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Perampanel Inhibits Neuroblastoma Cell Proliferation Through Down-regulation of AKT and ERK Pathways

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Abstract. *Background/Aim: Activation of AKT serine/ threonine kinase (AKT) predicts poor outcome in neuroblastoma, which highlights the potential of the AKT pathway as a promising target for neuroblastoma treatment. Several studies reported that blockade of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs) reduces proliferation in glioblastoma or lung cancer by inhibiting AKT and extracellular signal-related kinase (ERK) pathways. In this study, we examined the effect of the AMPAR antagonist perampanel on human neuroblastoma cells. Materials and Methods: Cell proliferation, caspase activity, and western blot assays were performed to determine the effect of perampanel on the KP-N-SI9s human neuroblastoma cell line. Results: Perampanel inhibited cell proliferation without triggering apoptosis in neuroblastoma cells. Down-regulation of AKT protein levels, AKT phosphorylation, and ERK1/2 phosphorylation were also observed in neuroblastoma cells with perampanel treatment. Conclusion: Perampanel inhibits neuroblastoma cell proliferation through down-regulation of AKT and ERK pathways and has potential for the treatment of neuroblastoma.*

Neuroblastoma is the most common extracranial solid tumour in children, accounting for approximately 8% of all childhood cancer and 15% of childhood cancer-related deaths. Despite the availability of multimodal therapy, high-risk neuroblastoma is difficult to cure and is associated with a 5-year survival rate of around 50% (1). The high incidence of resistance of high-risk neuroblastoma to conventional therapies has prompted the search for novel therapeutic approaches.

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The phosphatidylinositol-3-kinase (PI3K)/AKT serine/ threonine kinase (AKT) pathway plays critical roles in many human cancer types including neuroblastoma (2). Phosphorylated AKT (p-AKT) correlates with poor patient prognosis in neuroblastoma (3), and the PI3K/AKT pathway has been linked to augmented cell survival (4) and increased chemotherapy resistance in neuroblastoma (5). Therefore, targeting the PI3K/AKT pathway by inhibitors may be a promising strategy for overcoming therapy resistance in neuroblastoma (6).

Glutamate stimulates tumour cell proliferation and motility *via* activation of glutamate receptors (GluRs) (7). α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs) are widespread throughout the central nervous system and serve as synaptic receptors for fast excitatory synaptic transmission mediated by glutamate. AMPARs are heterotetramers containing at least two distinct GluR subunits (GluR1–4) (8). AMPARs are also expressed in various tumour types (9). Studies reported that blockade of AMPARs reduces proliferation in glioblastoma and lung cancer through down-regulation of AKT and extracellular signal-related kinase (ERK) pathways (10, 11). Therefore, AMPAR antagonists may inhibit AKT activation and be effective cytotoxic agents against neuroblastoma.

Perampanel, a non-competitive AMPAR antagonist, is a structurally novel anticonvulsant that is used to control epileptic seizures (12). In this study, we investigated the effects of perampanel on KP-N-SI9s human neuroblastoma cells and the AKT and ERK pathways. We discuss our findings as a rationale for the potential application of perampanel in the treatment of neuroblastoma.

Materials and Methods

Cell culture. The human neuroblastoma cell line KP-N-SI9s was purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and cultured in Roswell Park Memorial Institute medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% foetal bovine serum, 100 U/ml penicillin,

and 100 μg/ml streptomycin. Cells were cultured at 37˚C in a humidified incubator with 5% CO_2.

Cell proliferation assay. Cell proliferation was determined by the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). A total of 1×10^4 cells was seeded per well in 96-well plates and incubated with different concentrations of perampanel (AdooQ Bioscience, Irvine, CA, USA) or vehicle (dimethyl sulfoxide, DMSO) for 48 h. CCK-8 reagent was administered for 3 h, followed by absorbance measurement at 450 nm using a microplate reader (Tecan Sunrise; Tecan, Männedorf, Switzerland). The cell proliferation index of perampanel-treated cells was calculated from the mean absorbance (from triplicate wells), considering that of vehicle-treated cells as 100%.

