Anti-tumor effects of perampanel in malignant glioma cells

JURI TATSUOKA[1], EMIKO SANO[1], YUYA HANASHIMA[1], CHIHIRO YAGI[1], SHUN YAMAMURO[1], KOICHIRO SUMI[1], HIROYUKI HARA[2], KAZUHIDE TAKADA[3], KAZUNORI KANEMARU[4], SHIHOKO KOMINE-AIZAWA[3], YOICHI KATAYAMA[1,5] and ATSUO YOSHINO[1]

[1]Department of Neurological Surgery; [2]Division of Anatomical Science, Department of Functional Morphology; [3]Division of Microbiology, Department of Pathology and Microbiology; [4]Division of Biomedical Sciences, Department of Physiology, Nihon University School of Medicine, Tokyo 173-8610; [5]Center for Brain and Health Sciences, Aomori University, Aomori 038-0003, Japan

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Abstract. Glioblastoma has a poor prognosis even after multimodal treatment, such as surgery, chemotherapy and radiation therapy. Patients with glioblastoma frequently develop epileptic seizures during the clinical course of the disease and often require antiepileptic drugs. Therefore, agents with both antiepileptic and antitumoral effects may be very useful for glioblastoma treatment. Perampanel, an α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor antagonist, is an antiepileptic drug that is widely used for intractable epilepsy. The present study aimed to assess the potential antitumoral effects of perampanel using malignant glioma cell lines. The cell proliferation inhibitory effect was evaluated using six malignant glioma cell lines (A-172, AM-38, T98G, U-138MG, U-251MG and YH-13). A dose-dependent inhibitory effect of perampanel on cell viability was demonstrated; however, the sensitivity of cells to perampanel varied and further antitumoral effects were demonstrated in combination with temozolomide (TMZ) in certain malignant glioma cells. Furthermore, cell cycle distribution and apoptosis induction analyses were performed in T98G and U-251MG cells using a fluorescence activated cell sorter (FACS) and the expression levels of apoptosis-related proteins were evaluated using western blotting. No significant change was demonstrated in the proportions of cells in the G0/G1, S and G2/M phases under 1.0 μM perampanel treatment, whereas induction of apoptosis was demonstrated using FACS at 10 μM perampanel and western blotting at 1.0 μM perampanel in both glioma cell lines. Overexpression of SERPINE1 may be related to poor prognosis in patients with gliomas. The combination of 1.0 μM perampanel and 5.0 μM tiplaxtinin, a SERPINE1 inhibitor, demonstrated further reduced cell viability in perampanel-resistant U-138MG cells, which have high expression levels of SERPINE1. These results indicated that the antitumor effect of perampanel may not be expected for malignant gliomas with higher expression levels of SERPINE1. The findings of the present study suggested that the antiepileptic drug perampanel may also have an antitumor effect through the induction of apoptosis, which is increased when combined with TMZ in certain malignant glioma cells. These findings also suggested that SERPINE1 expression may be involved in perampanel susceptibility. These results may lead to new therapeutic strategies for malignant glioma.

Correspondence to: Professor Atsuo Yoshino, Department of Neurological Surgery, Nihon University School of Medicine, 30-1 Oyaguchi-Kamimachi, Itabashi, Tokyo 173-8610, Japan
E-mail: yoshino.atsuo@nihon-u.ac.jp

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Introduction

Standard postoperative radiotherapy with concomitant and adjuvant temozolomide (TMZ) has been shown to be beneficial for glioblastoma treatment, according to a randomized phase III trial by The European Organization for Research and Treatment of Cancer and The National Cancer Institute of Canada Clinical Trials Group in 2019 (1). Subsequently, radiotherapy with concomitant TMZ followed by adjuvant TMZ chemotherapy became the global standard treatment for malignant gliomas, particularly glioblastomas. Although these treatments improve the survival rate of patients with glioblastoma, the prognosis is unsatisfactory, as the 5-year survival rate remains <5% (2). Therefore, novel therapies are needed.

Glutamate is one of the main excitatory neurotransmitters in the central nervous system. Dysregulation of glutamate signaling is extremely important in seizure induction by initiating and synchronizing glutamatergic transmission (3,4). Epileptic seizures are a common symptom of primary brain tumors. Approximately 25-60% of patients with high-grade gliomas (World Health Organization grades 3 and 4) suffer from seizures (tumor-associated epilepsy) and ~15-25% of patients with high-grade gliomas develop drug-resistant epilepsy (5). Furthermore, glioma cells express α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which are stimulated by glutamate in autocrine and paracrine responses and subsequently tumor growth

and invasion, possibly through the activation of oncogenic signaling cascades and cytoskeletal remodeling (6-11).

