strikingly, we found that the co-administration of PD and TMZ resulted in the greatest reduction in tumor volume, underscoring the synergistic efficacy of this combination and surpassing the effects of PD or TMZ monotherapy (Figure 7A–C). Importantly, the absence of any noteworthy fluctuations in the body weights of the experimental mice within the treatment cohorts substantiated the relative safety profile of PD (Figure 7D).

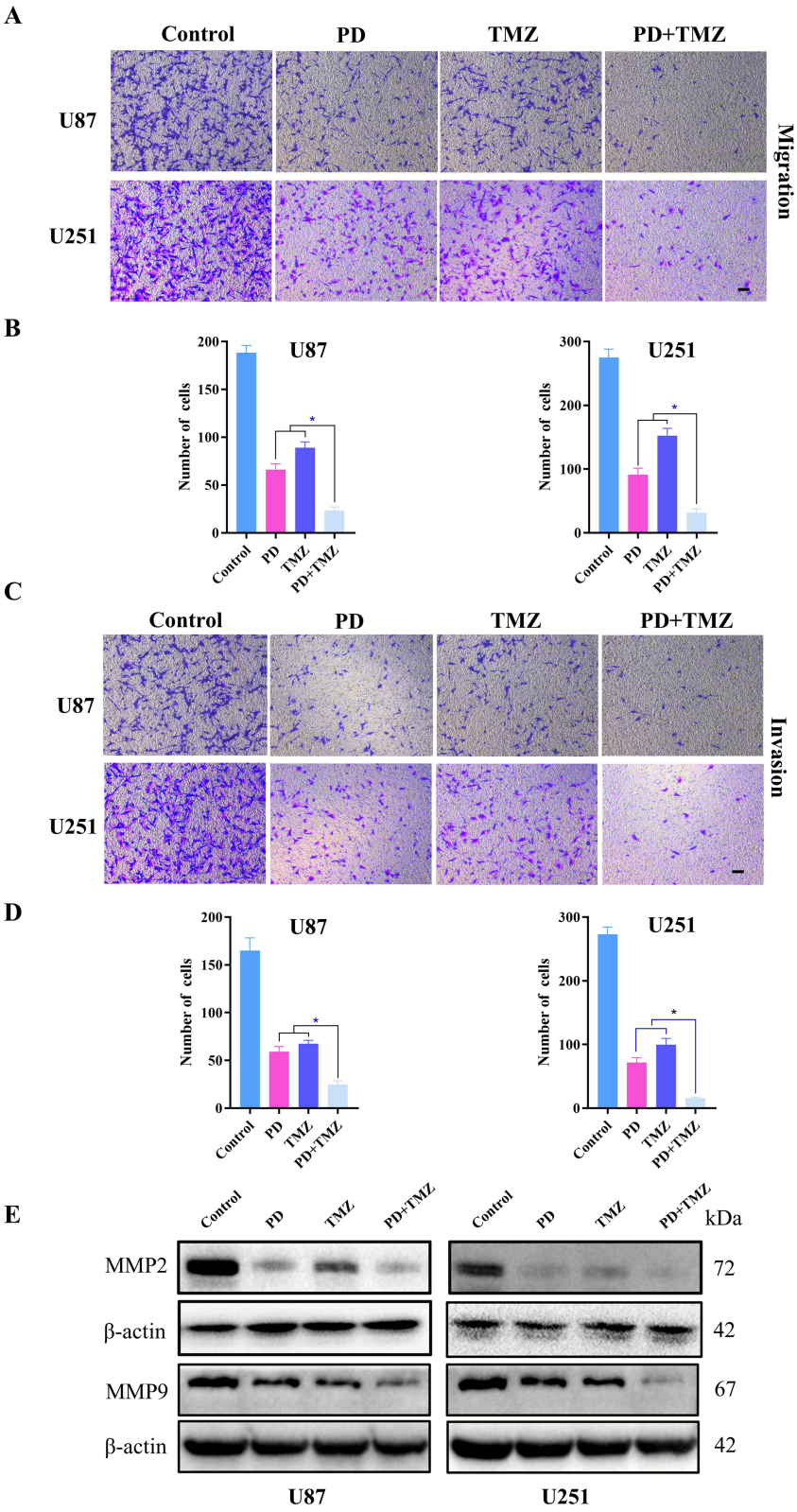

Figure 2 PD boosts the suppressive effects of TMZ on glioma cell motility. (**A** and **B**) Effects of PD in combination with TMZ on glioma cell migration. (**C** and **D**) Impact of PD in combination with TMZ on glioma cell invasion. (**E**) Impact of PD in combination with TMZ on MMP2 and MMP9 expression in glioma cells. Data are represented as mean ± SD (n= 5). *P < 0.05.

