Glutamate antagonists limit tumor growth

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Neuronal progenitors and tumor cells possess propensity to proliferate and to migrate. Glutamate regulates proliferation and migration of neurons during development, but it is not known whether it influences proliferation and migration of tumor cells. We demonstrate that glutamate antagonists inhibit proliferation of human tumor cells. Colon adenocarcinoma, astrocytoma, and breast and lung carcinoma cells were most sensitive to the antiproliferative effect of the *N*-methyl-D-aspartate antagonist dizocilpine, whereas breast and lung carcinoma, colon adenocarcinoma, and neuroblastoma cells responded most favorably to the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate antagonist GYKI52466. The antiproliferative effect of glutamate antagonists was Ca^{2+} dependent and resulted from decreased cell division and increased cell death. Morphological alterations induced by glutamate antagonists in tumor cells consisted of reduced membrane ruffling and pseudopodial protrusions. Furthermore, glutamate antagonists decreased motility and invasive growth of tumor cells. These findings suggest anticancer potential of glutamate antagonists.

The management of malignancies in humans remains a challenge for contemporary medicine (1). Progress has been achieved in chemotherapy, bone marrow transplantation, radiation technologies, surgical measures, adjunctive therapies, and in immunological and immunomodulatory approaches (2–4). Nevertheless, humans continue to succumb to cancer due to tumor recurrence and metastatic disease.

Glutamate is an essential amino acid and a transmitter in the mature mammalian nervous system (5). *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA), kainate, and metabotropic receptors are activated by glutamate (5). Glutamate has been pathogenetically linked to human psychiatric disorders such as anxiety or depression and to neurological disorders such as epilepsy, spasticity, stroke, or traumatic brain injury (6, 7). Glutamate antagonists were demonstrated to have anxiolytic, anticonvulsant, muscle relaxant, sedative/anesthetic, and neuroprotective properties (8).

During development of the nervous system, glutamate regulates proliferation, migration, and survival of neuronal progenitors and immature neurons (9–12). Certain characteristics of neuronal embryonic cells, including propensity to proliferate, migrate, and die, are shared by tumor cells, as is regulation of their invasive behavior by trophic factors (13). Although glutamate is present in nonneuronal tissues (14–16), including cancers, it is not known whether it may influence proliferation and migration of tumor cells.

To study the effect of glutamate on tumor growth, various tumor cell lines were exposed to NMDA and AMPA antagonists and glutamate, and the proliferation, morphology, and migration of tumor cells was evaluated. It was found that glutamate antagonists inhibit, whereas glutamate stimulates proliferation and migration of tumor cells.