The pathophysiology of malignant gliomas and tumor-associated epilepsy is not fully understood; however, AMPA receptor antagonists are promising candidates that may mediate both anticonvulsive and antitumoral effects on glioma cells. Furthermore, blocking AMPA receptors was reported to increase the effectiveness of conventional cytotoxic chemotherapy in certain cancer cell lines, including lung carcinoma, astrocytoma, neuroblastoma and rhabdomyosarcoma/medulloblastoma cells. (6,12). Talampanel is an orally administered non-competitive antagonist of AMPA receptor with good permeability across the blood-brain barrier and good tolerability in clinical trials for epilepsy and other neurologic disorders (9,13). A phase II trial of talampanel in combination with radiotherapy and TMZ demonstrated a survival advantage for patients with newly diagnosed glioblastoma compared to patients not treated with talampanel (14,15); however, talampanel failed to demonstrate significant antitumor activity as a single agent in patients with recurrent malignant glioma (9). Another non-competitive AMPA receptor antagonist, perampanel, has excellent brain penetration, a long half-life and a good drug concentration profile compared to talampanel (4,15-18). Perampanel is effective against drug-resistant epilepsy in patients with glioma (5,15,17,19). Recently, perampanel treatment was reported to be effective for uncontrollable epilepsy with gliomas, and magnetic resonance imaging was reported to demonstrate inhibition of tumor growth and/or reduction of peritumoral brain edema (17). Perampanel has antiproliferative effects on glioblastoma cell lines by reducing cell metabolism owing to a decrease in glucose uptake (15). Recently, the antitumoral effects of perampanel were reported to be mediated by the induction of apoptosis and a synergistic effect with TMZ in glioblastoma cell lines (20). The present study used much higher concentrations of perampanel than the usual maintenance blood concentration of perampanel when used as an anticonvulsant; the results suggested that perampanel may act as an anticonvulsant and may also have additional antitumoral effects on malignant gliomas. However, the detailed mechanisms of the antitumoral action remains unknown.

The poor prognosis for patients with glioblastoma may be partly due to the migratory and invasive behavior of tumor cells that exhibit widespread infiltration into the adjacent brain parenchyma (21). Recently, overexpression of SERPINE1, a clade E member of the serine protease inhibitor superfamily that is the main regulator of the plasminogen activator system, has been proposed as a factor for tumor migration and invasion in numerous types of cancer, including glioma, which results in poor patient prognosis (21). Furthermore, SERPINE1 has been reported to serve roles in CSF dissemination, angiogenesis, apoptosis, drug resistance, etc (21,22). Preliminary experiments (data not shown) suggested that *SERPINE1* expression was involved in perampanel susceptibility; perampanel, may also be associated with invasion and migration.

The present study evaluated the relationship between perampanel and SERPINE1, and assessed the mechanisms of their antitumoral activities.

Materials and methods

Cell lines, culture conditions and materials. Human malignant glioma cell lines A-172 (cat. no. JCRB0228; lot no. 021999), AM-38 (cat. no. IFO50492; lot no. 12082003), T98G (cat. no. IFO50303; lot no. 1007), U-251MG (cat. no. IFO50288; lot no. 12132002) and YH-13 (cat. no. IFO50493; lot no. 1164) were purchased from Health Science Research Resources Bank. The human glioblastoma U-138MG cell line (cat. no. HTB-16; lot no. 1104428) was purchased from the American Type Culture Collection.

Cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) (Nissui Pharmaceutical, Co., Ltd.) supplemented with 5% fetal calf serum (Thermo Fisher Scientific, Inc.) using plastic culture flasks (Corning, Inc.) in a 37˚C humidified incubator with an atmosphere containing 5% CO_2.

Perampanel was gifted by Eisai Co., Ltd. and TMZ was purchased from Tokyo Chemical Industry Co., Ltd.

Cell culture viability experiments. The inhibitory effect of perampanel on the viability of malignant glioma cells was evaluated by counting the number of cells following exposure to the drug. Briefly, cells were plated at $1x10^4$ cells/well in 24-well, flat-bottomed plates (Iwaki Cell Biology) and incubated with DMEM (Nissui Pharmaceutical) with 5% fetal calf serum (Thermo Fisher Scientific) at 37˚C for 24 h. The cells were subsequently washed twice with medium and further incubated for 72 h with fresh medium (control) or medium containing 0.01, 0.1, 1 or 10 μM perampanel. Subsequently, cells were detached by trypsinization and counted using a Coulter Counter Z1 (Beckman Coulter, Inc.). The experiment was repeated at each concentration in at least three independent systems and cell numbers were quantified at least eight times in total.

Subsequent experiments used 1.0 μM perampanel, based the blood concentrations previously reported as being achieved using oral administration of 4 mg or 8 mg perampanel as an antiepileptic drug of 0.84 μM or 1.48 μM, respectively (17).

Furthermore, as a low concentration of perampanel demonstrated significant anti-viability effects on the T98G and U-251MG cell lines, which are widely used in brain tumor experiments, these cell lines were used in the subsequent experiments (cell cycle distribution analysis and assessment of apoptosis). T98G expresses MGMT and U-251MG does not express MGMT (23).

Cell cycle distribution analysis. Perampanel-induced changes in the cell cycle distribution were assessed using flow cytometry. T98G and U-251MG cells were seeded in 6-well plates (Iwaki Cell Biology) at $1x10^6$ cells/plate and incubated at 37˚C for 24 h. The culture medium was replaced with fresh medium (control) or medium with 1.0 μM perampanel and incubated at 37˚C for 0, 6, 24 and 48 h. Then the cells were harvested using trypsin-EDTA solution and fixed in ice-cold 70% ethanol for 2 h. The fixed cells were treated with 0.5% RNase A (Roche Diagnostics GmbH) for 30 min and stained with 1.0 μg/ml propidium iodide (PI) solution (Miltenyi Biotech, Inc.) for 30 min at room temperature. Fluorescence was measured using a BD fluorescence activated cell sorter

(FACS)-Calibur Flow Cytometer (BD Biosciences) at a wavelength of 610 nm. The histograms were analyzed using FlowJo software (version 10.5.3. BioLegend, Inc.). The experiments were repeated at least four times.

Assessment of apoptosis. Apoptosis induced by perampanel in T98G and U-251MG malignant glioma cells was assessed using flow cytometry and western blotting.