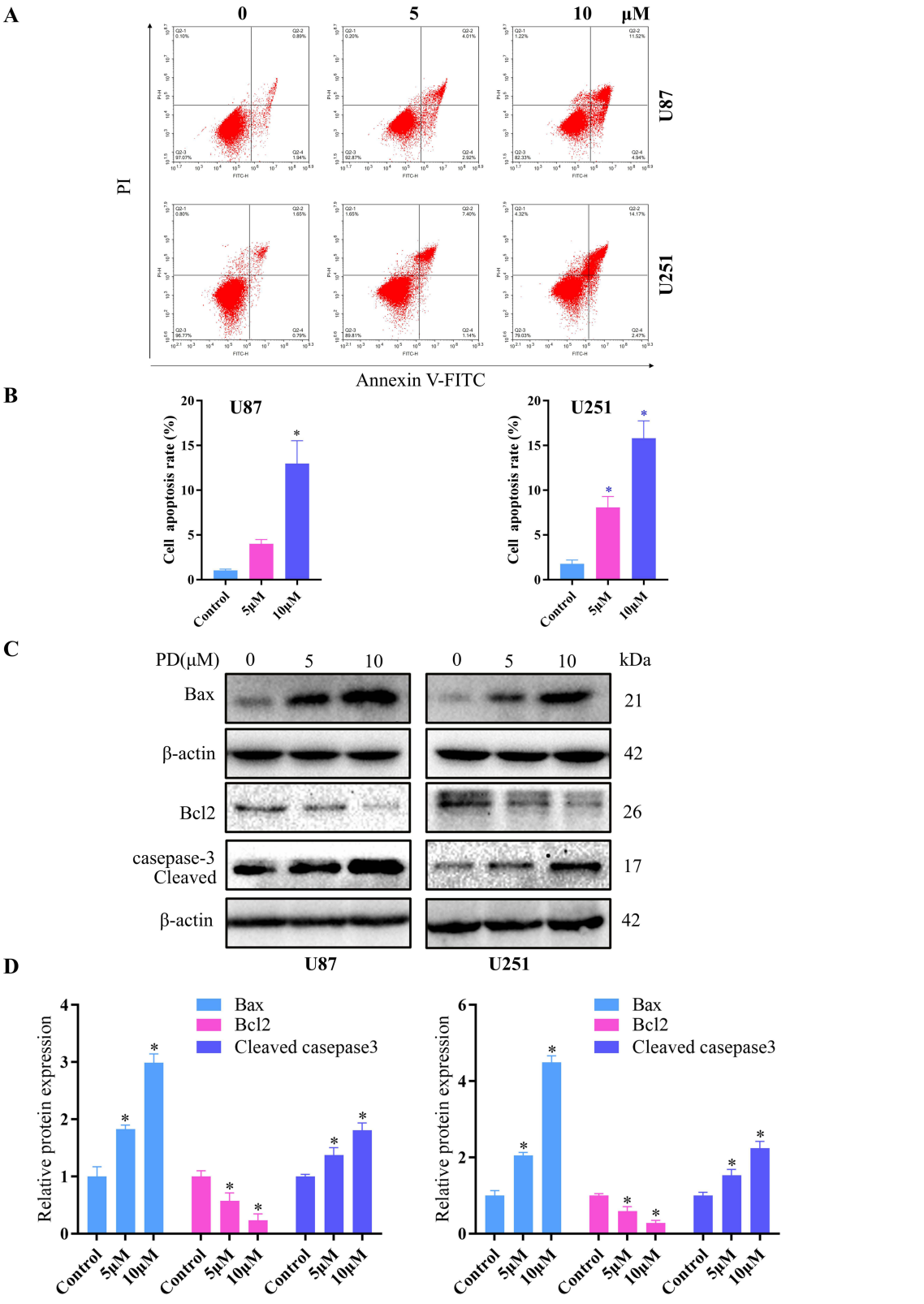

Figure 3 The Impact of PD on glioma cell apoptosis. (**A** and **B**) Cell apoptosis status after PD intervention. (**C**) Expression status of apoptosis-related proteins after PD