Western blotting. Control and perampanel-treated cells were lysed in cold lysis buffer (CytoBuster™ Protein Extraction Reagent; Novagen Inc., Madison, WI, USA) in the presence of a protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Mannheim, Germany). Protein samples were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were incubated with 5% skimmed milk solution and probed with the following primary antibodies at 4˚C overnight: human/mouse cross-reactive monoclonal antibodies against GluR-1 (sc-55509), GluR-2 (sc-517265), and GluR-4 (sc-271894) were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA); monoclonal antibodies against AKT (2920S) and polyclonal antibodies against phospho-AKT (p-AKT; 9271S) were purchased from Cell Signaling Technology (Boston, MA, USA); polyclonal antibodies against ERK1/2 (ab196883) and monoclonal antibodies against phospho-ERK1/2 (p-ERK1/2; ab201015) were purchased from Abcam (Cambridge, MA, USA). Membranes were washed and then incubated with secondary antibodies for 1 h at room temperature. Protein bands were visualized using the ECL Prime Western Blotting Detection Regent (GE Healthcare, Little Chalfont, UK) and a light-capture cooled CCD camera system (ATTO Corp., Tokyo, Japan). Protein expressions were normalized with respect to that for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc-25778; Santa Cruz Biotechnology). Whole mouse brain lysates from C57BL/6J mice (Japan SLC, Shizuoka, Japan) served as a positive control.

Caspase-3/7 activity assay. A total of 1×10^4 cells were seeded per well in 96-well plates and incubated with 100 or 200 μM of perampanel or vehicle (DMSO) for 48 h. Caspase-3/7 activity was investigated using Caspase-Glo 3/7 assay reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol. Flow cytometric analysis was performed using annexin-V and 7-aminoactinomycin D (7-AAD) double staining. Luminescence measurements were obtained using the TECAN infinite 200 plate reader (TECAN, Mannedorf, Switzerland). Caspase activity was calculated from the mean luminescence (from triplicate wells), considering that of the vehicle-treated cells as 1.0.

Statistical analysis. Results are expressed as the mean±standard error of the mean (SEM). The unpaired *t*-test was used to determine the significance of statistical differences, with values of $p<0.05$ being considered to indicate statistical significance. Statistical analyses were performed using GraphPad Prism 7 software (San Diego, CA, USA). All results were representative of at least three independent experiments.

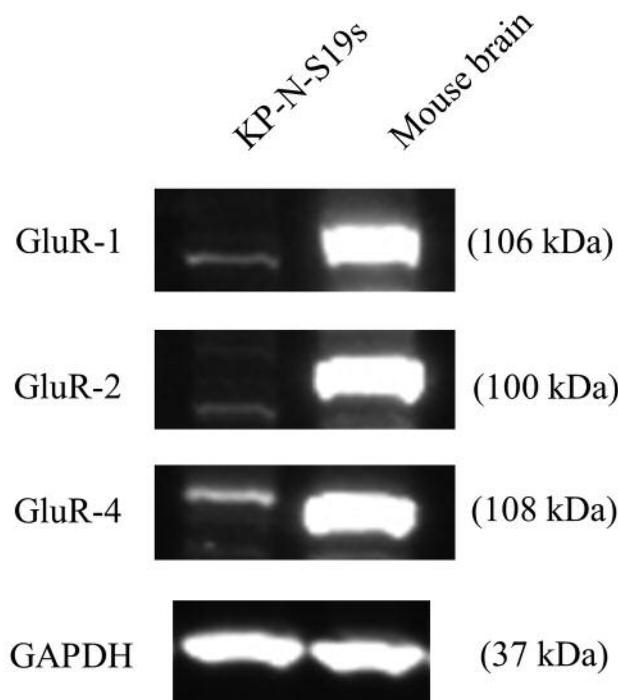

Figure 1. *Western blot analysis of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor subunits glutamate receptor-1 (GluR-1), GluR-2, and GluR-4 in KP-N-SI9s cells. Whole mouse brain lysates served as a positive control. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a control for protein loading efficiency.*