Flow cytometry. Apoptosis was assessed by flow cytometry, with Annexin V/PI double staining. Cells were seeded in 6-well plates (Iwaki Cell Biology) at $1x10^6$ cells/well and incubated at 37˚C for 24 h. The culture medium was then replenished with fresh medium (control) or with medium containing perampanel (0.01, 0.1, 1 or 10 μM) at 37˚C for 48 h. The cells were then washed with phosphate-buffered saline (PBS) and collected using trypsin-EDTA solution. Following centrifugation (two times at 15,300 x g for 5 min at 4˚C) and washing in PBS, the solution was agitated with 100 μl of binding buffer (Wako Pure Chemical Industries, Ltd.), into which 5 μl of Annexin V-Alexa Fluor 488 conjugate (Thermo Fisher Scientific, Inc.) and 10 μl of PI solution (Miltenyi Biotech, Inc.) were added and incubated at room temperature for 10 min. An additional binding buffer was added to give a total sample volume of 500 μl. Fluorescence was measured using a BD FACS-Calibur Flow Cytometer (BD Biosciences). The apoptotic cells were analyzed using FlowJo software (version 10.5.3. BioLegend, Inc). The experiments were repeated at least four times to confirm reproducibility.

Western blotting. Western blotting was performed after treatment with 1.0 μM perampanel at 37˚C for 0, 4, 8 or 24 h. Proteins were isolated from cells lysed in RIPA buffer (Wako Pure Chemical Industries, Ltd.) supplemented with protease inhibitor complex mix (cOmplete, Mini, EDTA-free. Roche Diagnostics GmbH). The protein concentrations were determined using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). A total of 50 μg of protein per lane was separated by 12% SDS-PAGE (Tefco Co., Ltd.) and transferred onto nitrocellulose membranes (Cytiva) for 60 min at 15 V using Bio-Rad Trans-Blot transfer system (Bio-Rad Laboratories, Inc.). The membranes were blocked using 1% skimmed milk dissolved in washing buffer (PBS +0.2% Tween-20) for 60 min at room temperature, then incubated with primary antibodies at 4˚C overnight as follows: Anti-caspase-3 mouse monoclonal antibody (mAb; 1:500; cat. no. sc-7272. Santa Cruz biotechnology, Inc.) and anti-β-actin mouse mAb (1:200; cat. no. sc-47778; Santa Cruz biotechnology, Inc.), which was utilized as a loading control. The membranes were incubated for 60 min at room temperature with the HRP conjugated goat anti-rabbit IgG secondary antibody (1:100). The band patterns were analyzed using an ImageQuant LAS-4000 (Cytiva) after treatment with ECL Prime Western Blotting Detection Reagent (Cytiva). The experiments were repeated three times.

Enhanced effects of TMZ by perampanel. Whether a combination of TMZ and perampanel could produce an additive antitumor effect compared with TMZ-only treatment in malignant glioma cells was assessed. Glioma cells, A-172, AM-38, T98G, U-138MG, U-251MG and YH-13, were plated at $1x10^4$ cells/well in 24-well, flat-bottomed plates (Iwaki Cell Biology) and incubated with DMEM (Nissui Pharmaceutical) with 5% fetal calf serum (Thermo Fisher Scientific) at 37˚C for 24 h. Subsequently, the cells were incubated with medium containing perampanel (0.01, 0.1, 1, 10, 100 μM) with or without 10 μM TMZ. The TMZ concentration was chosen to represent a clinically relevant concentration of TMZ (24). After exposure to the various concentrations of perampanel with or without TMZ for 72 h, cells were detached by trypsinization and counted using a Coulter Counter Z1 (Beckman Coulter, Inc.). The experiments were repeated at least four times at each concentration of perampanel.

Enhancement of perampanel by inhibitor of SERPINE1. Overexpression of SERPINE1 is an important factor for tumor migration and invasion in numerous types of cancer and is considered to indicate poor prognosis (21,22). In a preliminary experiment (data not shown), western blotting demonstrated that the SERPINE1 protein expression level was high in U-138MG and A-172 cells that were resistant to perampanel. Furthermore, RNA-sequencing was performed by the Bioengineering Lab. Co., Ltd. which demonstrated that the *SERPINE1* mRNA expression level in U-138MG cells was ~100 times higher compared with that in T98G cells, which were highly sensitive to perampanel (data not shown). Both T98G and U-138MG cells expressed MGMT (23).

The combination of perampanel and tiplaxtinin, a selective and orally efficacious inhibitor of SERPINE1, to produce an additive antitumor effect compared with perampanel-only treatment was evaluated in malignant glioma cells. U-138MG cells were plated at $1x10^4$ cells/well in 24-well, flat-bottomed plates (Iwaki Cell Biology) and incubated with medium for 24 h, as for the aforementioned cell viability studies, and then incubated at 37˚C in medium with or without 1.0 μM perampanel and with or without 5.0 μM tiplaxtinin (MilliporeSigma). After incubation for 72 h, the cells were detached by trypsinization and counted using a Coulter Counter Z1 (Beckman Coulter, Inc.). The experiments were repeated at least nine times for each condition.

Statistical analysis. Unpaired Student's t-test was used to compare the data between two groups, and one-way ANOVA followed by the Tukey-Kramer post hoc test was used for multiple comparisons, using the SPSS (version 21.0; IBM Corp.). Data are presented as the mean ± SEM and were considered significantly different at $P<0.05$.

Results

Our previous study demonstrated, by reverse transcription PCR and western blotting, that O^6-methylguanine-DNA methyltransferase (MGMT), a key factor of alkylating agents, was expressed in T98G, U-138MG and YH-13 cells (23). Consistent with an earlier study (25), the present study also confirmed in our laboratory that T98G (M237I) and U-251MG (R273H) both had a point mutation in the *p53* gene (data not shown).