intervention. (**D**) Quantitative analysis of results showed in (**C**). Data are represented as mean ± SD (n= 5). *P < 0.05.

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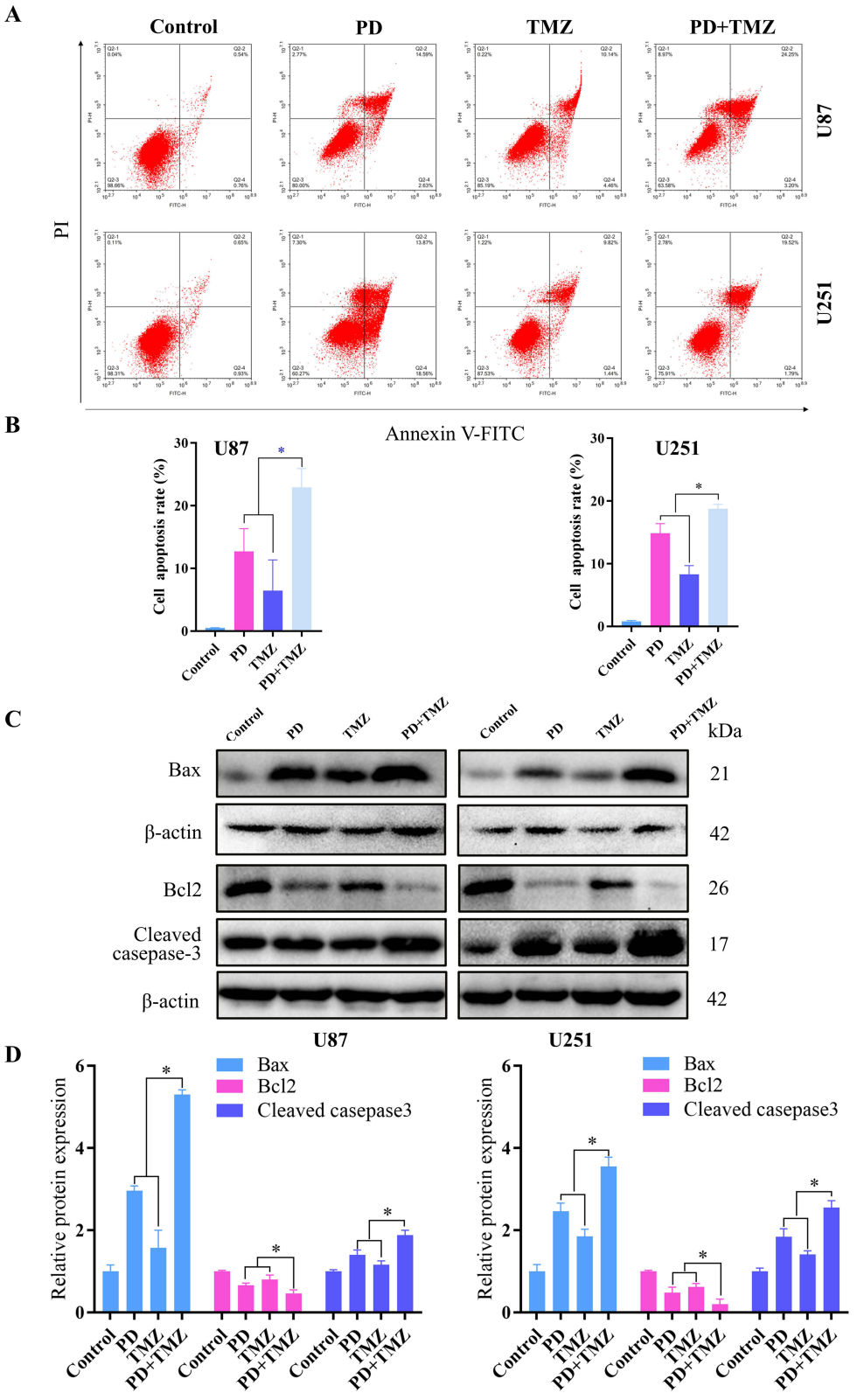

