Anti-tumor effects of anti-epileptic drugs in malignant glioma cells

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Abstract. Patients with glioblastoma frequently suffer epileptic seizures and often require anticonvulsant therapy during the treatment course. The present study investigated four common antiepileptic drugs, perampanel, carbamazepine (CBZ), sodium valproate (VPA) and levetiracetam (LEV), which are expected to have antitumor effects, and determined the most beneficial drug for the treatment of malignant glioma by comparing antitumor effects such as inhibition of cell proliferation and suppression of migration and invasion (using Transwell assays). The inhibition of cell growth was investigated using six malignant glioma cell lines (A-172, AM-38, T98G, U-138MG, U-251MG and YH-13). Significant inhibition of cell proliferation was observed in all six cell lines treated with perampanel, three cell lines treated with CBZ, four cell lines treated with VPA and two cell lines treated with LEV at the therapeutic blood concentration levels for the drugs to be used as antiepileptics. Further antitumor effects in combination with temozolomide were investigated using T98G and U-251MG cell lines, and were confirmed in both cell lines with perampanel and in T98G cells with LEV, but not observed with CBZ and VPA. Cell migration was significantly suppressed in both T98G and U-251MG cell lines with perampanel, but not with CBZ, VPA or LEV. To investigate the mechanisms by which perampanel suppresses the migration of malignant glioma cells, the expression of mRNA related to epithelial-mesenchymal transition following perampanel treatment was analyzed using reverse transcription-quantitative PCR in the T98G and U-251MG cell lines. The expression of *Rac1* and *RhoA*, which constitute the cytoskeleton that enhances cell motility, were reduced in both cell lines. Furthermore, the expression of the mesenchymal marker *N-cadherin*, which promotes cell migration and infiltration, was decreased, but the expression of the epithelial marker *E-cadherin*, which strengthens cell-cell adhesion and reduces cell motility, was increased. Furthermore, the expression of *matrix metalloproteinase-2*, a proteolytic enzyme, was reduced. These effects may reduce cell motility and increase adhesion between cells, suggesting that perampanel treatment suppressed cell migration. In conclusion, the present study suggests that perampanel may be more beneficial in terms of antitumor efficacy than other antiepileptic drugs for the treatment of malignant glioma.

Introduction

Epileptic seizures are the first symptom in 14% of patients with glioblastoma (1) and 40-60% of patients eventually suffer epileptic seizures even if none were observed at the time of diagnosis (2). Prevention of epileptic seizures is almost as important as the treatment of the glioblastoma and numerous patients receive antiepileptic therapy in actual clinical practice. Consequently, an agent that is able to prevent symptomatic epilepsy and exert antitumor effects may be beneficial to patients with glioblastoma.

Antiepileptic drugs reported to have antitumor effects include sodium valproate (VPA), carbamazepine (CBZ), levetiracetam (LEV), talampanel and perampanel, which achieve anticonvulsant effects through various mechanisms of action. In particular, VPA and LEV were indicated to improve overall survival in patients with glioblastoma (3-5), but no survival benefit was subsequently reported (6). Talampanel and perampanel are selective non-competitive antagonists of the *α-amino*-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)-type glutamate receptor and inhibit the influx of cations (Na^+, K^+) to suppress nerve excitement and exert antiepileptic activity (7).

Talampanel administration increased the overall survival time to 20.3 months compared to 14.6 months in the control group in a phase II clinical trial of primary glioblastoma (8). However, talampanel administered as a single agent achieved no significant prolongation of progression-free survival in a phase II study of recurrent glioblastoma (9).

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Investigation of the antitumor effects of perampanel, VPA, LEV and CBZ on glioblastoma cells indicated that only perampanel suppressed cell proliferation (10). The mechanism of action of perampanel depends on the reduction of cell metabolism caused by decreased glucose uptake (10). Perampanel exhibited a synergistic antitumor effect with temozolomide (TMZ), a standard chemotherapeutic agent for malignant glioma, on malignant glioma cell lines (11). Furthermore, perampanel suppressed cell proliferation by induction of apoptosis, but the synergistic effect with TMZ was not observed in all cell lines (11). However, the perampanel concentration used in those studies was 100 and 250 μM, respectively (10,11), which was much higher than the blood concentration of 1.48 μM achieved by administration of the perampanel maintenance dose of 8 mg (12).

A recent study by our group indicated that perampanel has a dose-dependent antitumor effect on malignant glioma cell lines, and induces apoptosis and inhibits cell proliferation in combination with TMZ at clinical blood concentrations as an antiepileptic drug (13). Clearly, the antitumor effect of perampanel on malignant glioma apparently depends on inhibition of cell proliferation, whereas any effects on cell migration or invasion have remained undetermined. Cell migration is the process of movement of cells from one location to another. Cell invasion implies that the cells denature the extracellular matrix (ECM) and settle in another location, which requires the secretion of proteolytic enzymes such as matrix metalloproteinases (MMPs) that denature the adjacent ECM (14).

Various signaling pathways are involved in cell migration and invasion of glioblastoma. Ca^{2+}-permeable AMPA receptors expressed in glioblastoma cells increase intracellular Ca^{2+} levels, allowing both tumor cell proliferation and migration through cascades such as phosphatidylinositol 3-kinase (PI3K)/Akt (15,16). In addition, activation of the AMPA receptors increases the expression of integrins and transmembrane receptors and enhances the focal adhesion kinase/steroid receptor coactivator (FAK/Src) pathway (17). These signals enhance the activity of molecules of the Rho family small GTP-binding protein, rac family small GTPase 1 (Rac1), cell division cycle 42 (Cdc42) and ras homolog family member A (RhoA), which are involved in the transformation of the cytoskeleton (18). On the other hand, the epithelial-mesenchymal transition (EMT) process is also important for the migration and invasion of glioblastoma cells. EMT-related molecules include E-cadherin and N-cadherin (19,20). E-cadherin is an epithelial marker and increases in its expression result in enhanced cell-cell adhesion and reduced cell motility. By contrast, N-cadherin is a mesenchymal marker and increased expression weakens cell-cell binding, facilitating cell separation and enhancing motility. EMT is induced by signaling pathways such as FAK/Src and PI3K/Akt. Glioblastoma cells, which exhibit mesenchymal morphology and migrate, secrete MMPs, destroy ECM and create pathways for migration (18,21). However, the mechanisms of cell migration and cell invasion remain to be fully elucidated due to the complex involvement of numerous factors in intracellular signaling pathways.

The present study compared the antitumor effects of perampanel, CBZ, VPA and LEV, which are commonly used antiepileptic drugs with different mechanisms of action, at therapeutic blood levels on the proliferation, migration and infiltration of malignant glioma cell lines. Changes in the expression of genes that affect cell migration and infiltration were evaluated using reverse transcription-quantitative (RT-q) PCR. Furthermore, the combination of these antiepileptic drugs with TMZ was also investigated to examine whether they exhibit any synergistic antitumor effect.

Materials and methods

Cell lines, culture conditions and materials. The 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 the Health Science Research Resources Bank of Japan. U-138MG (cat. no. HTB-16; lot no. 1104428) was purchased from the American Type Culture Collection. A previous study by our group confirmed that O^6-methylguanine-DNA methyltransferase (MGMT), a key factor of alkylating agents, was expressed in the T98G, U-138MG and YH-13 cell lines via RT-qPCR and western blot analyses (22). Consistent with an earlier study (23), it was also confirmed that the T98G (237 Met→Ile) and U-251MG (273 Arg→His) cell lines have a point mutation in the *TP53* gene (data not shown).

Cells were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical) supplemented with 10% fetal calf serum (Thermo Fisher Scientific, Inc.) using plastic culture flasks (Corning, Inc.) in a 37˚C incubator in a humidified (>95%) atmosphere containing 5% CO_2.