Antitumoral effects and cell sensitivity to perampanel in human malignant glioma cell lines. Fig. 1 demonstrates the inhibitory effects of perampanel on cell viability in a dose-dependent manner in all tumor cell lines, compared with

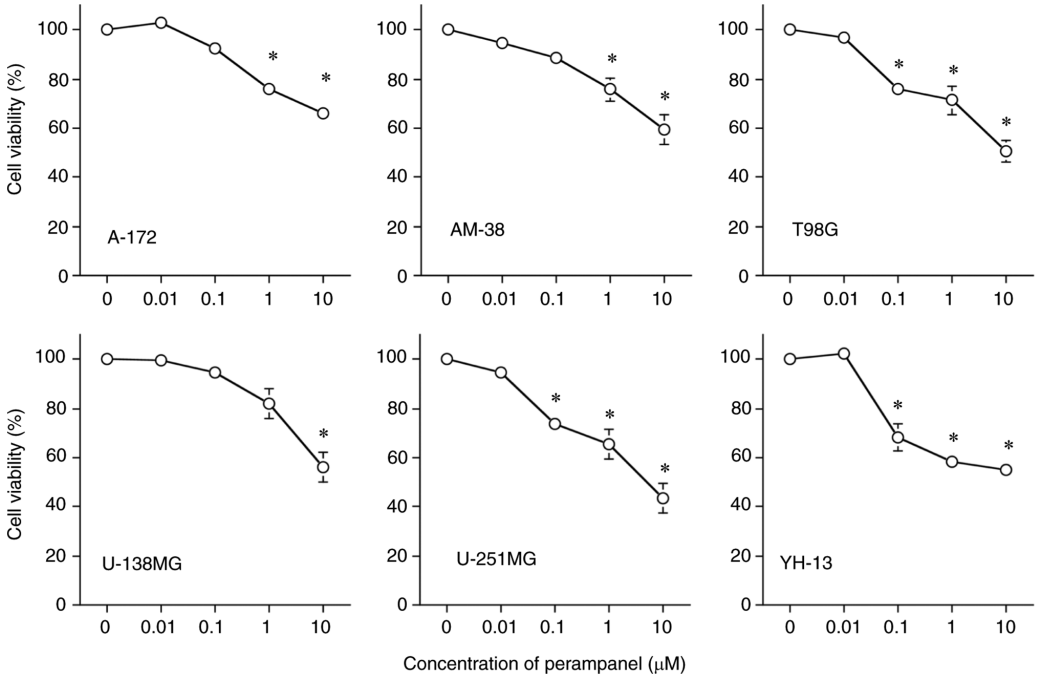

Figure 1. Inhibitory effect of perampanel on cell viability in six human glioma cell lines. Perampanel exhibited inhibitory effects on cell viability in a dose-dependent manner against all cell lines at 72 h. Significantly reduced cell numbers were observed in five cell lines, all except U-138MG, from treatment with 1.0 μM perampanel, which is the blood concentration achieved by oral administration as an antiepileptic drug. The IC_{50} of perampanel was ≤10 μM for U-251MG and >10 μM for the other five cell lines. Data are presented as the mean ± SEM. *P<0.05 vs. 0 μM.

the respective untreated control. However, the sensitivity of the cell lines to perampanel treatment varied, U-251MG was the most sensitive (lowest IC_{50} for perampanel among the 6 cell lines) and U-138MG was the least sensitive to perampanel. The IC_{50} of perampanel for U-251MG was <10 μM. The IC_{50} of perampanel for T98G was slightly higher than 10 μM, while the IC_{50} for the other four cell lines, A-172, AM-38, U-138MG and YH-13, was higher than 10 μM. Cell viability was significantly inhibited in five cell lines, excluding U-138MG, compared to the control when treated with perampanel at a concentration of 1.0 μM, which is the blood concentration achieved by oral administration as an antiepileptic drug (17).

Cell cycle distribution analysis. DNA histograms and the proportions of T98G or U-251MG cells in each cell cycle phase are presented in Fig. 2. Consistent results were obtained from at least four repeats; however, statistically significant differences were not observed between the control and perampanel groups. No significant increase in the populations of G0/G1, S or G2/M phases were observed in the cells following 1.0 μM perampanel treatment compared with the untreated control at 6, 24 and 48 h, which indicated that the antitumor effect of 1.0 μM of perampanel may not be due to the accumulation of cells at specific cell cycle phases.

Detection of apoptosis using flow cytometry and protein expression. The results presented in Fig. 3 demonstrate that 10 μM perampanel treatment induced significant apoptosis after 48 h, compared with the untreated control, in both T98G and U-251MG cells. The proportion of apoptotic cells (Annexin V-positive: early-stage apoptosis; Annexin V/PI-positive: late-stage apoptosis) following 10 μM perampanel treatment (T98G, 12.41±1.66%; U-251MG, 10.99±0.78%) was significantly higher compared with the proportion without treatment in both T98G and U-251MG cells (T98G, 5.03±0.44%; U-251MG, 4.16±0.41%). These findings did not contradict previously reported results (20).

Western blotting was used to evaluate the protein expression levels of caspase-3, an effector caspase related to apoptosis. Fig. 4 demonstrated that the protein expression level of cleaved caspase-3 was markedly greater after treatment with 1.0 μM perampanel for 24 h compared with the control in T98G and U-251MG cells.