Figure 4 PD potentiates TMZ-induced apoptosis. (**A** and **B**) Combined treatment with PD and TMZ significantly enhanced apoptosis compared with either single agent. (**C**) Detection of apoptosis-associated protein expression by Western blot analysis following combined treatment with PD and TMZ. (**D**) Quantitative analysis of results showed in (**C**). Data are represented as mean ± SD (n= 5). *P < 0.05.

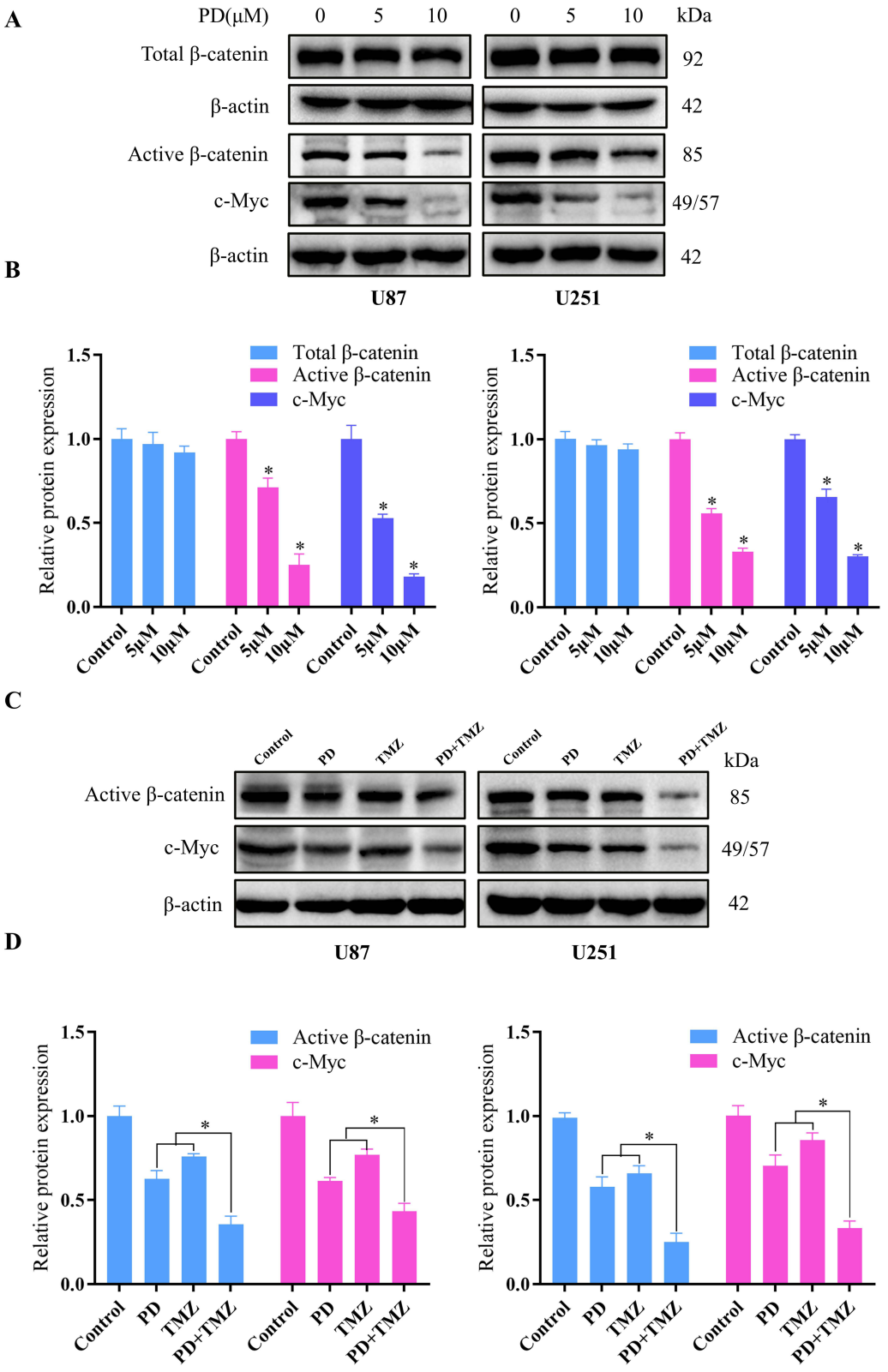

