ORIGINAL ARTICLE

Benzodiazepine-induced cell growth inhibition via GABA_A receptor- and TSPO-independent pathways

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Abstract

Our previous study showed that chronic administration of diazepam (DZP) induces changes in gene expression in the hippocampus, cortex, and amygdala. Pathway analysis indicated that cell cycle was altered by chronic DZP treatment. These results raise the concern that chronic DZP administration provokes alterations in cell proliferation. The aim of this study is to elucidate the mechanism by which benzodiazepines (BZDs) regulate cell cycle.

Cell growth curve analysis and MTT assays revealed that DZP treatment significantly reduced cell growth in the microglial cell line. Interestingly, cell growth was not affected by antagonizing the DZP target molecules, gamma-aminobutyric acid (GABA) Type A receptor (GABA_A-R), and 18 kDa translocator protein (TSPO). Similar to the results of DZP, other BZDs such as lorazepam (LZP) and midazolam (MDZ) showed a significant decrease in cell growth. In addition to the mouse microglial cell line results, mouse neuroblastoma cell line also showed an inhibition of cell growth. In contrast, only MDZ inhibited cell growth in human astrocytoma cell line, U373 cells. However, it is not clear whether these differences depend on species or cell types. Consequently, BZDs inhibit cell growth via GABA_A-R- and TSPO-independent pathways.

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Key words: GABA_A receptor; TSPO; benzodiazepine; cell cycle.

Introduction

Gamma-aminobutyric acid (GABA) Type A receptor (GABA_A-R) is a neurotransmitter receptor in the central nervous system (CNS) that is activated by the inhibitory neurotransmitter GABA. GABA_A-R is a chloride ion channel that modulates the excitability of neurons, resulting in an inhibitory effect on neurotransmission and plays a role in the regulation of anxiety and epilepsy [1, 2]. GABA_A-Rs are targeted by several clinically used drugs, including benzodiazepines (BZDs), barbiturates, and ethanol. BZDs are a class of psychoactive drugs that act on the CNS and are commonly used to treat a range of conditions, including anxiety, insomnia, and seizures by enhancing the effects of the neurotransmitter GABA, which reduces the activity of certain nerve cells in the brain [3]. BZDs are generally considered safe when taken as prescribed, but they can cause side effects such as drowsiness, dizziness, and confusion. BZDs can also be habit-forming; therefore, they are typically prescribed for short-term use. Long-term use can lead to tolerance, dependence, and withdrawal symptoms when discontinued [4, 5].

In addition to GABA_A-Rs, BZDs also bind to peripheral-type benzodiazepine receptor (PBR). PBR is currently known as 18-kDa translocator protein (TSPO) and is ubiquitously expressed in various organs and cells [6]. In contrast to GABA_A-Rs, TSPO is involved in the multicellular process such as cholesterol transport, cell growth, ATP production, and neuroinflammation [7-9]. Since TSPO expression correlates with microglial activation, its specific tracer and antagonist, PK-11195 is

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useful for visualizing neuroinflammation[10].

Previous studies have reported that BZDs and PK-11195 have the potential to inhibit cell growth in various cell lines and that inhibition is thought to be via TSPO-dependent manner[11, 12]. In addition, BZDs and PK-11195 induced a significant decrease in the percentage of cells in the S phase and an increase in the G2/M phase[13]. BZDs and PK-11195 also decreased UTP, CTP, and GTP, which are nucleotide resources; therefore, mitosis was inhibited after DNA synthesis. In human glioblastoma cells, diazepam (DZP) inhibited proliferation by G0/G1 cell cycle arrest through GABA$_A$-R- and TSPO-independent manner, and cell cycle arrest was controlled by alteration of cell cycle regulatory factors such as a decrease of CDKs[14]. Based on these results, there is no consensus on whether TSPO is mediated, and the detailed mechanism is unknown. Therefore, this study aimed to elucidate the target molecules and pathways using various cell lines and BZDs.