Antitumoral effects of combined perampanel and TMZ. Fig. 5 presents the results of combined treatment with perampanel and TMZ, which demonstrated further significant inhibition of cell viability compared with the same concentration of perampanel without TMZ co-treatment, in the A-172, T98G and U-251MG cell lines at ≥10 μM perampanel (a clinically relevant concentration). Significant inhibitory effects on cell viability were observed in the T98G and U-251MG cell lines co-treated with 1.0 μM perampanel with TMZ compared with 1.0 μM perampanel alone, which is achieved clinically by oral administration. Conversely, combined treatment with perampanel and TMZ demonstrated no further cell viability inhibitory effect in the AM-38, U-138MG and YH-13 cell lines.

Antitumoral effects of combined perampanel and tiplaxtinin. Cell viability was further inhibited following combined treatment for 72 h with 1.0 μM perampanel and 5.0 μM tiplaxtinin, a SERPINE1 inhibitor, compared with no treatment or either treatment alone (Fig. 6). However, no effect on viability was

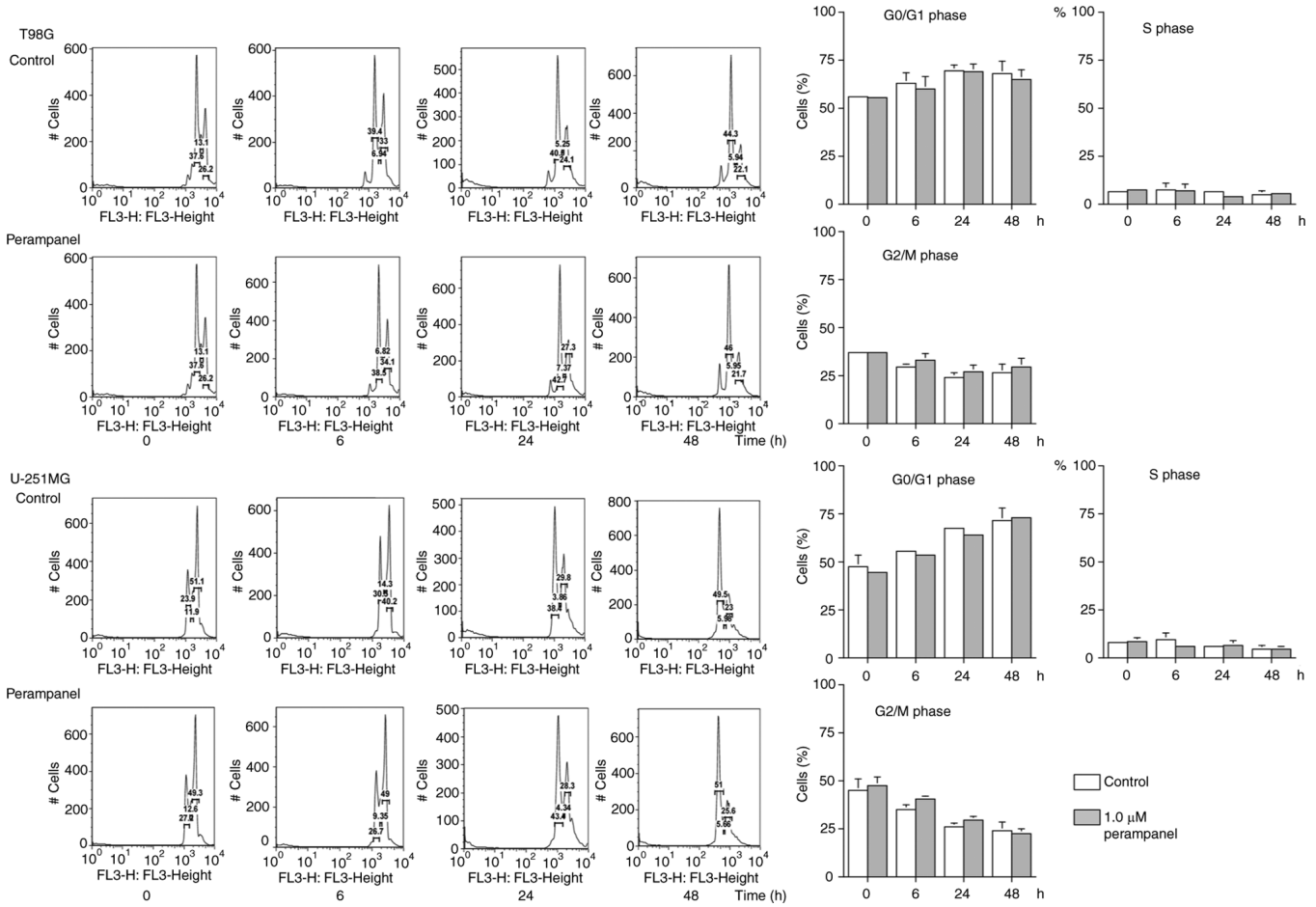

Figure 2. Cell cycle distribution analysis of T98G and U-251MG cells treated with perampanel. Untreated cells without perampanel served as the control. Fluorescence activated cell sorter data did not demonstrate any significant increase in the populations of cells in the G0/G1, S or G2/M phases following 1.0 μM perampanel treatment for 6, 24 and 48 h compared with the respective control. The results are presented as the mean ± SEM.

observed with only 1.0 μM perampanel or 5.0 μM tiplaxtinin alone compared with the untreated control in the perampanel-resistant U-138MG cell line.