The antiepileptic drugs perampanel (kindly gifted by Eisai Co., Ltd.), CBZ (Tokyo Chemical Industry), VPA (Tokyo Chemical Industry) and LEV (Tokyo Chemical Industry) and the anti-cancer agent TMZ (Tokyo Chemical Industry) were employed for this study.

Cell culture growth studies. The growth inhibitory effects of the four antiepileptic drugs (perampanel, CBZ, VPA and LEV) on malignant glioma cells were evaluated by quantifying the numbers of cells. In brief, cells were seeded in 24-well, flat-bottomed plates (Iwaki) at $1x10^4$ cells/well and incubated with medium for 24 h. The cells were subsequently washed twice with medium and further incubated with fresh medium (control) or medium containing perampanel (0.01, 0.1, 1.0 and 10 μM), CBZ (0.5, 5.0, 50 and 500 μM), VPA (1.0, 10, 100 and 1,000 μM) and LEV (1.0, 10, 100 and 1,000 μM). After exposure to the various concentrations of antiepileptic drugs 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 12 times at each concentration. For certain experiments regarding the effect of perampanel on cell growth, certain experimental repeats included the research results of another study of our group by Tatsuoka *et al* (13).

The molecular weights of perampanel, CBZ, VPA and LEV are 349.4, 236.3, 166.2 and 170.2 g/mol; the maintenance doses are 8-12, 400-1,200, 400-1,200 and 1,000-3,000 mg; and the therapeutic ranges of blood concentrations are 0.14-1.14, 17-50, 300-600 and 70-270 μM, respectively (24). The treatment dose of each anticonvulsant was determined using this information.

Since T98G and U-251MG cells display a marked antitumor response after being treated with a small amount of perampanel (13) and are widely used in brain tumor experiments, these cells were employed in the subsequent experiments to investigate the effects of perampanel combined with TMZ on cell migration and invasion. The 50% inhibitory concentration (IC_{50}) for perampanel on U-251MG cells was close to the blood concentration used for antiepileptic therapy (see Results section). In addition, as mentioned above, T98G cells expressed MGMT, but U-251MG cells did not.

Enhanced effects of TMZ and antiepileptic drugs. The additive antitumor effect of the combination of TMZ and antiepileptic drugs compared to TMZ alone in malignant glioma cells was assessed. T98G and U-251MG cells were plated at $1x10^4$ cells/well in 24-well, flat-bottom plates and incubated with medium for 24 h. Subsequently, they were incubated with medium containing various concentrations of perampanel (0-10 μM), CBZ (0-500 μM), VPA (0-1,000 μM), and LEV (0-1,000 μM) with or without TMZ (10 μM). The TMZ concentration (10 μM) was chosen as a representative of a clinically relevant concentration (25). After exposure to the various concentrations of antiepileptic drugs with/without TMZ for 72 h, cells were detached using 0.25% Trypsin-EDTA solution (Invitrogen; Thermo Fisher Scientific, Inc.) and counted. The experiments were repeated at each concentration in at least four independent systems and cell numbers were quantified at least four times in total. Certain experiments regarding the effect of a combination perampanel with TMZ on cell growth were included; those experiments were reported in part (but not in detail) by co-author Tatsuoka *et al* (13).

Inhibitory effect of antiepileptic drugs on cell migration and cell infiltration. Cell migration was evaluated in the T98G and U-251MG cell lines using the CytoSelect 24-well Cell Migration Assay (Cell Biolabs, Inc.). Among the cells seeded in the upper chamber filled with serum-free medium, only migrating cells pass through the membrane with pores and move to the lower chamber side filled with serum-containing medium. Cell migration may be evaluated by quantifying the migrated cells. The cell migration study was performed using the following method: T98G or U-251MG cells, adjusted to $1x10^5$ cells/300 μl/well in serum-free medium, were seeded in the upper chamber equipped with the polycarbonate membrane with 8-μm pores. The antiepileptic drug concentration was set to the blood concentration level within the maximum therapeutic range of 1.0 μM for perampanel, 50 μM for CBZ, 600 μM for VPA and 270 μM for LEV. Medium containing 10% calf serum was placed in the lower chamber. The following was performed according to the manufacturer's instructions for the Cell Migration Assay (Cell Biolabs, Inc.). After culturing for 24 h, the non-migrating cells remaining in the upper chamber were wiped off with a cotton swab and the migrating cells that had passed through the pores of the upper chamber and moved to the bottom surface of the filter were treated with a cell stain solution. After drying, the stain was extracted with extraction solution and measured for absorbance at 560 nm using a microplate reader (Model 680; Bio-Rad Laboratories, Inc.). The experiments were repeated for each antiepileptic drug in at least five independent systems and quantified at least five times in total.

Cell invasion was evaluated using the CytoSelect 24-well Cell Invasion Assay (Cell Biolabs, Inc.). Unlike that in the Transwell assay used in the cell migration experiment, the polycarbonate membrane was coated with an extracellular matrix layer. This allows the cell invasion ability to be measured if extracellular matrix degradation occurs. Apart from this, the same procedure as that for the cell migration experiment was used.

mRNA expression analysis of genes involved in cell migration and infiltration using RT-qPCR. Perampanel was observed to cause significant suppression of cell migration and tended to suppress cell invasion. Therefore, the changes in mRNA expression levels after perampanel treatment were evaluated using RT-qPCR to identify factors related to the induction of EMT, which is an important process for cell migration and infiltration. Cells were cultured in 75 cm^2 flasks, treated with 1.0 μM of perampanel for 4 h, and total RNA was extracted from $1x10^6$ cells by employing the RNeasy Mini kit (Qiagen, Inc.). After determination of the RNA contents with a NanoDrop (Thermo Fisher Scientific, Inc.), mRNA expression levels were analyzed using the Step-One Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with RNA-direct SYBR Green Realtime PCR Master Mix (Toyobo Life Science) according to the manufacturer's instructions. The primer sequences for each gene are presented in Table I. In the Step-One Real time PCR system, real-time PCR assay, RT and PCR amplification were performed in the same reaction tube. The total reaction volume of 20 μl containing 0.8 μg of RNA, 2.0 μl each of forward and reverse primers (10 pmol), 1.0 μl of 50 mM $Mn(OAc)_2$, 10 μl of RNA-direct SYBR Green Realtime PCR Master Mix (Toyobo Life Science) and RNase-free water. The thermocycling conditions were as follows: 1st stage, 95˚C for 30 sec, 61˚C for 20 min, and 95˚C for 1 min; 2nd stage, 45 cycles at 95˚C for 15 sec, 55˚C for 15 sec, and 74˚C for 45 sec; and 3rd stage, 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec. GAPDH mRNA expression levels were employed as the quantitative internal control. The expression levels were calculated employing the following equations by comparing the threshold cycle (Cq): ΔCq=Cq of β1 integrin, FAK, Src, PI3K, Akt, Rac1, RhoA, Cdc42, MMP-2, E-cadherin or N-cadherin, -Cq of GAPDH, ΔΔCq (target cell line)-ΔCq (reference cell line), and ratio=$2^{-\Delta\Delta Cq}$ (26). The experiments were repeated three times for each condition.

Statistical analysis. Values are expressed as the mean ± standard error of the mean. Student's t-test (unpaired) was used to compare the data between two groups and one-way analysis of variance followed by the Tukey-Kramer method was used to compare three or more groups using the software SPSS (v.21.0; IBM Corporation). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Antitumor effect of antiepileptic drugs in human malignant glioma cells. Fig. 1 indicates that the cell growth inhibitory effects of perampanel were dose-dependent in all tumor cell lines. However, the sensitivity of the cell lines varied, as also suggested by the previous study (13). The therapeutic blood concentration

Table I. Primer sets.