Figure 5 PD enhances the responsiveness of glioma cells to TMZ by regulating the Wnt/β-catenin pathway. (**A** and **B**) Effects of PD on β-catenin, active β-catenin, and c-Myc expression. (**C** and **D**) Concurrent administration of PD and TMZ and its effect on active β-catenin and c-Myc expression in glioma cells. Data are represented as mean ± SD (n= 5).*P < 0.05.

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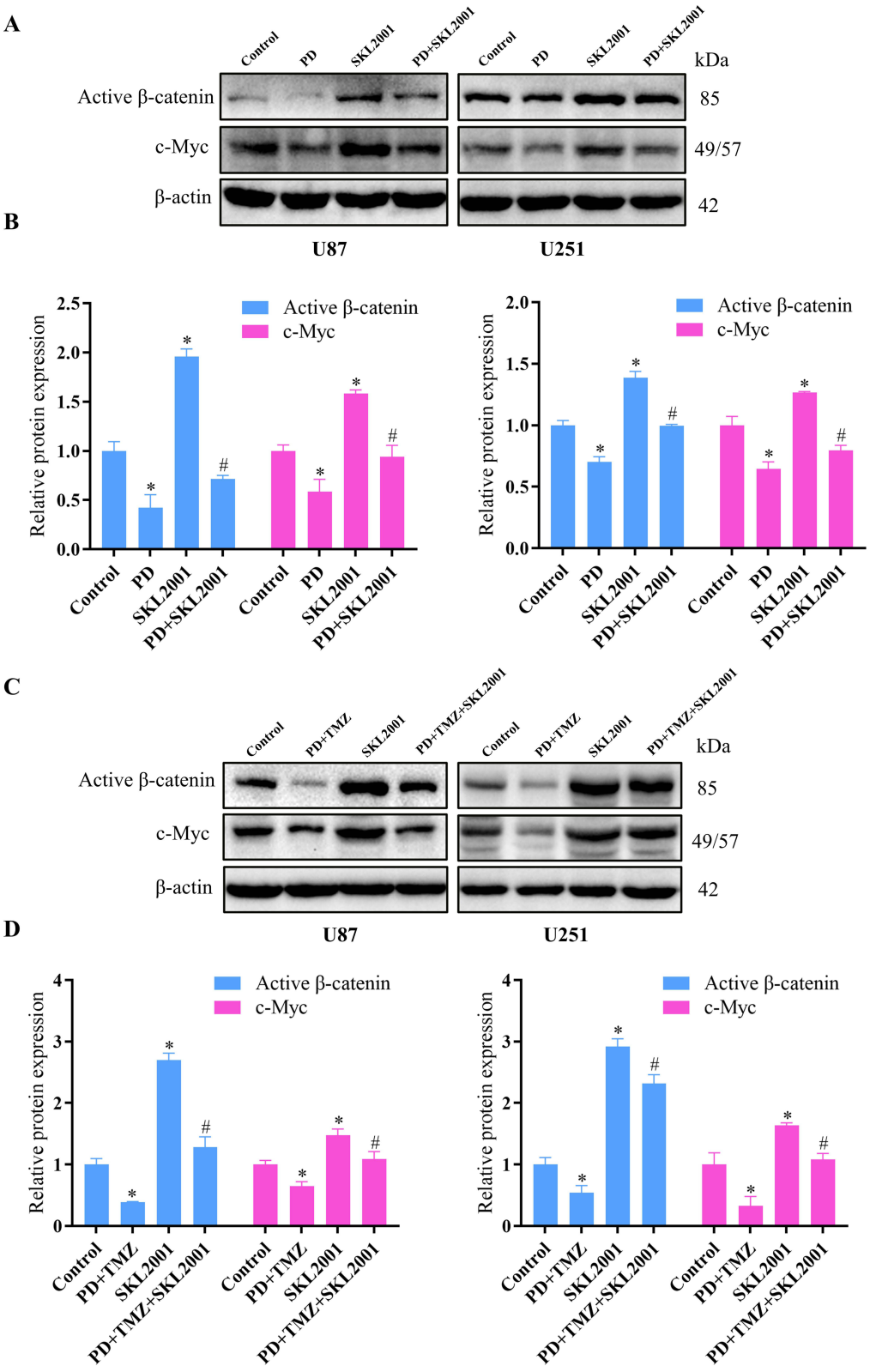