Discussion

The present study demonstrated that the AMPA receptor non-competitive antagonist perampanel, an antiepileptic drug, had a dose-dependent inhibitory effect on cell viability in the six human malignant glioma cell lines examined. The clinical blood concentration levels of perampanel administered as an antiepileptic drug were reported to be 0.84 μM at 4 mg dosage and 1.48 μM at 8 mg dosage (17). In the present study, 5 of 6 cell liens exhibited significantly reduced cell viability with 1.0 μM perampanel treatment; the IC_{50} of perampanel was ≤10 μM in the U-251MG cell line. Therefore, perampanel may have an antitumor effect on malignant glioma in certain cases, even if only given at antiepileptic drug dosages.0

In the present study, the antitumoral effects of perampanel in malignant glioma were further evaluated by assessing the effects on the cell cycle and the induction of apoptosis. No significant change was observed in the proportions of cells in the G0/G1, S and G2/M phases under 1.0 μM perampanel treatment in T98G and U-251MG cells, despite observing the inhibitory effect of perampanel on cell viability in this study. The protein expression levels of the apoptosis-related protein caspase-3 demonstrated the induction of apoptosis in both glioma cell lines under 10 μM perampanel treatment. Conversely, Lange *et al* (15) reported that the antitumor effect of perampanel was not due to cell cycle-related viability inhibition or apoptosis induction in malignant glioma cell lines established from patients. In their study, caspase activation analysis indicated no induction of apoptosis even using 30 and 100 μM perampanel. Therefore, it was concluded that the ultimate antitumor effect of perampanel was due to a decrease in cell metabolism (15). Salmaggi *et al* (20) reported that cell proliferation was suppressed by perampanel owing to induction of apoptosis in four types of malignant glioma cell lines (U87, U138, A172 and SW1783) and also indicated negative results for the cell cycle-related effects of perampanel, but reported that perampanel induced apoptosis. These differences in apoptosis findings may have been due to different cell lines and measurement methods (20). Furthermore, both studies used perampanel at higher concentrations than in the present study, so a different mechanism of action from the present antitumor effect may have been involved. These results indicated that the antitumor effect of perampanel may vary between cell lines, but the differences in susceptibility and