Gene/ direction	Sequence
GAPDH	
Forward	5'-CAGAACATCATCCCTGCCTCT-3'
Reverse	5'-GCTTGACAAAGTGGTCGTTGAG-3'
β1 integrin	
Forward	5'-AATGGGAACAACGAGGTCATGGTT-3'
Reverse	5'-TTGTGGGATTTGCACGGGCAGTAC-3'
FAK	
Forward	5'-GGTGCAATGGAGCGAGTATT-3'
Reverse	5'-GCCAGTGAACCTCCTCTGA-3'
Src	
Forward	5'-GGGTAGCAACAAGAGCAA-3'
Reverse	5'-GAGTTGAAGCCTCCGAACAG-3'
PI3K	
Forward	5'-CCCTGCTCATCAACTAGGAAACC-3'
Reverse	5'-TTGCCGTAAATCATCCCCATT-3'
Akt	
Forward	5'-TGCCCTTCTACAACCAGGAC-3'
Reverse	5'-ACACGATACCGGCAAAGAAG-3'
Rac1	
Forward	5'-CTGCCAATGATATGGTAGATG-3'
Reverse	5'-CCGCACCTCAGGATACCA-3'
RhoA	
Forward	5'-TCAAGCCGGAGGTCAACAAC-3'
Reverse	5'-ACGAGCTGCCCATAGCAGAA-3'
Cdc42	
Forward	5'-GAAGGCTGTCAAGTATGTGG-3'
Reverse	5'-CTCTTCTTCGGTTCTGGAGG-3'
MMP-2	
Forward	5'-CCGTCGCCCATCATCAAGTTC-3'
Reverse	5'-GCAGCCATAGAAGGTGTTCAGG-3'
E-cadherin	
Forward	5'-ATTGCTCACATTTCCCAACTCC-3'
Reverse	5'-CTCTGTCACCTTCAGCCATCCT-3'
N-cadherin	
Forward	5'-TTTGATGGAGGTCTCCTAACACC-3'
Reverse	5'-ACGTTTAACACGTTGGAAATGTG-3'

FAK, focal adhesion kinase; PI3K, phosphatidylinositol 3-kinase; Rac1, rac family small GTPase 1; RhoA, ras homolog family member A; Cdc42, cell division cycle 42; MMP-2, matrix metalloproteinase-2.

range of perampanel as an antiepileptic drug is 8-12 μM (24). Only U-251MG cells had an IC_{50} of <10 μM for perampanel.

CBZ inhibited the cell proliferation in all six cell lines, but suppressed the cell proliferation only at a concentration of 500 μM in the A-172, AM-38 and YH-13 cell lines. CBZ exhibited a concentration-dependent cell proliferation inhibition in the T98G, U-138MG and U-251MG cell lines. The U-138MG cell line was particularly sensitive, with an IC_{50} of 25 μM. The therapeutic blood concentration range of CBZ as an antiepileptic drug is 17 to 50 μM (24). Only the U-138MG cell line had IC_{50} of 50 μM or less for CBZ (Fig. 2).

VPA inhibited cell proliferation in all cell lines except U-138MG. Growth inhibition was observed only at a concentration of 1,000 μM in the A-172 cell line, which is higher than the maximum therapeutic range of the blood concentration of 600 μM (24). The cell proliferation inhibition was concentration-dependent in the other four cell lines, with IC_{50} values below the therapeutic blood level range of 300-600 μM for VPA, including 91 μM in AM-38, 463 μM in T98G, 546 μM in U-251MG and 139 μM in YH-13 cells (Fig. 3).

LEV inhibited cell proliferation of the A-172, T98G, U-138MG and YH-13 cell lines, but had no statistically significant effect on the AM-38 and U-251MG cell lines. Cell proliferation inhibition was suppressed only at 1,000 μM in the A-172 and U-138MG cell lines, which is higher than the maximum therapeutic blood concentration of 270 μM for LEV. Cell proliferation was dependent on the concentration of LEV in the T98G and YH-13 cell lines (Fig. 4). The IC_{50} of each cell line exceeded the therapeutic blood concentration of 70 to 270 μM as an antiepileptic drug.

Cell proliferation inhibition by combined TMZ and antiepileptic drug. As presented in Fig. 5, perampanel in combination with 10 μM of TMZ produced a significantly enhanced cell proliferation inhibition compared with only TMZ from the concentration of 1.0 μM in T98G (1.0 μM of perampanel without TMZ vs. with TMZ: 73.08±9.77 vs. 59.89±1.75%; 10 μM of perampanel without TMZ vs. with TMZ: 51.92±7.76 vs. 38.55±3.57% compared to the control; $P<0.05$), and from the concentration of 0.1 μM in U-251MG cells (0.1 μM of perampanel without TMZ vs. with TMZ: 80.58±2.56 vs. 66.92±2.56%; 1.0 μM of perampanel without TMZ vs. with TMZ: 78.21±4.13 vs. 57.58±2.06%; and 10 μM of perampanel without TMZ vs. with TMZ: 66.50±1.97 vs. 56.6±1.59% compared to the control; $P<0.05$). These results, for perampanel in combination with TMZ, were partially reported (but not in detail) in the previous study by our group (13). Other than perampanel, the combination of 10 μM of LEV and 10 μM of TMZ had enhanced cell proliferation inhibition in the T98G cell line compared to only TMZ (10 μM of LEV without TMZ vs. with TMZ: 88.08±4.33 vs. 67.78±5.23% compared to the control, $P<0.05$).

Effect of antiepileptic drugs on cell migration and invasion. Fig. 6 demonstrates a significant suppression of cell migration ability in the perampanel-treated group (T98G: 89.11±3.29% and U-251MG: 74.17±7.98% compared to the control; $P<0.05$), but none in the other antiepileptic drug-treated groups.

All four antiepileptic drugs had a tendency to suppress cell invasion, but no significant difference was observed in the T98G cell line (Fig. 7). Perampanel and LEV tended to suppress cell invasion compared with the other antiepileptic drugs, but no significant difference was observed in the U-251MG cell line.