Materials and Methods

Transcriptome analysis

Our previous expression profiling data[15], deposited in the Gene Expression Omnibus, #GSE76700) were subjected to pathway analysis using Partek® Pathway™ (Partek, Chesterfield, MO). Cell cycle-related genes were selected and examined for alterations following chronic diazepam treatment. Fold change and *P*-value were presented as a volcano plot.

Cell Culture, chemicals, and growth curve

Mouse microglial cell line, BV-2 was obtained and maintained as described previously[16]. The human astrocytoma cell line, U373 MG, mouse neuroblastoma cell line, Neuro2a cell, and human embryonic kidney cells (HEK293T) were purchased from ATCC (Manassas, VA). Cells were maintained according to the manufacturer's

standard protocol. The chemicals used in this study are listed in Table. 1, and the structures of the compounds were drawn using Marvin Sketch (ChemAxon, Budapest, Hungary). Cells were seeded in 3.5 cm dishes at a density of 5.0 ×10^4 cells/dish for BV-2 and U373 cells or 1.0× 10^5 cells/dish for Neuro2a cells. Cell numbers were counted after chemical treatment using a TC20™ Automated Cell Counter (BIO-RAD, Hercules, CA).
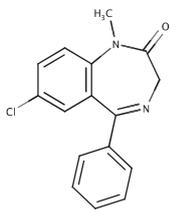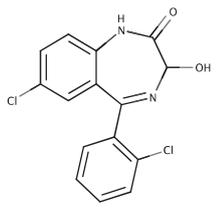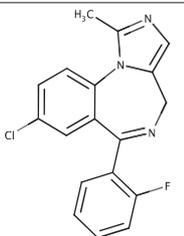
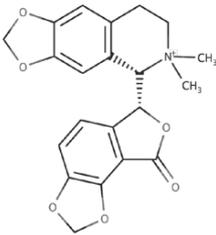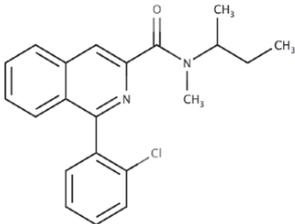### Preparation of whole-cell extracts, SDS-PAGE and Western blotting

As a positive control for GABA$_A$-R β3 subunit, the expression vector (pPyCAG-cHA-IP, RIKEN BRC, #RDB07100, Tsukuba, Ibaraki, Japan) of mouse GABA$_A$-R β3 subunit was transfected to HEK293T cells. Forty-eight hours after transfection, cells were harvested and subjected to whole-cell extracts. Preparation of whole-cell extracts, SDS-PAE and Western blotting analyses were performed as described previously[16]. Antibodies used in this study were shown as below; anti-GABA$_A$-R β3 subunit (Abcam, Cambridge, UK, #ab104659, 1:5,000), anti-TSPO (Abcam, #ab109497), anti-GAPDH (ThermoFisher Scientific, Waltham, MA, #MA5-15738, 1:10,000), Goat anti-Rabbit IgG HRP-conjugated (Agilent, Santa Clara, CA, #P044801-2, 1:5,000), Rabbit anti-Mouse IgG HRP-conjugated (Agilent, #P026002-2, 1:5,000). The full-size images were shown as supplementary data.

MTT assay

MTT Cell Viability assay (Biotium, Fremont, CA) was performed to measure cell viability and toxicity. Approximately 1.0×10^3 cells were seeded in a 96-well culture plate (Corning, #3610, Corning, NY) and subjected to MTT assay in accordance with manufacturer's instructions. The absorbance was measured on a plate reader (FlexStation 3, Molecular Devices) at 570 nm (signal) and 630 nm (background).

Table 1. Chemicals used in this study.

Compound	structure	Supplier (product #)	concentration (μM)
diazepam (DZP)		FUJIFILM Wako Chemicals (045-18901)	1 nM to 1 mM (in 10-fold increments)
lorazepam (LZP)		FUJIFILM Wako Chemicals (122-04591)	100
midazolam (MDZ)		FUJIFILM Wako Chemicals (135-13791)	100
bicucullin methiodide (BMI)		abcam (ab120108)	10
PK-11195		AdipoGen (AG-CR1-0008)	10

Statistics

All values are presented as the mean ± standard deviation (S.D). The unpaired t-test and one-way analysis of variance (ANOVA) were performed to compare activity data. Differences were considered statistically significant at $p < 0.05$. A dose-response curve was drawn by Prism 10 (GraphPad Software, Boston, MA) and calculated half-maximal inhibitory concentration (IC_{50}).