Figure 6 SKL2001 reverses the effects of PD and the combination of PD and TMZ on the Wnt/β-catenin signaling pathway. (**A** and **B**) SKL2001 counteracts the effect of PD on the Wnt/β-catenin signaling pathway. (**C** and **D**) SKL2001 reverses the effects of the PD and TMZ combination on the Wnt/β-catenin signaling pathway. Data are represented as mean ± SD (n= 5). *P < 0.05. #P < 0.05, compared with the PD group or PD+TMZ group.

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Subsequently, we conducted immunohistochemical analyses to assess the expression levels of Ki67, active β-catenin, and c-Myc in the tumor tissues. Intriguingly, the combination treatment group exhibited discernibly diminished expression of Ki67, active β-catenin, and c-Myc compared with the PD and TMZ monotherapy groups (Figure 7E). Western blotting was used to investigate the expression profiles of active β-catenin and c-Myc in the subcutaneous tumor tissues

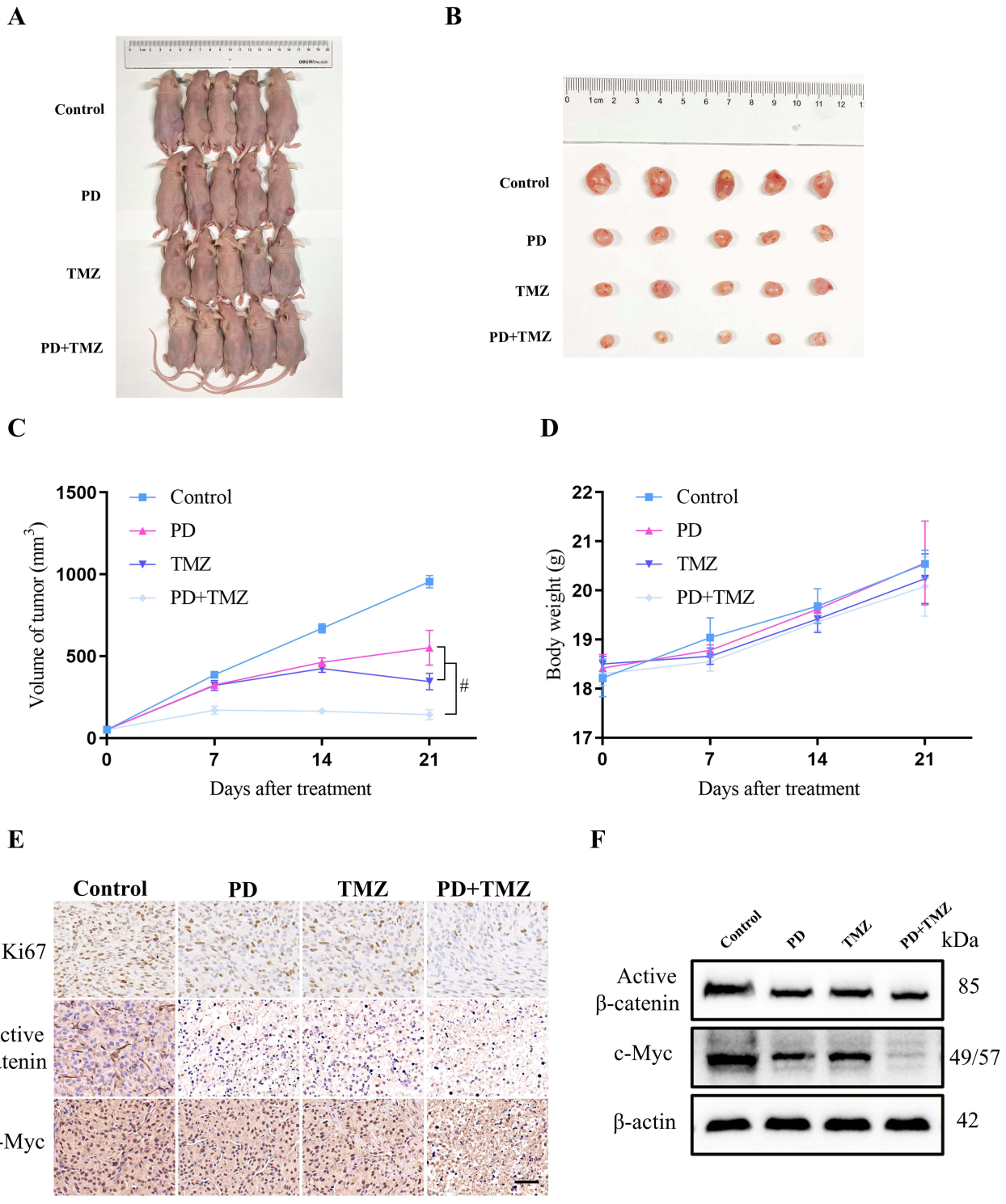

Figure 7 PD enhances the responsiveness of glioma cells to TMZ by regulating the Wnt/β-catenin pathway in vivo. (**A**) Images of post-treatment mice; (**B**) Tumor images; (**C**) Tumor volume; (**D**) Body weight; (**E**) Assessment of Ki67, active β-catenin, and c-Myc expression by immunohistochemistry (scale bar = 50 μm); (**F**) Evaluation of active β-catenin and c-Myc expression by Western blotting. n = 5 in each group. $^{\#}P < 0.05$, compared with the PD group or TMZ group.

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obtained from each experimental group. Interestingly, the combination treatment group exhibited notably reduced expression of active β-catenin and c-Myc compared to the PD and TMZ monotherapy cohorts (Figure 7F).