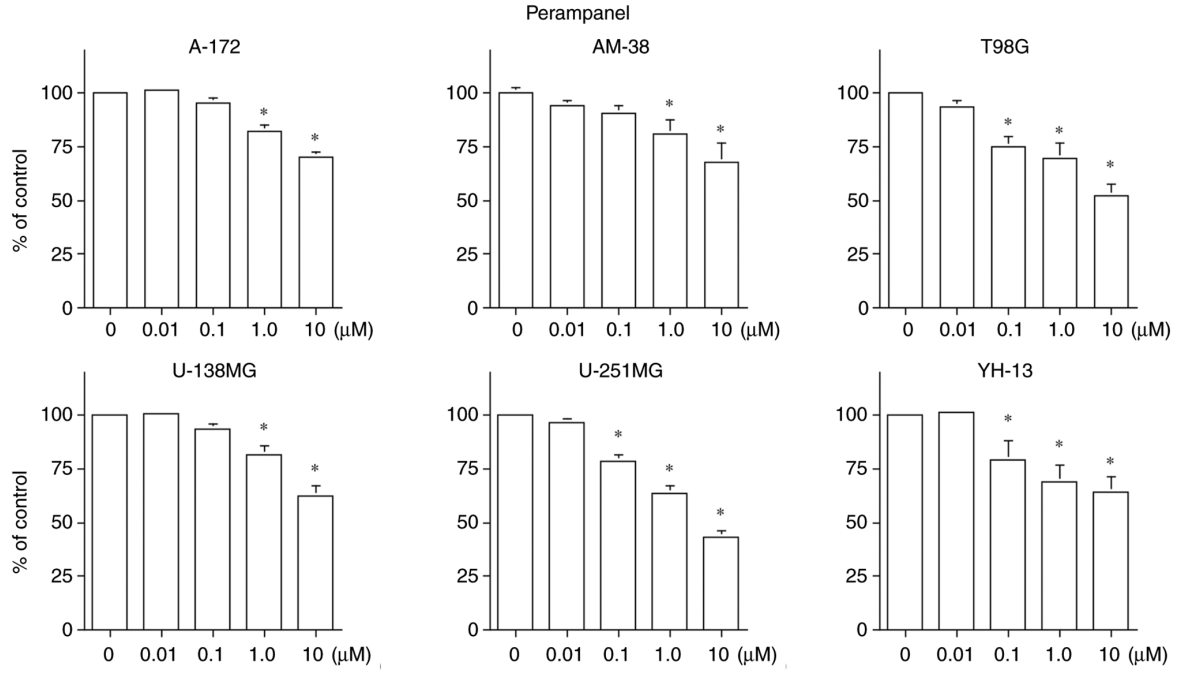

Figure 1. Antitumor effects of perampanel in six human glioma cell lines. Malignant glioma cells were treated with 0, 0.01, 0.1, 1.0 or 10 μM perampanel and cultured for 72 h. Similar results have been previously reported by co-author Tatsuoka *et al* (13). Values are expressed as the percentage (mean ± standard error) of the untreated control. *P<0.05.

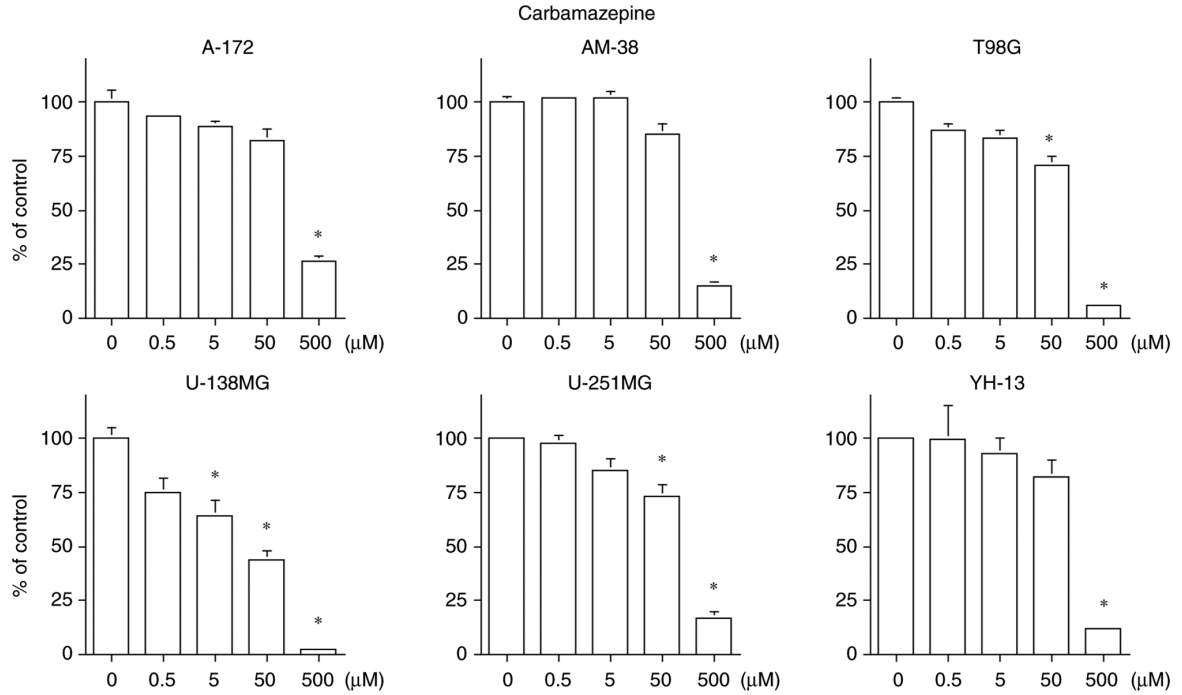

Figure 2. Cell proliferation inhibition using CBZ on six malignant glioma cell lines. Malignant glioma cells were treated with 0, 0.5, 5, 50 or 500 μM of CBZ, cultured for 72 h and the number of surviving cells at each concentration was counted. Values are expressed as the percentage (mean ± standard error) of the untreated control. *P<0.05. CBZ, carbamazepine.

Perampanel suppresses EMT of malignant glioma cells. No significant change was observed in the expression of *β1 integrin* after treatment with perampanel in both T98G and U-251MG cell lines, but downstream of the FAK/Src pathway, the expression of *Src* was decreased only in the T98G cell line (0.60±0.19 compared to the control; P<0.05). Further downstream, the expression of *Rac1* and *RhoA*, which reconstruct the cytoskeleton that enhance cell motility, was reduced in both T98G (*Rac1*: 0.80±0.03 and *RhoA*: 0.10±0.06 compared to the control; P<0.05) and U-251MG cells (*Rac1*: 0.20±0.39 and *RhoA*: 0.80±0.03 compared to the control; P<0.05), but *Cdc42* expression was unchanged in both cell lines (Fig. 8).

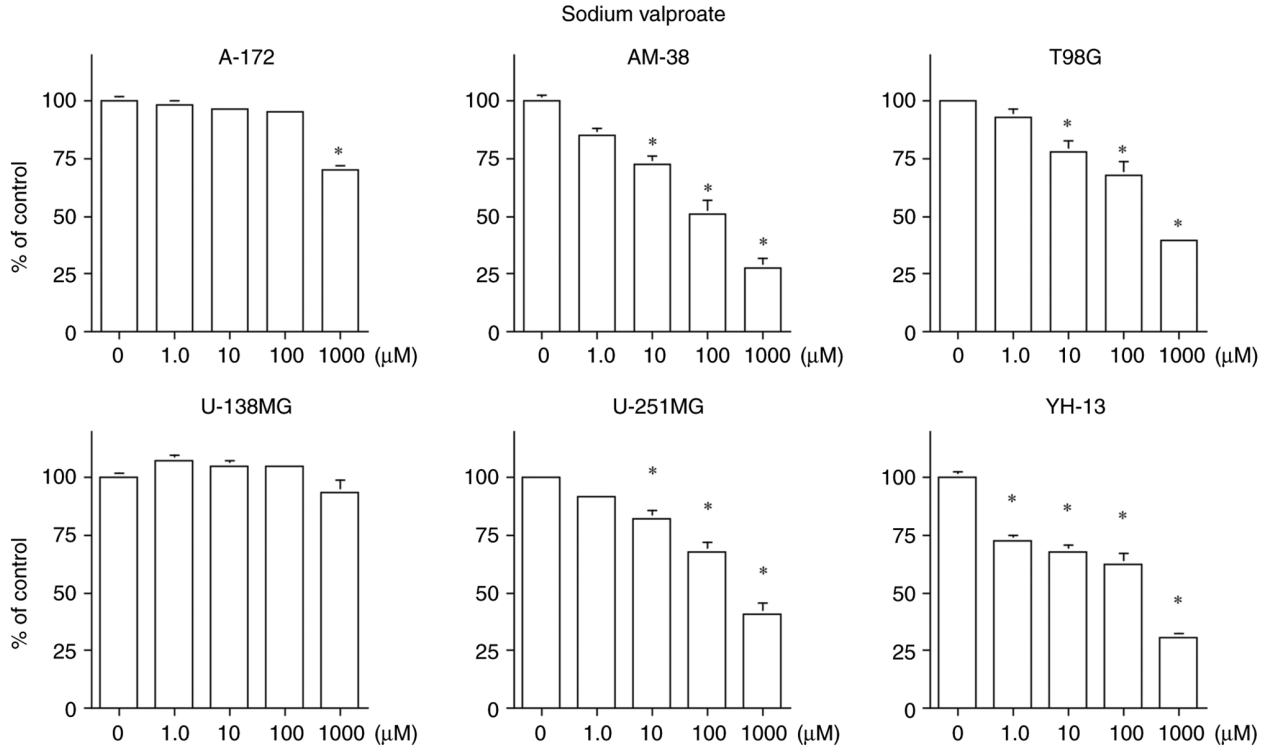

Figure 3. Cell proliferation inhibition using VPA on six malignant glioma cell lines. Malignant glioma cells were treated with 0, 1.0, 10, 100 or 1,000 μM of VPA, cultured for 72 h and the number of surviving cells at each concentration was counted. Values are expressed as the percentage (mean ± standard error) of the untreated control. *P<0.05. VPA, sodium valproate.