Materials and Methods

Cell Lines and Cultures. Human brain astrocytoma (MOGGCCM), human rhabdomyosarcoma/medulloblastoma (TE671), human neuroblastoma (SKNAS), and human thyroid carcinoma (FTC238) were obtained from the European Collection of Cell Cultures (Center for Applied Microbiology and Research, Salisbury, U.K.). Human Caucasian colon adenocarcinoma (LS180) and human breast carcinoma (T47D) were gifts from Lublin Medical University, Lublin, Poland. Human Caucasian lung carcinoma (A549) and human colon adenocarcinoma (HT29) were obtained from the Institute of Immunology and Experimental Therapy (Polish Academy of Sciences, Wroclaw, Poland). SKNAS, TE671, T47D, and LS180 were grown in 1:1 mixture of DMEM (D6421, Ca^{2+} concentration of 1.05 mM) (Sigma) and nutrient mixture F-12 Ham (Ham's F-12) (Sigma) supplemented with 10% FBS (Life Technologies, Paisley, Scotland). FTC238 was grown in the same culture medium supplemented with 5% FBS. HT29 was grown in DMEM (D6046, Sigma; Ca^{2+} concentration of 1.8 mM) supplemented with 10% FBS. MOGGCCM and A549 were grown in 2:1 mixture of DMEM (D6046)/Ham's F-12 (Sigma) supplemented with 10% FBS. Human skin fibroblasts was a laboratory strain obtained by outgrowth technique from a skin explant, embedded in 2% agar and cultured in 1:1 mixture of DMEM and nutrient mixture Ham's F-12. Bone marrow stromal cell culture was established from bone marrow obtained from a healthy donor, mixed 1:1 with Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies) containing 0.2% methyl cellulose (Sigma), and left for 45 min in room temperature. After completion of erythrocyte sedimentation, nuclear cells were collected, washed twice in IMDM containing 2% FBS (Life Technologies), and centrifuged at 1,500 rpm for 5 min. Cells were suspended in growth medium consisting of IMDM supplemented with 10% FBS, 10% horse serum (Life Technologies), 10 μg/ml hydrocortisone (Sigma), and antibiotic–antimycotic solution (Life Technologies, 1 ml/100 ml of culture medium). Human skin fibroblasts and bone marrow stromal cells were used as nonmalignant proliferating cell lines. Cultures were kept at 37°C in a 95% air/5% CO_2 atmosphere. As Ca^{2+}-free medium, a minimum essential medium M8167 (Sigma) was used. (+)Dizocilpine and GYKI52466 were obtained from Research Biochemicals. (−)Dizocilpine, ketamine, memantine, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX), and 1-(4′-aminophenyl)-3,5-dihydro-7,8-dimethoxy-4H-2,3-benzodiazepin-4-one (CFM-2) were obtained from Tocris (Bristol, U.K.). Cyclophosphamide was obtained from ICN Biomedicals (Eschwege, Germany). Cisplatin and vinblastin were obtained from Sigma and thiotepa from Lederle Laboratories (Pearl River, NY). To determine the time course of antiproliferative action of glutamate antagonists, tumor cell lines such as MOGGCCM, T47D, A549, TE671, or FTC238 were exposed for 24, 48, 72, 96, and 120 h to different concentrations of (+)dizocilpine or GYKI52466. For three-

Abbreviations: NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; GYKI52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline; CFM-2, 1-(4′-aminophenyl)-3,5-dihydro-7,8-dimethoxy-4H-2,3-benzodiazepin-4-one.

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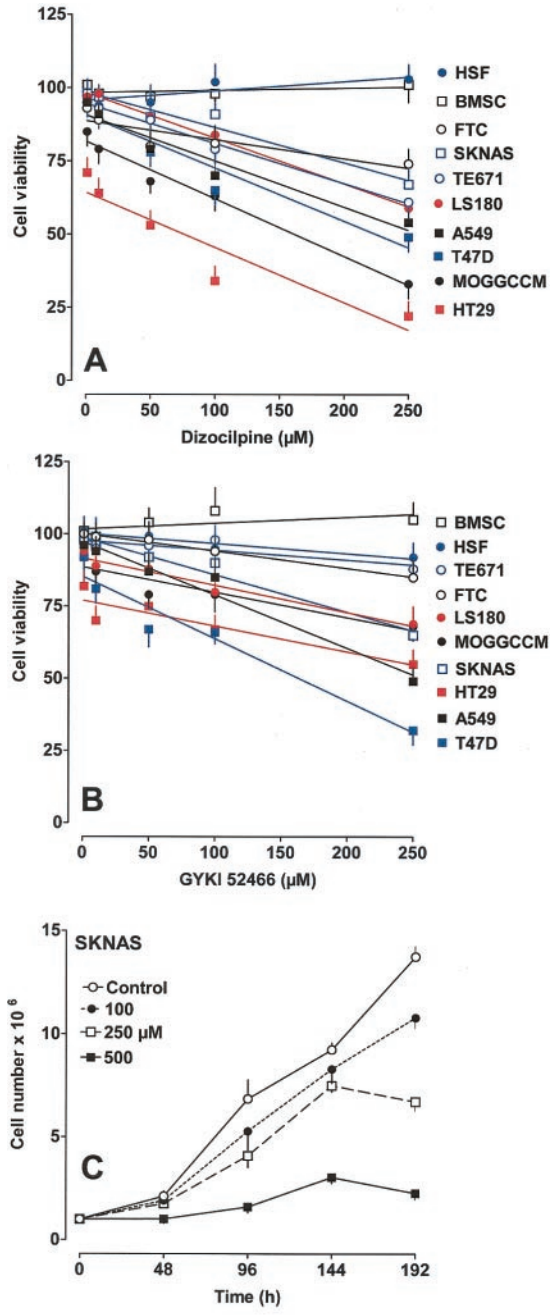