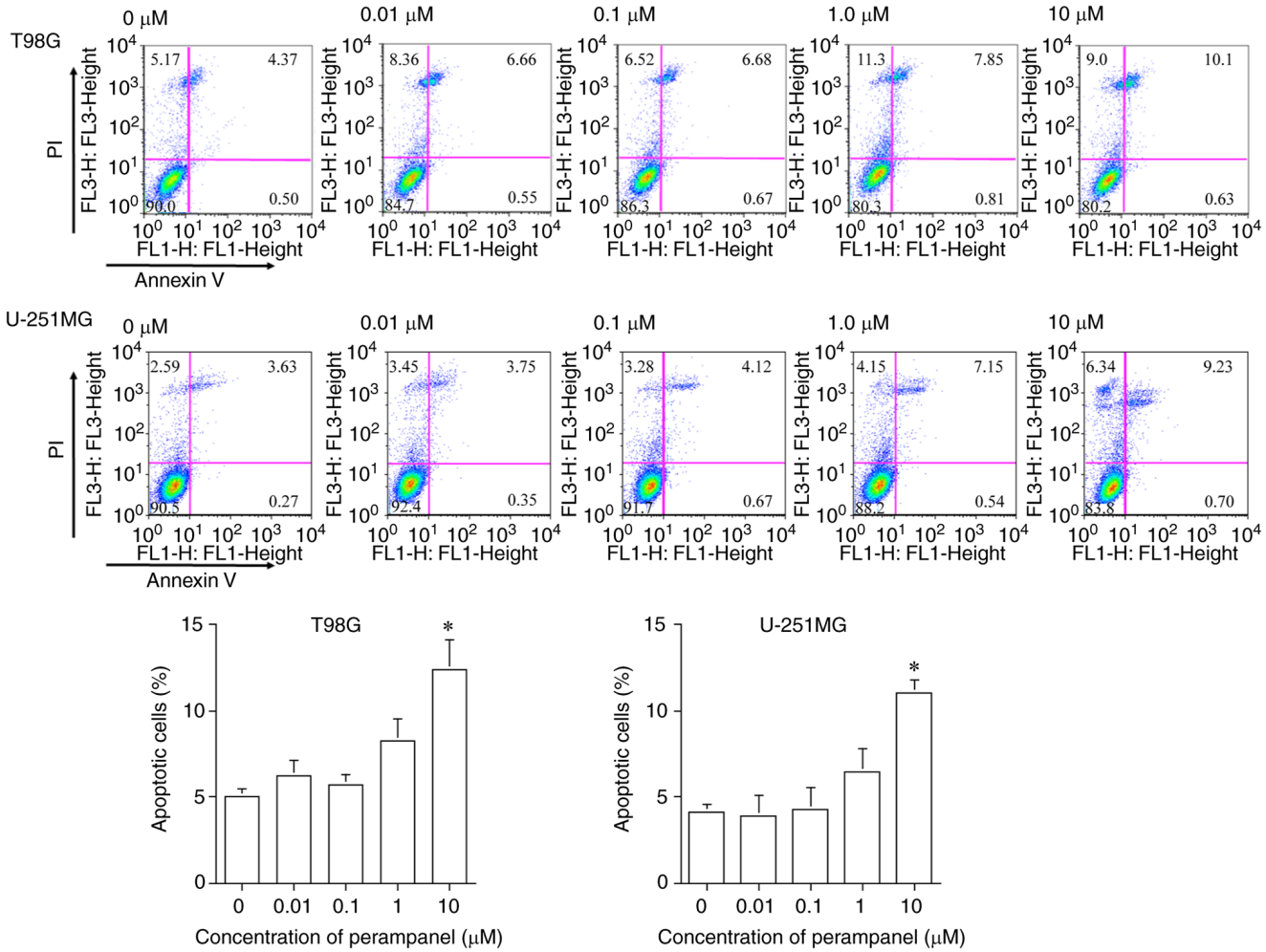

Figure 3. Induction of apoptosis by perampanel in T98G and U-251MG cells assessed using a fluorescence activated cell sorter. Proportions of apoptotic cells (Annexin V-Alexa Fluor 488-positive, early-stage apoptosis; Annexin V-Alexa Fluor 488-positive/PI-positive, late-stage apoptosis) were increased after 48 h of treatment with 10 μM perampanel. The results are presented as the mean ± SEM. *P<0.05 vs. 0 μM. PI, propidium iodide.

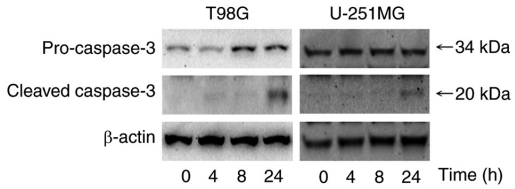

Figure 4. Western blotting of apoptosis-associated proteins in T98G and U-251MG cells. The protein expression levels of cleaved caspase-3 were markedly greater after treatment with 1.0 μM perampanel for 24 h compared to the control in both T98G and U-251MG glioma cells.

mechanism of action need further investigation. Assessment of the expression of cleaved caspase-8 (initiator caspase in the extrinsic apoptotic pathway), caspase-9 (initiator caspase in the intrinsic mitochondrial pathway) and PARP are required to further elucidate this mechanism of action; however, in the present study, only the protein expression levels of the effector caspase, caspase-3/cleaved caspase-3, was assessed using western blotting. Furthermore, it is also important to evaluate the anti-tumor effects of perampanel on glioma cells in the presence of caspase inhibitors such as Z-VAD-FMK.

The antitumor effect of perampanel combined with TMZ, a standard chemotherapeutic drug for malignant glioma, was also assessed. Certain cell lines demonstrated significantly increased inhibition of cell viability when treated with ≤10 μM perampanel and 10 μM TMZ (a clinically relevant concentration) compared with perampanel alone; therefore, perampanel given at antiepileptic drug dosages in addition to TMZ may have further antitumoral effects in some patients. The mechanism of the combined antitumor effect of TMZ and perampanel remains unknown, but further effects were demonstrated in two (T98G and U-251MG) of the three cell lines in which perampanel demonstrated a good antitumor effect. A previous study, using different doses and regimens from the present study, reported synergistic antitumoral effects of a combination of perampanel and TMZ in three cell lines (U87, U138 and A172) (20). A172 cells were reported to be the most sensitive, and this cell line also demonstrated statistically significant effects from the co-treatment with 10 μM perampanel and 10 μM TMZ in the present study. MGMT is expressed in 45-75% of malignant gliomas (26). MGMT expression is strongly involved in the sensitivity of cells to TMZ; T98G cells express MGMT and are resistant to TMZ (23). However, in the present study, the co-treatment of