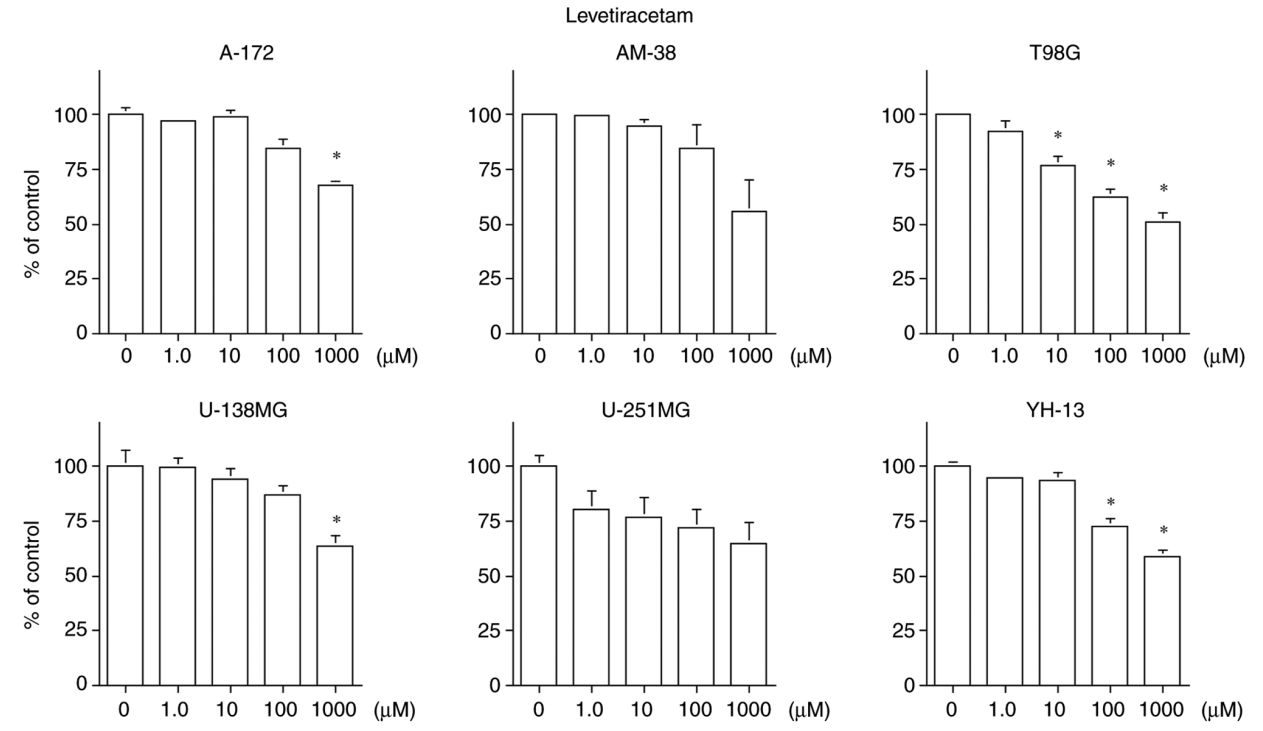

Figure 4. Cell proliferation inhibition using LEV on six malignant glioma cell lines. Malignant glioma cells were treated with 0, 1.0, 10, 100 or 1,000 μM of LEV, cultured for 72 h and the number of surviving cells at each concentration was counted. Values are expressed as the percentage (mean ± standard error) of the untreated control. *P<0.05. LEV, levetiracetam.

PI3K/Akt is another pathway that induces EMT, and a significant decrease in *PI3K* expression was observed in the U-251MG cell line after treatment with perampanel (0.40±0.33 compared to the control; P<0.05), but the decrease was not significant in the T98G cell line. The expression of *Akt* exhibited no change in both cell lines compared with

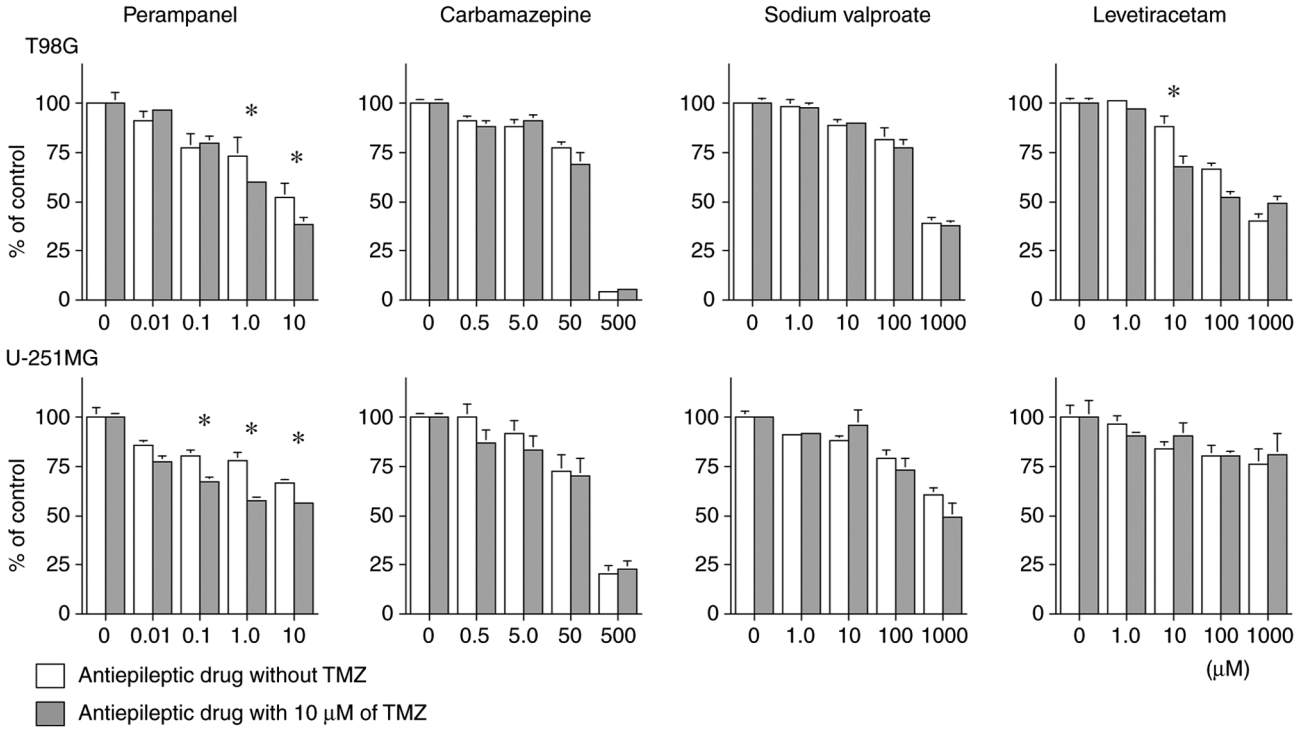

Figure 5. Growth inhibition by various antiepileptic drugs in combination with 10 μM of TMZ. After exposure to the various concentrations of antiepileptic drugs with/without TMZ for 72 h, cells were counted. The effect of a combination perampanel with TMZ on cell growth are included the results in our previous study reported in part by co-author Tatsuoka *et al* (13). Values are expressed as the percentage (mean ± standard error) of the untreated control. *P<0.05. TMZ, temozolomide.