Results

Our previous work revealed that chronic DZP administration induced alterations of gene expression in hippocampus, cortex, and amygdala[15]. First, we performed an additional
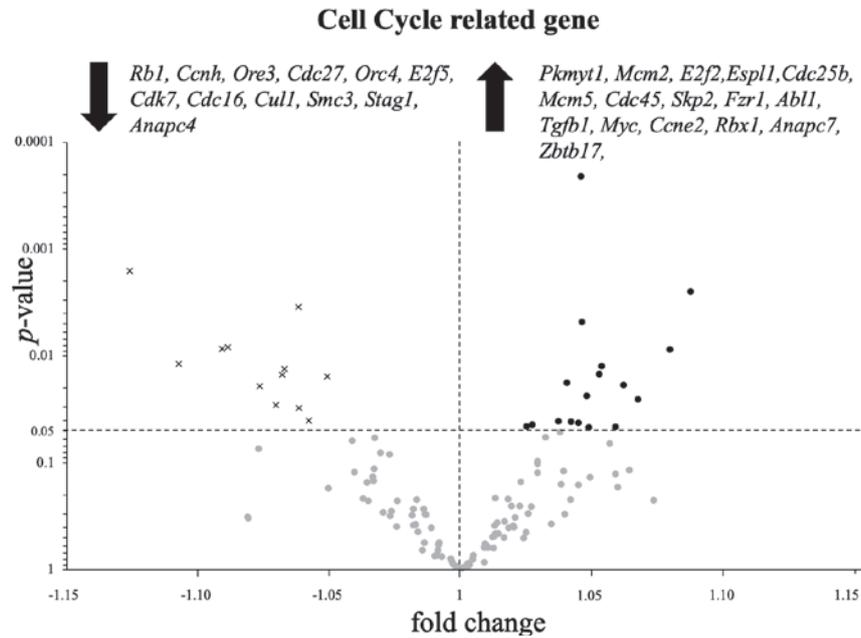
Cell Cycle related gene

Fig. 1 Expression of Cell Cycle related genes was altered by DZP administration.

Transcriptome analysis data were subjected to pathway analysis as described in Materials and Methods. The fold change and p-value of cell cycle-related genes were shown as a volcano plot. Dashed lines were criteria for significantly ($p<0.05$) altered genes and showed gene symbols.

further analysis using gene expression profiling data, which indicated that the expression of cell cycle-related genes was altered (Fig.1). Following chronic DZP administration, 12 genes were downregulated and 16 were upregulated. However, the state of the cell cycle is unclear, and its promotion or inhibition is yet to be determined.

In our previous study, DZP was administered intraperitoneally to mice, and punched out brain tissue samples, including both neurons and glial cells, were used for gene expression analysis. In this study, the effects of BZDs on cell proliferation were examined using cultured cell lines, BV-2, U373, and Neuro2a. The cell growth curve was drawn using a cell counter and the cell number was measured at the various concentrations of DZP in BV-2 cells. As shown in Fig.2A, DZP treatment drastically inhibited cell growth. Based on this result, a dose-response curve was drawn and the IC_{50} was determined to be 16.27 μM (Fig.2B). Since $GABA_A$-R β3 subunit