Fig. 1. The NMDA antagonist dizocilpine (*A*) and the AMPA antagonist GYKI52466 (*B*) exert concentration-dependent antiproliferative effect in human tumor cell lines but not in human skin fibroblasts and bone marrow stromal cells. Cells were exposed to either culture medium alone (control), dizocilpine (1–250 μM), or GYKI52466 (1–250 μM) for 96 h, and viability was measured photometrically by means of the MTT assay. Data represent mean normalized optical densities ± SEM of 6–8 trials and were analyzed by means of linear regression. (*C*) Growth inhibition of neuroblastoma cells in three-dimensional cultures by dizocilpine (100–500 μM) is shown. Numbers of cells were assessed by counting, after trypsinization. ANOVA showed that the effect of treatment was significant [$F(3,36) = 49.33$, $P < 0.001$], with multiple comparisons revealing that dizocilpine induced antiproliferative action in a dose-dependent manner. The effect of time was also significant [$F(4,36) = 296.69$, $P < 0.001$], indicating that the antiproliferative effect of dizocilpine was more pronounced with time. SKNAS, human neuroblastoma; TE671, human rhabdomyosarcoma/medulloblastoma; MOGGCCM, human brain astrocytoma; FTC238, human thyroid carcinoma; A549, human Caucasian lung carcinoma; LS180, human Caucasian colon adenocarcinoma; T47D, human breast carcinoma; HT29, human colon adenocarcinoma; HSF, human skin fibroblasts; BMSC, human bone marrow stromal cells.

dimensional cultures, spongostan biodegradable gelatin sponge (Spongostan; Ferrosan, Copenhagen) was used. Tumor cells were immobilized on spongostan discs (0.06 cm^3) at a density of 1×10^5 in 5-ml tubes. Tubes were subjected to constant rotary shaking at 150 rpm at 37°C. Culture medium was renewed every 48 h, and tumor growth was assessed by cell counting after trypsinization of the gelatin mix.

Cell Viability Assay. The yellow tetrazolium salt 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is metabolized by viable, metabolically active cells to purple formazan crystals. Tumor cells grown in 96-well multiplates were incubated for 4 h with MTT solution (Cell proliferation kit I; Roche Molecular Biochemicals). Formazan crystals were solubilized overnight, and the product was quantified spectrophotometrically by measuring absorbance at 570 nm wave length by using a Victor Multilabel Counter (Perkin–Elmer-Wallac, Freiburg, Germany).

Cell Proliferation Assay. Tumor cells were incubated with BrdUrd (BrdUrd labeling and detection kit III; Roche Molecular Biochemicals) for 18 h. Cells were subsequently fixed with 0.5 M ethanol/HCl and then were incubated with nucleases to partially digest DNA. Monoclonal anti-BrdUrd antibodies conjugated to peroxidase were subsequently added and detected by using 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate. Quantitation was performed colorimetrically at 405–490 nm wavelength.

Cytotoxicity Assay. A cytotoxicity detection kit based on measurement of lactate dehydrogenase (LDH) activity was used (Roche Molecular Biochemicals). The culture supernatant was collected and incubated with substrate mixture. LDH activity was determined by a coupled enzymatic reaction whereby the tetrazolium salt p-iodonitrotetrazolium violet is reduced to formazan. The amount of formazan formed is directly related to LDH activity in the supernatant. Absorption was measured at 490 nm. For visualization of degenerating cells, cultures were treated with 0.4% trypan blue (Sigma).

Immunocytochemistry and Light Microscopy. Cells fixed in 4% paraformaldehyde were incubated for 24 h with primary antibody against the NR1 (AB 1516, 1:200; Chemicon) or GluR2/3 (AB 1506, 1:100; Chemicon) glutamate receptor subunits. Antibody detection was performed by using a multilink streptavidin-biotin complex method, and visualization was achieved by using diaminobenzidine chromagen method (PK-6100, Vector; Boehringer Ingelheim). Negative control samples were incubated in primary antibody diluent only. For light microscopy, cells plated on plastic round coverslips at a density of 1×10^4 were allowed to grow for a 72- to 96-h period and were subsequently fixed in 4% paraformaldehyde in phosphate buffer, dehydrated, and stained with methylene blue/azure II.