Results

KP-N-SI9s human neuroblastoma cells express AMPARs. We evaluated the AMPAR subunit expressions in KP-N-SI9s cells by western blot (Figure 1). Whole mouse brain lysates were used as a positive control. The results demonstrated that KP-N-SI9s cells expressed all three AMPAR subunits examined, GluR-1, GluR-2, and GluR-4.

Perampanel inhibits proliferation of KP-N-SI9s cells. To determine the effect of perampanel on proliferation of human neuroblastoma cells, we examined the growth of KP-N-SI9s cells treated with several concentrations of perampanel for 48 h using CCK-8 assays. Perampanel treatment inhibited cell proliferation compared with controls in a concentration-dependent manner (Figure 2).

Perampanel suppresses the AKT pathway. The AKT signalling pathway is one of the most critical pathways in regulating cell survival (13). AKT and p-AKT protein expressions were measured in KP-N-SI9s cells treated with perampanel using western blot. Both AKT and p-AKT protein expressions were significantly down-regulated in KP-N-SI9s cells treated with 200 μM perampanel for 48 h compared with controls (Figure 3).

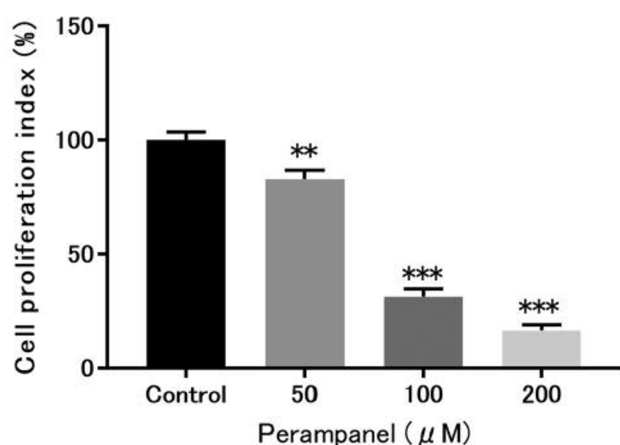

Figure 2. *Effects of perampanel on proliferation of KP-N-SI9s cells. Cells were treated with 50, 100, or 200 μM of perampanel for 48 h and evaluated by CCK-8 assays. Results are expressed as the percentage of cell proliferation relative to that of vehicle control. Data are shown as the mean±SEM from three independent experiments. Significantly different at **p<0.01 and ***p<0.001 versus vehicle control.*