and TSPO are the molecular targets of DZP, their expression levels were examined by Western blotting analysis. The expression of $GABA_A$-R β3 was extremely low or not expressed in the cell lines used in this study (Fig.2C). On the other hand, TSPO was expressed in these cell lines (Fig.2D) as reported previously[6]. In addition, the effects of antagonizing $GABA_A$-R and TSPO were examined. As we expected, $GABA_A$-R antagonist, bicuculine methiodide (BMI) had no effect on DZP-mediated inhibition of cell growth (Fig.2E). Interestingly, TSPO antagonist, PK-11195, also had no effect on DZP-mediated cell growth inhibition. Furthermore, DZP, BMI and PK-11195 had no effect on cell death because the number of cells was slightly increased. MTT assay also indicated that DZP-mediated inhibition of cell growth was not affected by $GABA_A$-R and TSPO antagonists (Fig.2F). Next, other BZDs, lorazepam (LZP) and midazolam (MDZ), which have the same action and target molecules as DZP but have different structures and
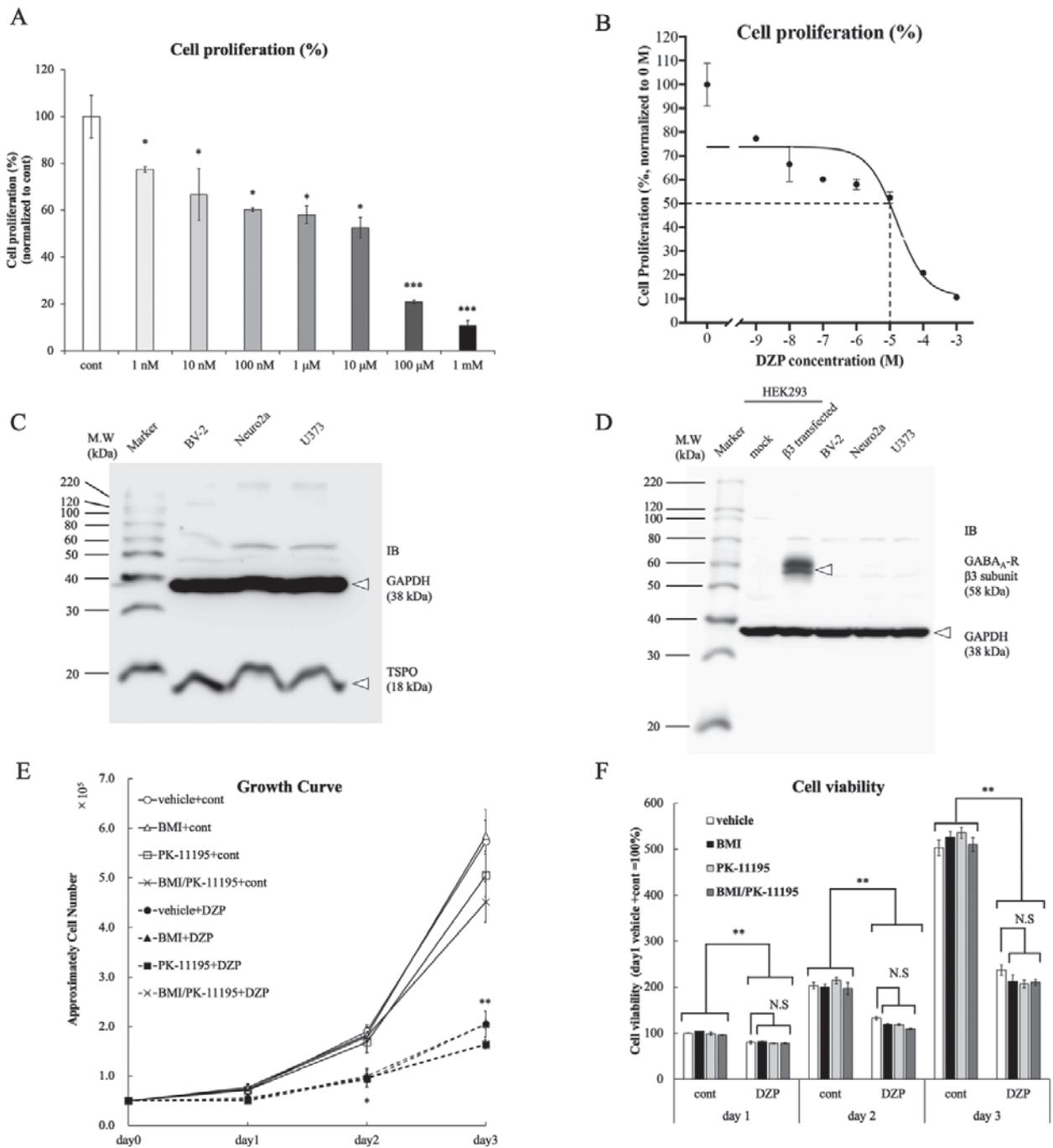
Fig. 2   DZP treatment significantly reduced cell growth via GABA$_A$-R- and TSPO-independent pathways.