Scanning Electron Microscopy. Cells were seeded at a density of $0.5–1 \times 10^4$ on 13-mm round plastic coverslips (Thermanox, Nunc) or at a density of $3–6 \times 10^5$ on 24-mm round polycarbonate membrane filters (0.4-μm pore size) in 6-well transwells (Costar) and grown for 96 h in the presence or absence of dizocilpine (100 μM) or GYKI52466 (100 μM). Cells were fixed with 2% glutaraldehyde in PBS, pH 7.4, and processed for scanning electron microscopy.

Migration Assay. Tumor cells were seeded on polycarbonate filters, 3 μm size, at a density of 1×10^5 in 12-well transwell plates (Costar) in the absence or presence of dizocilpine (25 or 50 μM) or GYKI52466 (25 or 50 μM) and allowed to migrate to the

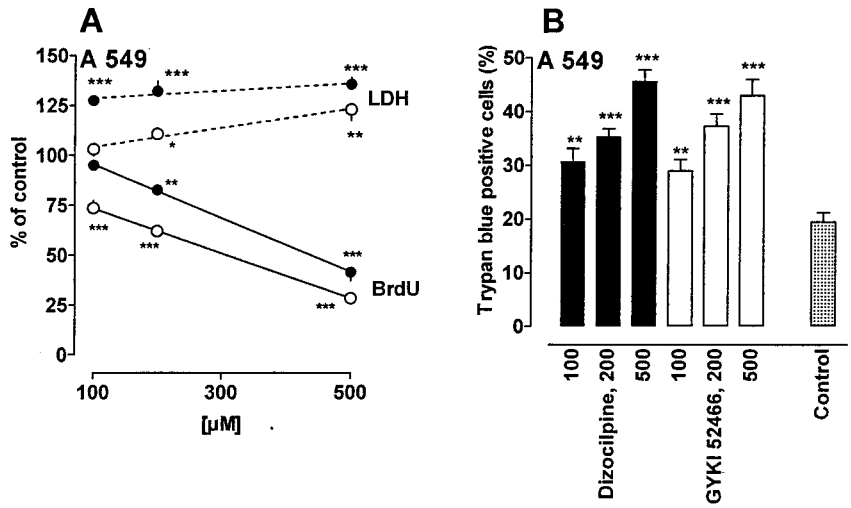

Fig. 2. The antiproliferative effect of dizocilpine (●) and GYKI52466 (○) is attributed to decreased cell division and to increased cell death. (*A*) Human lung carcinoma (A549) cells were grown in culture medium only (control), in the presence of dizocilpine (100–500 μM), or in the presence of GYKI52466 (100–500 μM) for 96 h. BrdUrd incorporation was used as a marker for cell division. Cell death was quantified by measuring LDH release from damaged cells after 96 h in culture. The data represent mean normalized optical densities ± SEM of 6–8 trials. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. control, Student's *t* test. (*B*) The effect of exposure of human lung carcinoma (A549) cells to different concentrations of dizocilpine or GYKI52466 on the numbers of trypan blue-accumulating cells is presented. Bars represent mean ± SEM number of trypan blue-positive cells/100 counted cells after 96 h in culture in the absence (control) or in the presence of different concentrations (μM) of glutamate antagonists ($n = 6$). **, $P < 0.01$; ***, $P < 0.001$, Student's *t* test.

lower compartment for 96 h. Number of cells in the lower compartment was estimated.

Results

Glutamate Antagonists Decrease Tumor Cell Proliferation. Proliferation of tumor cells was decreased in cultures exposed to the NMDA antagonist (+)dizocilpine (Fig. 1*A*) or the AMPA antagonist GYKI52466 (Fig. 1*B*) in a concentration-dependent manner as measured by means of the MTT assay, whereas proliferation of human skin fibroblasts and bone marrow stromal cells was unaffected by exposure to both antagonists (Fig. 1 *A* and *B*). Time-course studies revealed that antiproliferative effect was already established after 24 h and did not differ in intensity between 24- and 120-h exposure of MOGGCCM, T47D, A549, TE671, or FTC238 tumor cells to dizocilpine or GYKI52466. The antiproliferative effect of dizocilpine was reproduced in a three-dimensional neuroblastoma cell culture grown on spongostan (Fig. 1*C*).