These results provide compelling evidence that, in vivo, PD enhances the responsiveness of glioma cells to TMZ by reducing the expression of active β-catenin and c-Myc. This multifaceted investigation not only highlighted promising therapeutic implications but also accentuated the intricate molecular interplay underlying the observed outcomes.

Discussion

Gliomas are the predominant primary intracranial tumors and are associated with poor prognosis. Due to factors such as EGFR overexpression, MGMT promoter methylation, 1p/19q co-deletion, and dysregulated autophagy, approximately half of glioma patients exhibit intrinsic or acquired resistance to TMZ therapy.[23–25] Temozolomide is widely used in the treatment of both primary and recurrent gliomas; however, formidable drug resistance poses a substantial hurdle to therapeutic strategies. Numerous investigations have focused on augmenting the responsiveness of gliomas to TMZ, with the overarching goal of extending patient survival. Within the confines of this study, our in vitro experiments revealed that PD amplifies glioma cell responsiveness to TMZ. Furthermore, we demonstrated that PD exerts its influence through intricate modulation of the Wnt/β-catenin pathway. In vivo experiments in nude mice showed that animals receiving a combination of PD and TMZ exhibited the smallest tumors. This observation underscores and authenticates the assertion that PD, through the regulation of the Wnt/β-catenin pathway, heightens the sensitivity of glioma cells to TMZ.