（A）The cell numbers of BV-2 cells with or without various concentrations of DZP treatment for 2 days were counted. The cell proliferation of BV-2 cells was normalized to "cont" and expressed percentages.

（B）Dose-response curve was drawn based on Fig.2A by Prism 10 as described in Materials and Methods section.

（C），（D）The expression level of β3 subunit of GABA$_A$-R（C）and TSPO（D）in each cell line used in this study was visualized by Western blotting analysis. GAPDH was used as an internal control. Arrowheads showed the target molecule. M.W: molecular weight

（E）BV-2 cells were treated with 100 μM DZP for 1-3 days following treatment with bicuculline methiodide（BMI）or PK-11195 at the concentration of 10 μM for 2 hours. The cell number was counted as indicated above.

（F）MTT assay was performed as described in Materials and Methods section. Cells were treated with chemicals as indicated above. n = 3, Asterisks indicated statistically significant differences between cont（0.1% DMSO）and DZP. n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$
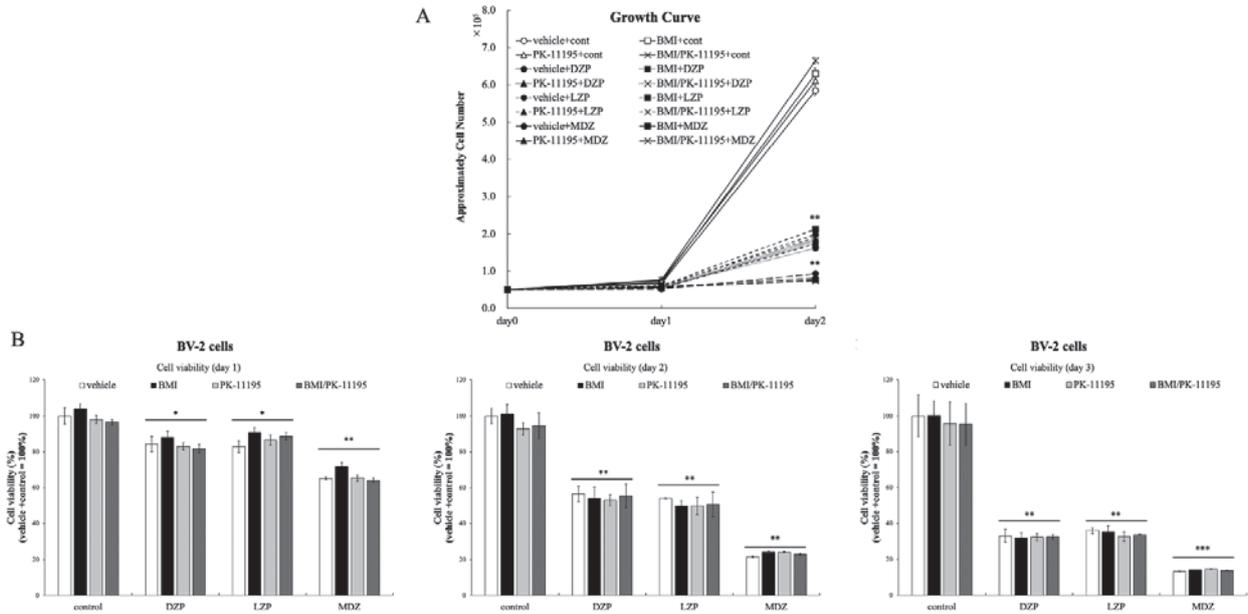
Fig. 3 DZP, LZP, and MDZ significantly reduced cell growth via GABA$_A$-R and TSPO-independent pathways.
（A）BV-2 cells were treated with 100 μM BZDs following treatment with BMI or PK-11195 at the concentration of 10 μM for 2 hours. The cell number was counted as described in Materials and Methods.
（B）Cells were treated with chemicals as indicated above and MTT assay was performed as described in Materials and Methods. n=3, * $p<0.05$, **$p<0.01$, *** $p<0.001$

hemodynamics were investigated. LZP and MDZ were administered in culture medium, and these compounds also inhibited cell growth via GABA$_A$-R- and TSPO-independent pathways （Fig.3A, B）. These results suggested that GABA$_A$-R and TSPO-independent pathways might be involved in BZD-induced cell growth inhibition.