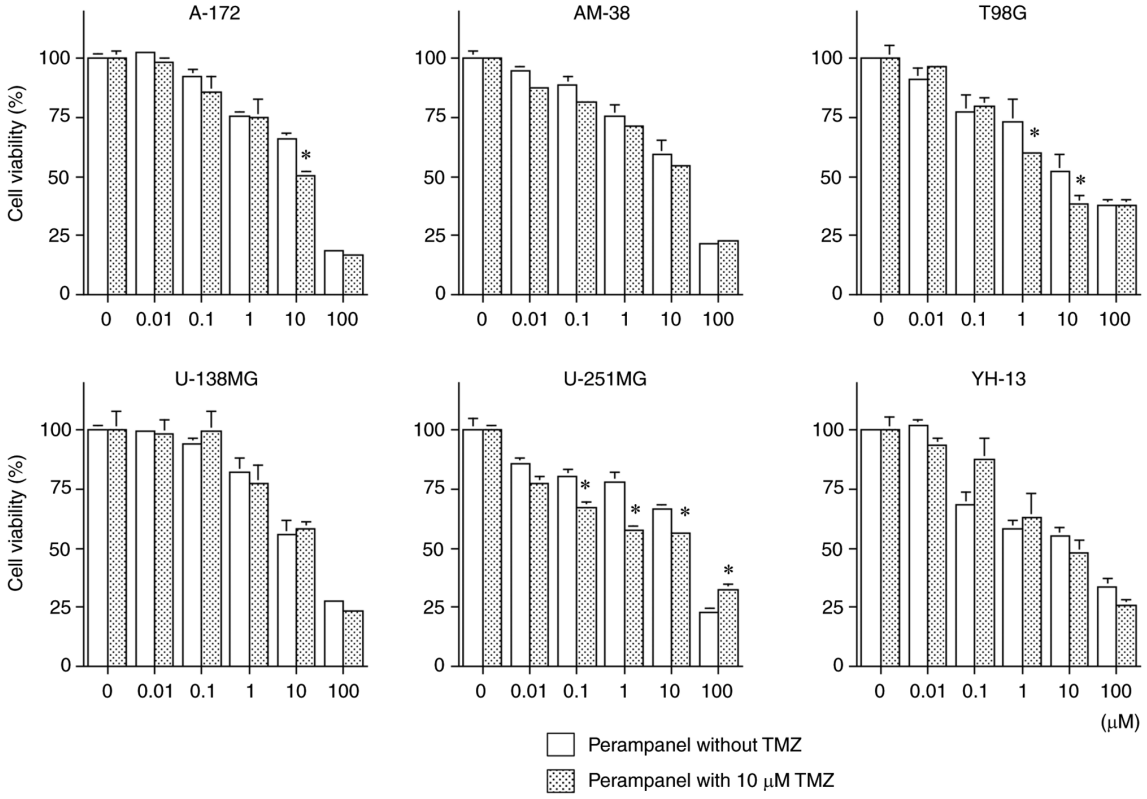

Figure 5. Inhibitory effect of a combination of perampanel and TMZ on cell viability in six glioma cell lines. Cells were cultured in media containing perampanel (0, 0.01, 0.1, 1, 10 and 100 μM) with or without 10 μM TMZ for 72 h. The combination of TMZ and perampanel demonstrated significant additive inhibitory effects on cell viability in the A-172, T98G and U-251MG cell lines, but not in the AM-38, U-138MG and YH-13 cell lines. Data are presented as the mean ± SEM. *P<0.05 vs. respective perampanel without TMZ. TMZ, temozolomide.

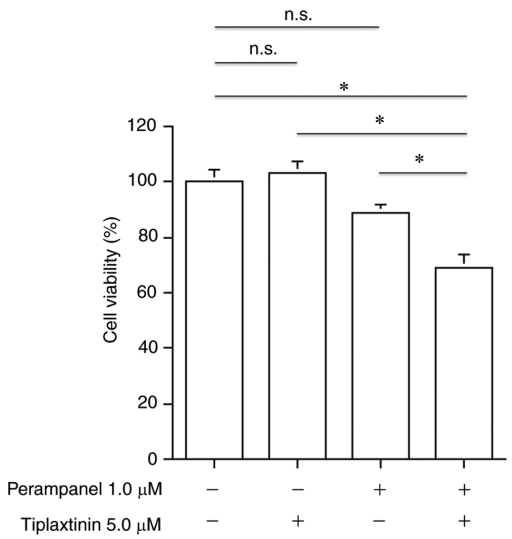

Figure 6. Inhibitory effect of a combination of perampanel and tiplaxtinin on cell viability in U-138MG cells. U-138MG cells were exposed to media containing 1.0 μM perampanel, 5.0 μM tiplaxtinin (an inhibitor of SERPINE1) or both perampanel and tiplaxtinin for 72 h. The combination of perampanel and tiplaxtinin demonstrated a significant additive inhibitory effect on cell viability, whereas perampanel or tiplaxtinin treatment alone demonstrated no inhibitory effect on viability. Data are presented as the mean ± SEM. *P<0.05. n.s., not significant.

perampanel with TMZ enhanced the antitumor effect against T98G cells. Further research is needed, but the results of the present study indicated that perampanel may exert antiepileptic as well as antitumor effect, which would be beneficial clinically.

Preliminary RNA-sequencing experiments demonstrated ~100-fold higher mRNA expression levels of *SERPINE1* in U-138MG, with low sensitivity to perampanel, compared with T98G which demonstrated high sensitivity. *SERPINE1* expression was reported to be correlated with worse prognosis in patients with malignant glioma and knockdown of *SERPINE1* in malignant glioma cells was reported to have suppressed tumor growth and invasion (21). Therefore, in the present study, how the sensitivity of perampanel changed in perampanel-resistant cells as SERPINE1 was suppressed by tiplaxtinin, a selective inhibitor of SERPINE1, was evaluated. Significantly decreased cell viability was observed when cells were treated with a combination of perampanel and tiplaxtinin compared with perampanel or tiplaxtinin alone in the U-138MG cell line. Therefore, the antitumoral effect of perampanel may not occur in malignant gliomas with high protein expression levels of SERPINE1. The present study did not assess how the action of SERPNE1 was involved in susceptibility to the perampanel, but increased understanding of this may lead to improved treatment of malignant glioma.

In the present study the antitumoral effect of perampanel on glioblastoma cell lines was assessed. The results did not examine the relationship between malignant glioma and related epilepsy, which is also an important therapeutic challenge. Therefore, the study of malignant glioma and associated

epilepsy is crucial for malignant glioma patients and requires further study in the future.

The present study demonstrated marked differences in susceptibility to perampanel among glioma cells, but the results suggested that perampanel, an anticonvulsant, exhibited antitumoral effects, such as cell viability inhibition and apoptosis induction, which were greater when used in combination with TMZ in some malignant glioma cells. The results also suggested that *SERPINE1* expression may be involved in perampanel susceptibility. These results may lead to a new therapeutic strategy for malignant glioma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JT and ES developed the experimental design, performed experiments and data analysis, and drafted the manuscript. YH, CY, SY, KS, KT, KK and SKA were involved in the conception and design of the study, performed experiments, analyzed the data and drafted the manuscript. HH and YK supervised the study including the development of the experimental design and proofread the manuscript. AY contributed to the experimental design, data analysis and writing of the manuscript. JT, ES and AY confirm the authenticity of all raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent to participate

Not applicable.

Competing interests

AY, the corresponding author, has received research funds for other research projects from Medtronic Japan Co., Ltd. and Eisai Co., Ltd. Note that Eisai Co., Ltd. provided the antiepileptic agent perampanel used in the present study. AY has also, in accordance with the rules, reported any competing interests (including a small amount of research funds for other research projects) to his main academic society, The Japan Neurosurgical Society.

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