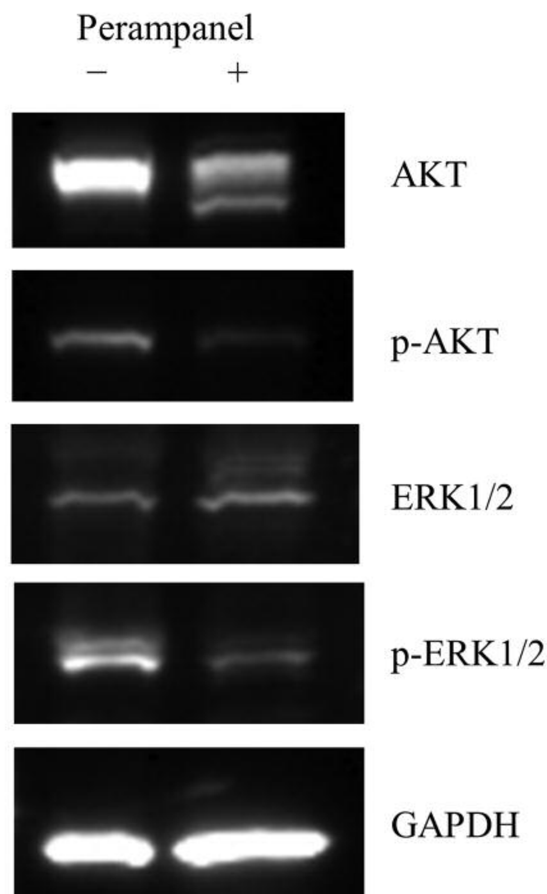

Figure 3. *Western blot analysis of AKT serine/threonine kinase (AKT) and extracellular signal-related kinase (ERK) in KP-N-SI9s cells treated with perampanel. Cells were treated with 200 μM perampanel or vehicle for 48 h, and AKT, phospho-AKT (p-AKT), ERK1/2, and p-ERK1/2 were assessed by western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.*

Perampanel reduces ERK1/2 phosphorylation. The ERK pathway regulates cell proliferation and cell differentiation (14). ERK1/2 and p-ERK1/2 protein expression was measured in perampanel-treated KP-N-SI9s cells using western blot. Perampanel significantly reduced the phosphorylation of ERK1/2 following treatment with 200 μM perampanel compared with control cells (Figure 3).

Perampanel does not induce caspase-mediated apoptosis. We assessed the ability of perampanel to induce apoptosis of KP-N-SI9s cells by measuring the activity of caspase-3/7. At concentrations of 100 or 200 μM, perampanel had no effect on caspase-3/7 activation (Figure 4A). In addition, perampanel did not induce apoptosis of KP-N-SI9s cells as assessed by flow cytometric analysis using annexin-V and 7-aminoactinomycin D double staining (Figure 4B).

Discussion

In this study, we demonstrated that perampanel inhibited proliferation of a human neuroblastoma cell line. This effect was found to be associated with suppression of the AKT and ERK pathways. Our data suggest a potential approach for the use of perampanel in the treatment of neuroblastoma.

AMPARs play critical roles in cancer cell proliferation in several types of tumour (9), and AMPAR antagonists have been shown to inhibit proliferation of various types of tumour cells (15). Recently, the first selective AMPA receptor antagonist perampanel was approved as an adjunctive therapy for the treatment of seizures (12). Some studies reported the use of perampanel in the management of brain tumours in both preclinical and clinical settings (16, 17). Izumoto *et al.* evaluated the clinical impact of perampanel on tumour progression in patients with glioma and found tumour growth was inhibited in those treated with perampanel (17). However, the effect of perampanel on neuroblastoma cells has never been reported to our knowledge. In the present study, we found that AMPARs are indeed expressed in neuroblastoma cells, and that perampanel, a AMPAR antagonist, inhibited the growth of neuroblastoma cells. To the best of our knowledge, this is the first report demonstrating anticancer activities of perampanel on neuroblastoma cells.

AKT plays a major role in both cell survival and resistance to tumour therapy (4, 5). Pathological activation of AKT also frequently occurs in neuroblastoma and is correlated with poor prognosis (2, 3, 13). ERK1/2 responds to a wide variety of