Similar experiments were performed using a mouse neuroblastoma cell line, Neuro2a cell, and a human glioblastoma cell line, U373. The effect of BZDs on Neuro2a cells was similar to the results of BV-2, BZDs significantly inhibited cell growth via GABA$_A$-R- and TSPO-independent pathways （Fig.4A, B）. On the other hand, the proliferation of U373 cells was inhibited by only MDZ on day 3 （Fig.4C, D）.

Discussion

Previous studies have reported that BZDs have the potency to inhibit the cell cycle, and this effect could be applied to the treatment of gliomas[12]. Our results also showed that treatment with BZDs inhibited cell growth in neuroblastoma and microglial cell lines. Furthermore, BZDs-induced inhibition of cell growth was not decreased by GABA$_A$-R or TSPO antagonists. The TSPO antagonist, PK-11195, significantly reduced cell proliferation in murine B16 melanoma cells and rat breast tumor cells in a dose-dependent manner[13, 17], however in this study, PK-11195 at the concentration of 10 μM did not show inhibition of cell growth in Neuro2a, BV-2, and U373 cells. In addition to the experimental conditions, such as cell types, species, and concentration of PK-11195, the effect was also thought to be related to the expression level of TSPO.

BZDs used in this study have several common molecular targets in addition to GABA$_A$-R and TSPO according to BioAssay Results in PubChem （https://pubchem.ncbi.nlm.nih.gov/）. However, there are currently no molecular
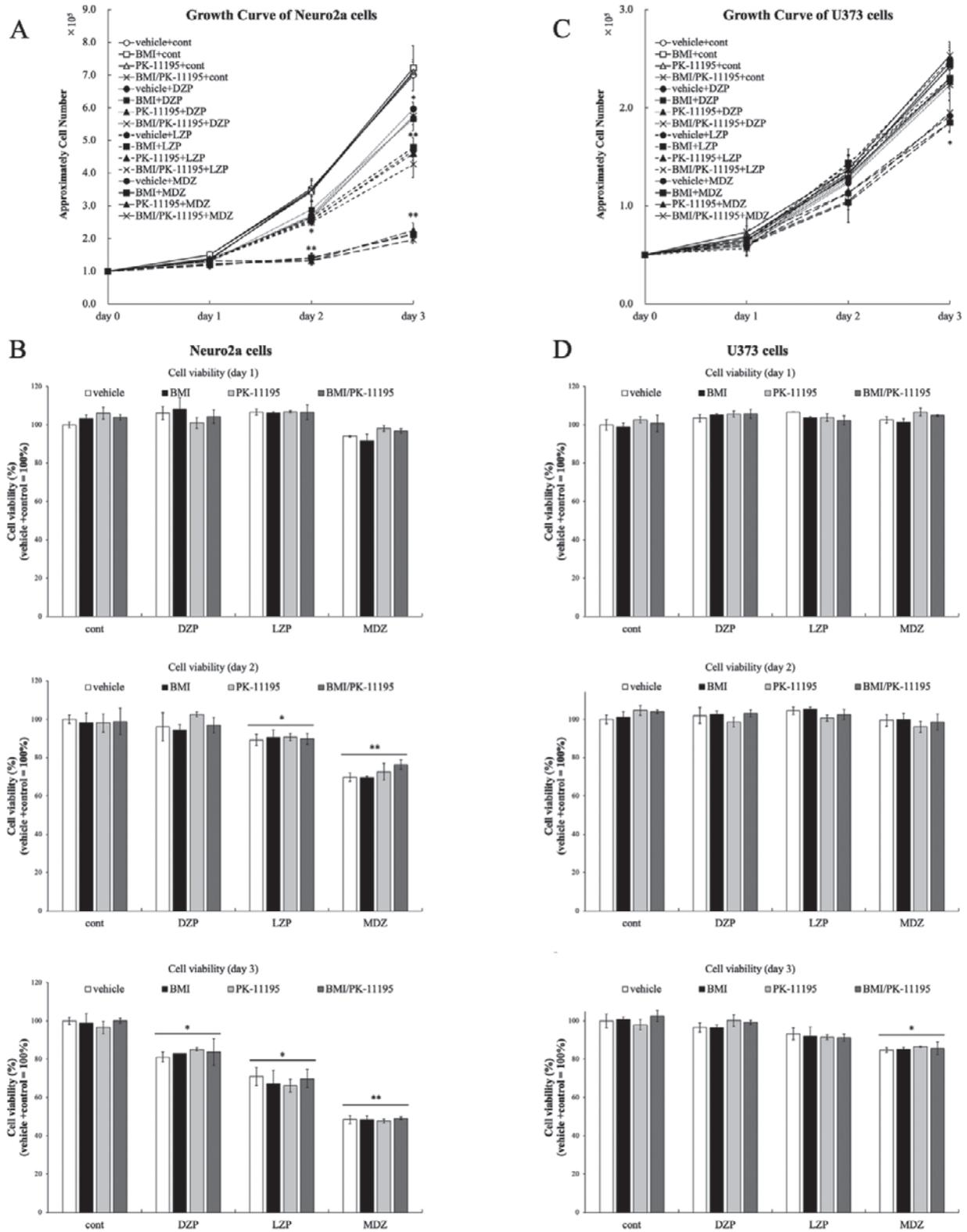
Fig. 4 The Effect of BZDs on cell proliferation was different between cell types.