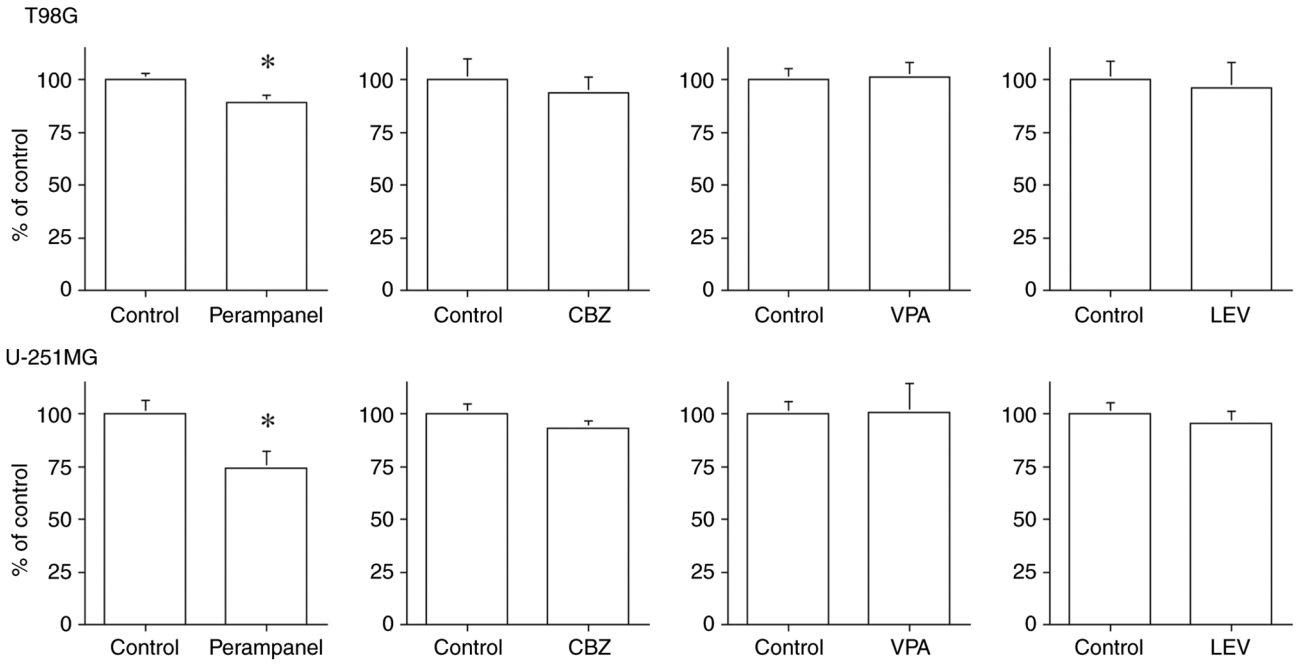

Figure 6. Cell migration inhibition by perampanel, CBZ, VPA and LEV in T98G and U-251MG cell lines using the Transwell migration assay. Values are expressed as the percentage (mean ± standard error) of the untreated control. *P<0.05. CBZ, carbamazepine; VPA, sodium valproate; LEV, levetiracetam.

the control group. However, the expression of the mesenchymal marker *N-cadherin*, which promotes cell migration and infiltration, was decreased in both cell lines (T98G: 0.20±0.42 and U-251MG: 0.40±0.03 compared to the control; P<0.05). On the other hand, the expression of the epithelial marker *E-cadherin*, which strengthens cell-cell adhesion and reduces cell motility, was increased in both cell lines (T98G: 2.00±0.20 and U-251MG: 1.30±0.03 compared to the control; P<0.05). Furthermore, the expression of *MMP-2*, a proteolytic enzyme, was reduced in both cell lines (T98G: 0.80±0.05

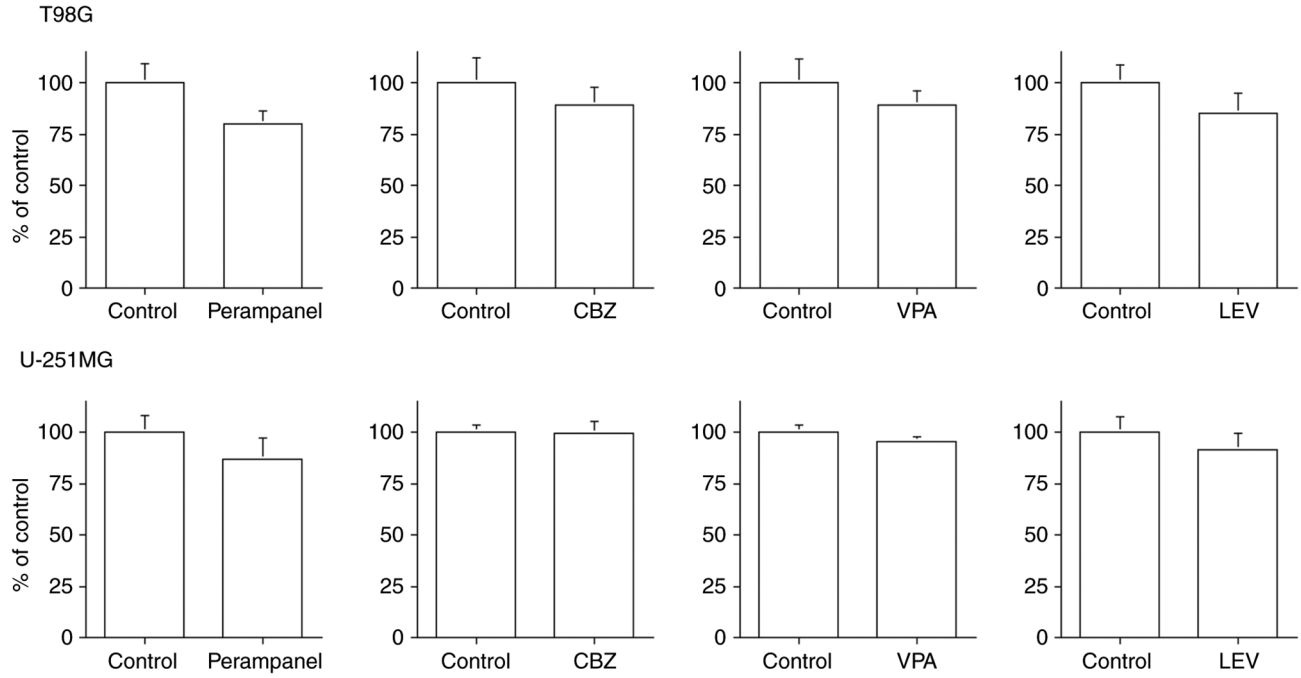

Figure 7. Cell infiltration inhibition by perampanel, CBZ, VPA and LEV in T98G and U-251MG cell lines using the Transwell migration assay. Values are expressed as the percentage (mean ± standard error) of the untreated control. CBZ, carbamazepine; VPA, sodium valproate; LEV, levetiracetam.

and U-251MG: 0.30±0.05 compared to the control; P<0.05) (Fig. 8).