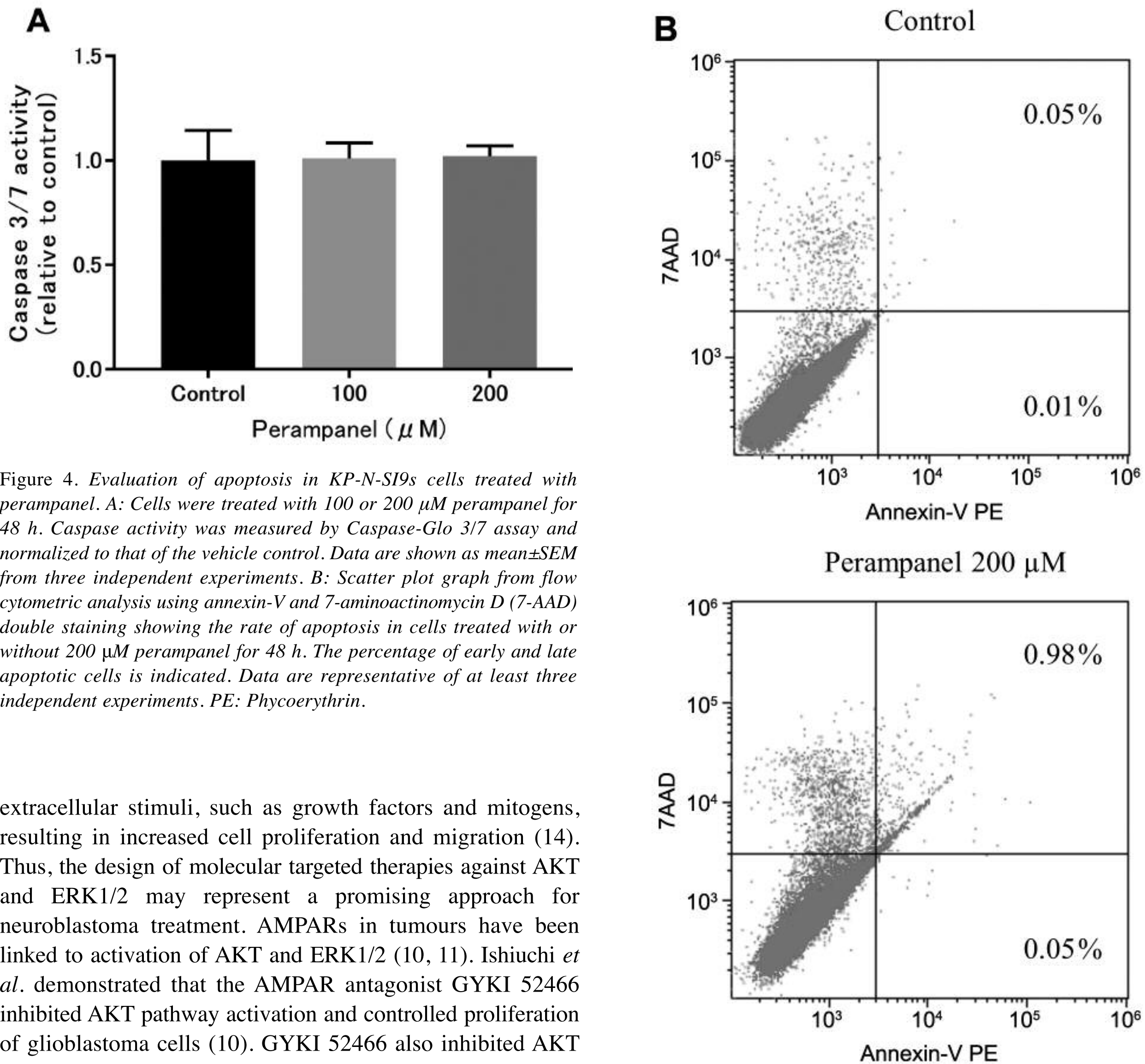

Figure 4. *Evaluation of apoptosis in KP-N-SI9s cells treated with perampanel. A: Cells were treated with 100 or 200 μM perampanel for 48 h. Caspase activity was measured by Caspase-Glo 3/7 assay and normalized to that of the vehicle control. Data are shown as mean±SEM from three independent experiments. B: Scatter plot graph from flow cytometric analysis using annexin-V and 7-aminoactinomycin D (7-AAD) double staining showing the rate of apoptosis in cells treated with or without 200 μM perampanel for 48 h. The percentage of early and late apoptotic cells is indicated. Data are representative of at least three independent experiments. PE: Phycoerythrin.*