The growth curve and the results of MTT assay in Neuro2a cells（A, B）and U373 cells（C, D）were indicated. Cells were treated with 100 μM BZDs following treatment with BMI or PK-11195 at the concentration of 10 μM for 2 hours. n=3, * *p*<0.05, **p*<0.01

targets related to cell cycle regulation. On the other hand, transcription factors such as MEN1, RORG, and VDR were common candidate targets of BZDs. It is possible that these transcription factors may regulate gene expression of cell cycle-related proteins as shown in Fig. 1. However, our speculation has a limitation because the BioAssay Results are the results of high-throughput screening and are not validated results.

Different from Neuro2a cell and BV-2 cell, the growth of U373 cell showed significant inhibition only in MDZ treatment. In addition to benzene and diazepine rings, midazolam has a condensate imidazole ring, which provides different physical properties than BZDs. For example, midazolam has slightly higher water solubility under acidic conditions and a shorter half-life than other BZDs, according to PubChem and DrugBank (https://go.drugbank.com/). These differences in physical properties probably generated cell type-specific effects.

Gliomas are malignant tumors derived from glial cells that are extremely undifferentiated and have a high proliferative potential. Because the boundary between normal and cancerous cells is blurred, it is difficult to resect the tumor tissue completely; thus, chemotherapy with temozolomide and radiation therapy are used. The mechanism of action of temozolomide is inducing apoptosis by alkylation and methylation of DNA. However, the expression of O^6-methylguanine–DNA methyltransferase (AGT protein, *MGMT* gene) causes resistance to temozolomide treatment because it repairs DNA damage induced by alkylation or methylation[18]. For this reason, there is an urgent need for the development of a therapeutic alternative to temozolomide. When considering the use of BZDs in glioma therapy, it is essential to evaluate cell proliferation in both *ex vivo* and *in vivo*. Although BZDs have a long history of use and are known to be safe for short-term use, there are concerns regarding the adverse effects of long-term use. In addition, their potential use in the treatment of glioma is not clear and requires further *in vivo* research in future studies.

Conflict of interest

The authors declare that they have no competing interests.

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Author's contributions

SS and TF contributed equally to this work and are regarded as co-first authors. SS and SU designed this study and acquired the funding. SS and TF conducted all experiments, analyzed the

data, and wrote the first draft. All authors read and approved the final manuscript.

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