The Wnt/β-catenin pathway is known to have a crucial role in the progression of glioma, influencing cell proliferation and invasion.[26–29] Furthermore, this pathway has been documented to instigate resistance to TMZ in human glioma through autophagic mechanisms.[30] Zhang et al discovered that LGR5 induced epithelial-mesenchymal transition (EMT) by activating the Wnt/β-catenin pathway and was indicative of an adverse outlook for individuals with glioma.[31] In their investigation, Wang et al found that attenuation of Wnt/β-catenin expression amplified the responsiveness of glioma cells towards TMZ, resulting in heightened sensitivity in this context.[32] Ma et al revealed that depletion of OLFML2A hindered glioma proliferation by suppressing the Wnt/β-Catenin pathway.[33] Other signaling pathways, such as the PI3K/Akt/mTOR pathway, also play a critical role in glioma resistance. Wu et al reported that FK228 enhanced glioma cell sensitivity to temozolomide by inhibiting PI3K/Akt/mTOR signaling.[34] Similarly, Yin et al demonstrated that daurisoline suppressed autophagy and increased TMZ sensitivity by modulating the PI3K/Akt/mTOR pathway, thereby inhibiting glioma progression.[35] Moreover, the PI3K/Akt pathway has been implicated in the regulation of β-catenin stability and degradation, further underscoring its role in glioma pathophysiology.[36]

Numerous studies have strategically increased glioma sensitivity to temozolomide (TMZ) by modulating Wnt/β-catenin pathway expression dynamics. Fei et al discovered that mannose hindered glioma cell proliferation while promoting apoptosis, thereby augmenting sensitivity to temozolomide, by modulating the Wnt/β-catenin signaling cascade.[37] Resveratrol has been reported to reinstate the responsiveness of glioma cells to temozolomide by modulating the Wnt signaling cascade.[38] Tsai et al discovered that NBM-BMX effectively addressed temozolomide resistance in GBM by suppressing the β-catenin/c-Myc pathway.[39] Intriguingly, within the scope of our investigation, it became apparent that PD exhibited a nuanced effect on β-catenin levels, specifically by mitigating its activation rather than affecting overall expression (see Figure 5A). Notably, the concurrent administration of PD and TMZ substantially reduced the expression of active β-catenin and c-Myc (Figure 5B). The introduction of the β-catenin activator SKL2001 effectively counteracted the impact of PD, as well as the combined PD and TMZ treatment on active β-catenin and c-Myc expression. These findings suggest that PD heightens the susceptibility of glioma cells to TMZ through modulation of the Wnt/β-catenin pathway, shedding light on intricate regulatory dynamics in glioblastoma biology.

Study Limitations: This study utilized a subcutaneous glioma model to assess the anti-glioma effects of PD and TMZ. While this model offers advantages, such as ease of operation and real-time tumor growth monitoring, it does not fully recapitulate the pharmacokinetic and pharmacodynamic properties of these agents in the native glioma microenvironment, primarily due to the absence of blood-brain barrier (BBB) simulation. To address this limitation, future studies will employ an orthotopic glioma model, which more accurately mimic the physiological and pathological conditions of gliomas. Additionally, we plan to investigate whether PD can overcome TMZ resistance in patient-derived glioma cells with defined

MGMT methylation status or mismatch repair deficiencies, which will provide further insights into its therapeutic potential and clinical applicability.

Conclusion

To the best of our knowledge, this study provides the first evidence that PD augments glioma cell response to TMZ. Both animal experiments and in vitro cell studies robustly supported the assertion that PD achieves heightened sensitivity by intricately modulating the Wnt/β-catenin pathway. These findings not only contribute a novel dimension to the field but also suggest potential translational implications for enhancing therapeutic strategies in glioblastoma.

Data Sharing Statement

Upon request, the data will be provided.

Acknowledgments

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors have no conflicts of interest to disclose.

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