extracellular stimuli, such as growth factors and mitogens, resulting in increased cell proliferation and migration (14). Thus, the design of molecular targeted therapies against AKT and ERK1/2 may represent a promising approach for neuroblastoma treatment. AMPARs in tumours have been linked to activation of AKT and ERK1/2 (10, 11). Ishiuchi *et al*. demonstrated that the AMPAR antagonist GYKI 52466 inhibited AKT pathway activation and controlled proliferation of glioblastoma cells (10). GYKI 52466 also inhibited AKT and ERK1/2 pathway activation and controlled proliferation of lung cancer cells (11). In the present study, we showed that the AMPAR antagonist perampanel also inhibited AKT and ERK pathways in neuroblastoma cells, suggesting that perampanel may be a potential therapeutic approach for neuroblastoma.

Our results showed that perampapanel did not induce apoptosis of KP-N-SI9s cells. Similarly, perampanel did not exhibit apoptotic activity against glioblastoma cells (16). In contrast, YM872, a competitive antagonist of AMPAR, induced apoptosis of glioblastoma cells (18). Ca^{2+} influx through AMPARs seems to be required for stimulation of the anti-apototic signalling cascade (18). Therefore, apotosis-inducing activity by AMPAR antagonists may be associated with differences in inhibition of Ca^{2+} influx through AMPARs. Further investigation is required to elucidate the molecular mechanisms underlying the anticancer activities of AMPAR antagonists.

In conclusion, as far as we are aware of, this is the first study to report the antitumour effect of the AMPAR antagonist perampanel on neuroblastoma cells. Perampanel inhibited cell proliferation and survival in neuroblastoma cells *via* inhibition of the AKT and ERK pathways. These findings suggest the potential clinical application of perampanel in the treatment of neuroblastoma.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in regard to this study.

Authors' contributions

A. N. designed the study, and wrote the initial draft of the article. M. O., M. N., H. O., and T. F. contributed to analysis and interpretation of data, and assisted in the preparation of the article. A. N., M. M., S. Y., S. E., and N. K. carried out the experiments. All other Authors contributed to data collection and interpretation, and critically reviewed the article. All Authors approved the final version of the article, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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References

1 Irwin MS and Park JR: Neuroblastoma: Paradigm for precision medicine. Pediatr Clin North Am *62*: 225-256, 2015. PMID: 25435121. DOI: 10.1016/j.pcl.2014.09.015

2 Hennessy BT, Smith DL, Ram PT, Lu Y and Mills GB: Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov *4*: 988-1004, 2005. PMID: 16341064. DOI: 10.1038/nrd1902

3 Opel D, Poremba C, Simon T, Debatin KM and Fulda S: Activation of AKT predicts poor outcome in neuroblastoma. Cancer Res *67*: 735-745, 2007. PMID: 17234785. DOI: 10.1158/0008-5472.CAN-06-2201

4 Boller D, Schramm A, Doepfner KT, Shalaby T, von Bueren AO, Eggert A, Grotzer MA and Arcaro A: Targeting the phosphoinositide 3-kinase isoform p110delta impairs growth and survival in neuroblastoma cells. Clin Cancer Res *14*: 1172-1181, 2008. PMID: 18281552. DOI: 10.1158/1078-0432.CCR-07-0737

5 Li Z1 and Thiele CJ: Targeting AKT to increase the sensitivity of neuroblastoma to chemotherapy: Lessons learned from the brain-derived neurotrophic factor/TrkB signal transduction pathway. Expert Opin Ther Targets *11*: 1611-1621, 2007. PMID: 18020981. DOI: 10.1517/14728222.11.12.1611

6 Paz-Ares L, Blanco-Aparicio C, García-Carbonero R and Carnero A: Inhibiting PI3K as a therapeutic strategy against cancer. Clin Transl Oncol *11*: 572-579 2009. PMID: 19775996.