Discussion

The previous study by our group on cell proliferation inhibitory effects using six malignant glioma cell lines indicated perampanel has a concentration-dependent inhibitory effect on proliferation in all cell lines (13). Although perampanel has been reported to have antitumor effects, the amount of perampanel used in those studies was much higher than the blood concentration for its clinical use as an antiepileptic drug (10,11). However, in the present study, much like our previous study, perampanel demonstrated significant inhibition of cell proliferation in most cell lines at the therapeutic blood concentration level as an antiepileptic agent. By contrast, CBZ, a voltage-gated Na^+ channel inhibitor, had a cell growth inhibitory effect only on U-138MG cells. Furthermore, the IC_{50} of CBZ for U-138MG was <50 μM and the therapeutic blood concentration range of CBZ as an antiepileptic drug is 17-50 μM (24). VPA is a gamma-aminobutyric acid (GABA) metabolism inhibitor and the antiepileptic action is mainly due to the increased concentration of GABA, an inhibitory neurotransmitter, which binds to the receptor and promotes the influx of Cl^-, causing the suppression of nerve excitement (27). Therapeutic concentrations of VPA had no inhibitory effect on cell proliferation in U-138MG cells and almost none in A172 cells. However, AM-38, T98G, U-251MG and YH-13 cells demonstrated an inhibitory effect of 50% or more at a maximum therapeutic blood concentration of 600 μM of VPA. LEV acts as an antiepileptic drug by binding to the synaptic vesicle protein SV2A and reducing the release of synaptic vesicles (27). LEV inhibited cell proliferation at a concentration of 100 μM (blood concentration range as anticonvulsant, 70-270 μM) in only T98G and YH-13 cells, and no cell line exhibited a cell proliferation inhibitory effect of 50% or more at the therapeutic range of the blood concentration as an antiepileptic drug. A significant cell growth inhibitory effect of perampanel was observed within each concentration as anticonvulsant in all 6 cell lines, while half of the cell lines were sensitive to CBZ, four to VPA and two to LEV. In addition, the IC_{50} values of certain cells for penampanel, CBZ and VPA were within the concentrations of the drugs used as anticonvulsants. CBZ only exerted a specific cell growth inhibitory effect on U-138MG. Only perampanel and VPA were observed to have antiproliferative effects on malignant glioma cells. In the present study, the mechanism of action of the cell growth inhibitory effect of each anticonvulsant was not examined in detail. The previous study by our group reported that the mechanism of the antitumor effect of perampanel was not cell cycle-related, but was associated with induction of apoptosis (13), which is somewhat consistent with previous findings (10,11). However, VPA has numerous reported effects on tumor cells, including cell proliferation inhibition and apoptosis induction associated with histone deacetylase and cell cycle inhibition through glycogen synthase kinase 3β inhibition (28,29).

In the present study, the antitumor effect of the antiepileptic drugs combined with 10 μM of TMZ, a therapeutic level of a standard chemotherapeutic drug for glioblastoma, was investigated using the T98G and U-251MG cell lines, which tended to be sensitive to the antiepileptic drugs at therapeutic levels. It was investigated which anticonvulsants elicit further antitumor effects when used with TMZ. Perampanel produced a significant inhibition of cell growth from 1.0 μM in both cell lines, and LEV also demonstrated a significant inhibition of cell growth at 10 μM in the T98G cell line. These antiepileptic drugs may have further antitumor effects in combination with

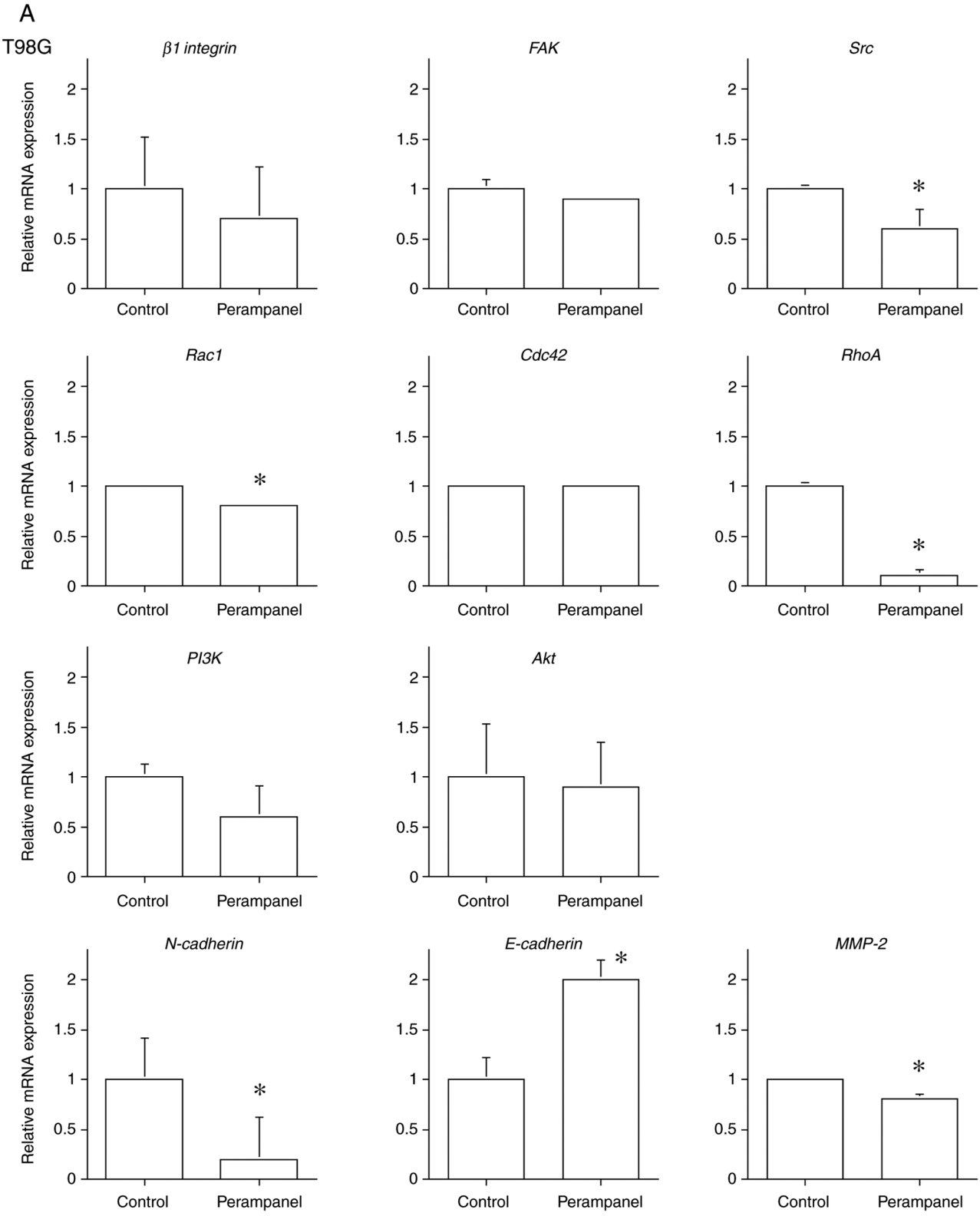

Figure 8. Continued.