7 Takano T, Lin JH, Arcuino G, Gao Q, Yang J and Nedergaard M: Glutamate release promotes growth of malignant gliomas. Nat Med *7*: 1010-1015, 2001. PMID: 11533703. DOI: 10.1038/nm0901-1010

8 Nicoll RA, Malenka RC and Kauer JA: Functional comparison of neurotransmitter receptor subtypes in mammalian central nervous system. Physiol Rev *70*: 513-565, 1990. PMID: 1690904. DOI: 10.1152/physrev.1990.70.2.513

9 Stepulak A, Rola R, Polberg K and Ikonomidou C: Glutamate and its receptors in cancer. J Neural Transm *121*: 933-944, 2014. PMID: 24610491. DOI: 10.1007/s00702-014-1182-6

10 Ishiuchi S, Yoshida Y, Sugawara K, Aihara M, Ohtani T, Watanabe T, Saito N, Tsuzuki K, Okado H, Miwa A, Nakazato Y and Ozawa S: Ca^{2+}-permeable AMPA receptors regulate growth of human glioblastoma *via* AKT activation. J Neurosci *27*: 7987-8001, 2007. PMID: 17652589. DOI: 10.1523/JNEUROSCI.2180-07.2007

11 Stepulak A, Sifringer M, Rzeski W, Brocke K, Gratopp A, Pohl EE, Turski L and Ikonomidou C: AMPA antagonists inhibit the extracellular signal regulated kinase pathway and suppress lung cancer growth. Cancer Biol Ther *6*: 1908-1915, 2007. PMID: 18059166. DOI: 10.4161/cbt.6.12.4965

12 Krauss GL, Perucca E, Kwan P, Ben-Menachem E, Wang XF, Shih JJ, Patten A, Yang H, Williams B and Laurenza A: Final safety, tolerability, and seizure outcomes in patients with focal epilepsy treated with adjunctive perampanel for up to 4 years in an open-label extension of phase III randomized trials: Study 307. Epilepsia *59*: 866-876, 2018. PMID: 29574701. DOI: 10.1111/epi.14044

13 Song G, Ouyang G and Bao S: The activation of AKT/PKB signaling pathway and cell survival. J Cell Mol Med *9*: 59-71. PMID: 15784165. DOI: 10.1111/j.1582-4934.2005.tb00337.x

14 Murphy DA, Makonnen S, Lassoued W, Feldman MD, Carter C and Lee WM: Inhibition of tumor endothelial ERK activation, angiogenesis, and tumor growth by sorafenib (BAY43-9006) Am J Pathol *169*: 1875-1885, 2006. PMID: 17071608. DOI: 10.2353/ajpath.2006.050711

15 Rzeski W, Ikonomidou C and Turski L: Glutamate antagonists limit tumor growth. Biochem Pharmacol *64*: 1195-1200, 2002. PMID: 12234599.

16 Lange F, Weßlau K, Porath K, Hörnschemeyer J, Bergner C, Krause BJ, Mullins CS, Linnebacher M, Köhling R and Kirschstein T: AMPA receptor antagonist perampanel affects glioblastoma cell growth and glutamate release *in vitro*. PLoS One *14*: e0211644, 2019. PMID: 30716120. DOI: 10.1371/journal.pone.0211644

17 Izumoto S, Miyauchi M, Tasaki T, Okuda T, Nakagawa N, Nakano N, Kato A and Fujita M: Seizures and tumor progression in glioma patients with uncontrollable epilepsy treated with perampanel. Anticancer Res *38*: 4361-4366, 2018. PMID: 29970574. DOI: 10.21873/anticanres.12737

18 Ishiuchi S, Tsuzuki K, Yoshida Y, Yamada N, Hagimura N, Okado H, Miwa A, Kurihara H, Nakazato Y, Tamura M, Sasaki T and Ozawa S: Blockage of Ca(2+)-permeable AMPA receptors suppresses migration and induces apoptosis in human glioblastoma cells. Nat Med *8*: 971-978, 2002. PMID: 12172 541. DOI: 10.1038/nm746

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