TMZ. Synergistic effects between perampanel and TMZ have also been reported by another study (11). On the other hand, the expression of MGMT is strongly associated with susceptibility to TMZ and 45-75% of malignant gliomas express MGMT (30). T98G cells express MGMT and are resistant to TMZ treatment, whereas U-251MG cells do not express MGMT (22). The present study did not further elucidate the mechanisms of the cell growth inhibitory effect of perampanel in combination with TMZ on T98G and U-251MG cells, including how perampanel alters MGMT implicated in TMZ resistance. However, it was previously reported that LEV suppresses the expression of MGMT and enhances the

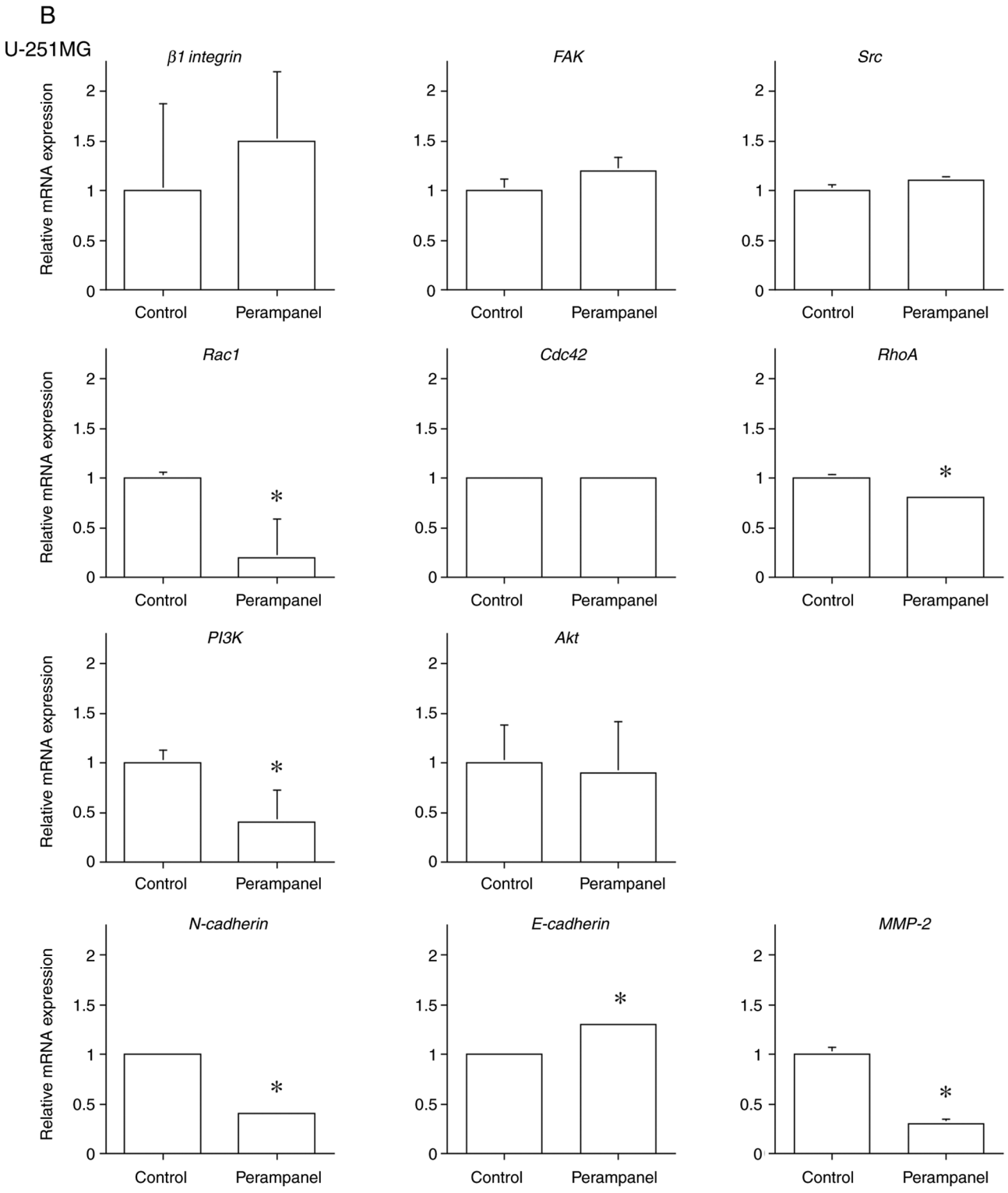

Figure 8. Expression of mRNA related to epithelial-mesenchymal transition following 4 h of perampanel (1.0 μM) treatment using reverse transcription-quantitative PCR in the (A) T98G and (B) U-251MG cell lines. *GAPDH* was employed as the internal control. The relative expression level of the genes was calculated using the $2^{-\Delta\Delta Cq}$ method. Values are expressed as the mean ± standard error. *P<0.05. FAK, focal adhesion kinase; PI3K, phosphatidylinositol 3-kinase; Rac1, rac family small GTPase 1; RhoA, ras homolog family member A; Cdc42, cell division cycle 42; MMP-2, matrix metalloproteinase-2.

effect of TMZ by activating the apoptotic pathway (31). The present study indicated that the combination of LEV and TMZ enhanced the inhibition of cell proliferation in T98G cells. Therefore, LEV may increase TMZ sensitivity of certain glioma cells, which may be due to a variety of mechanisms, including an inhibitory effect on MGMT.

Only perampanel significantly suppressed cell migration and perampanel tended to suppress cell invasion more than the other antiepileptic drugs. Thus, further experiments (mRNA expression of EMT-related molecules) were subsequently conducted for only perampanel. The present results indicated that perampanel downregulated the *Rac1* and *RhoA*, as well

as the β1 integrin/FAK/Src pathways; furthermore, it upregulated the *E-cadherin* and downregulated the *N-cadherin* and PI3K/Akt pathways. These effects may inhibit changes in cell morphology or reduce cell motility and increase intercell adhesion. The present study suggested that perampanel suppressed the EMT and inhibited cell migration. Further studies, including western blot analysis, such as phosphorylation (activation) of Akt, are required, since protein analysis is critical. Such studies would make the present results more conclusive.

Various antiepileptic drugs are clinically used to treat and control symptomatic epilepsy caused by malignant glioma. Controlling epileptic seizures is important for maintaining the patients' quality of life and it is vital to continue the treatment for brain tumors. However, which antiepileptic drug has the best seizure-suppressing effect remains to be determined. A systematic review comparing the effects of antiepileptic drugs on patients with grade 2-4 gliomas reported that the 12-month seizure-free rate was 43% for CBZ, 37% for VPA and 74% for LEV (32). Perampanel monotherapy data were not included, but combination therapy with another anticonvulsant achieved a 45% 12-month seizure-free rate. The treatment failure rate for epilepsy in 12 months was 0% for perampanel, 26% for CBZ, 21% for VPA and 24% for LEV. The present study indicated that perampanel and LEV had a high seizure-control effect on brain tumor-related epilepsy, but perampanel has a low risk of side effects and is generally more effective (32). Therefore, perampanel may be selected as a higher-priority therapeutic drug, for both antiepileptic and antitumor effects.

As a limitation of the present study, DMSO was used as the vehicle for the anticonvulsants perampanel and CBZ. DMSO concentrations of <10% are considered to be of low toxicity (33). The concentrations of DMSO in the 10 μM perampanel and 500 μM CBZ (highest concentration of DMSO for each drug) groups were 0.1 and 0.5% DMSO in the present study, respectively. Therefore, no DMSO vehicle control was included in the present study to check for solvent toxicity.

In conclusion, the present study on the four common antiepileptic drugs, perampanel, CBZ, VPA and LEV, indicated that perampanel not only suppresses cell proliferation but also enhances the cell proliferation inhibitory effect when used in combination with TMZ in certain malignant glioma cell lines. In addition, perampanel had an antitumor effect that inhibited cell migration. Therefore, perampanel may be more beneficial in terms of antitumor efficacy than other antiepileptic drugs in the treatment of 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

CY, JT and ES developed the experimental design, performed most of the experiments and analysis, and drafted the manuscript. YH, YO, SYo, SYa, KS, HH and YK were involved in the conception and design of the study, performed parts of the experiments, analyzed the data and contributed to the writing of the manuscript. AY contributed to the experimental design, analyzed the data and was involved in writing the manuscript. CY, JT, ES, YH 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 has received research funds for other research projects from Medtronic Japan Co., Ltd. and Eisai Co., Ltd., Tokyo, Japan. He has also, in accordance with the rules, reported their competing interests (including a small amount of research funds for other research projects) to his main academic society, the Japan Neurosurgical Society. The other authors have no competing interests to declare.

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