The effect of glutamate antagonists on tumor cell proliferation was attributed both to decreased cell division, as determined by measurements of incorporation of BrdUrd, and increased cell death, as determined by measurements of LDH activity (Fig. 2*A*) and trypan blue exclusion (Fig. 2*B*). Colon adenocarcinoma (HT29), astrocytoma (MOGGCCM), breast carcinoma (T47D), and lung carcinoma (A549) were most sensitive to the cytostatic effect of the NMDA antagonist dizocilpine (Fig. 1*A*), whereas breast carcinoma (T47D), lung carcinoma (A549), colon adenocarcinoma (HT29), and neuroblastoma (SKNAS) were most sensitive to the AMPA antagonist GYKI52466 (Fig. 1*B*). The threshold concentrations of dizocilpine or GYKI52466 required to elicit antiproliferative effects ranged between 1 and 50 μM (Fig. 1*A* and *B*). Significant antiproliferative effect of dizocilpine was detected at concentrations as low as 1 μM in colon adenocarcinoma cells (HT29) and as low as 10 μM in astrocytoma (MOGGCCM) cells. In lung carcinoma (A549), neuroblastoma (SKNAS), breast carcinoma (T47D), Caucasian colon adenocarcinoma (LS180), rhabdomyosarcoma/medulloblastoma (TE671), and thyroid carcinoma (FTC238) cells, antiproliferative effects of dizocilpine were seen at 50 μM (Fig. 1*A*). Similarly, GYKI52466 significantly inhibited proliferation of colon adenocarcinoma cells (HT29) at concentrations as low as 1 μM and of breast carcinoma (T47D) and Caucasian colon adenocarcinoma (LS180) cells at concentrations as low as 10 μM (Fig. 1*B*). In the remaining tumor cell lines, antiproliferative effects of GYKI52466 were detected at 50 μM (Fig. *1B*). Such concentrations of glutamate antagonists are required to modulate NMDA- or AMPA-mediated currents in nonneuronal tissues such as osteoblasts and osteoclasts (17, 18) or to inhibit migration of embryonic cortical neurons (12). The antiproliferative effect of dizocilpine or GYKI52466 was absent in lung carcinoma (A549) and rhabdomyosarcoma/medulloblastoma (TE671) cells grown in the Ca^{2+}-free medium (Fig. 3 *A–D*), indicating that blockade of Ca^{2+} influx was involved in mediating the antiproliferative effect of glutamate antagonists.

To confirm that the antiproliferative effects of dizocilpine or GYKI52466 were mediated via NMDA or AMPA receptor/ion channel complexes, several NMDA and AMPA antagonists were tested in lung carcinoma (A549) and rhabdomyosarcoma/medulloblastoma (TE671) cells. The NMDA antagonists ketamine and memantine reproduced the antiproliferative effect of dizocilpine (Fig. 3 *A* and *C*). The AMPA antagonists NBQX and CFM-2 reproduced antiproliferative effects of GYKI52466 in lung carcinoma and rhabdomyosarcoma/medulloblastoma cells (Fig. 3 *B* and *D*). (−)Dizocilpine, the less active enantiomer of dizocilpine, was significantly less effective than (+)dizocilpine in lung carcinoma [$F_{A549}(1,80) = 128.52$, $P < 0.001$] or rhabdomyosarcoma/medulloblastoma [$F_{TE671}(1,80) = 268.60$, $P < 0.001$] cells, indicating stereoselectivity for the NMDA channel blockade (Fig. 3 *A* and *C*).

Tumor Cell Proliferation and Glutamate. To test whether glutamate may stimulate proliferation of tumor cells, lung carcinoma (A549) and rhabdomyosarcoma/medulloblastoma (TE671) cells were exposed to different concentrations of glutamate (1–10 mM) and proliferation was evaluated after 96 h by means of the MTT assay. Glutamate did not affect proliferation of lung carcinoma or rhabdomyosarcoma/medulloblastoma cells in a medium containing 10% serum. However, glutamate stimulated the proliferation of lung carcinoma cells in the serum-deprived medium or medium supplemented with serum-replacement me-

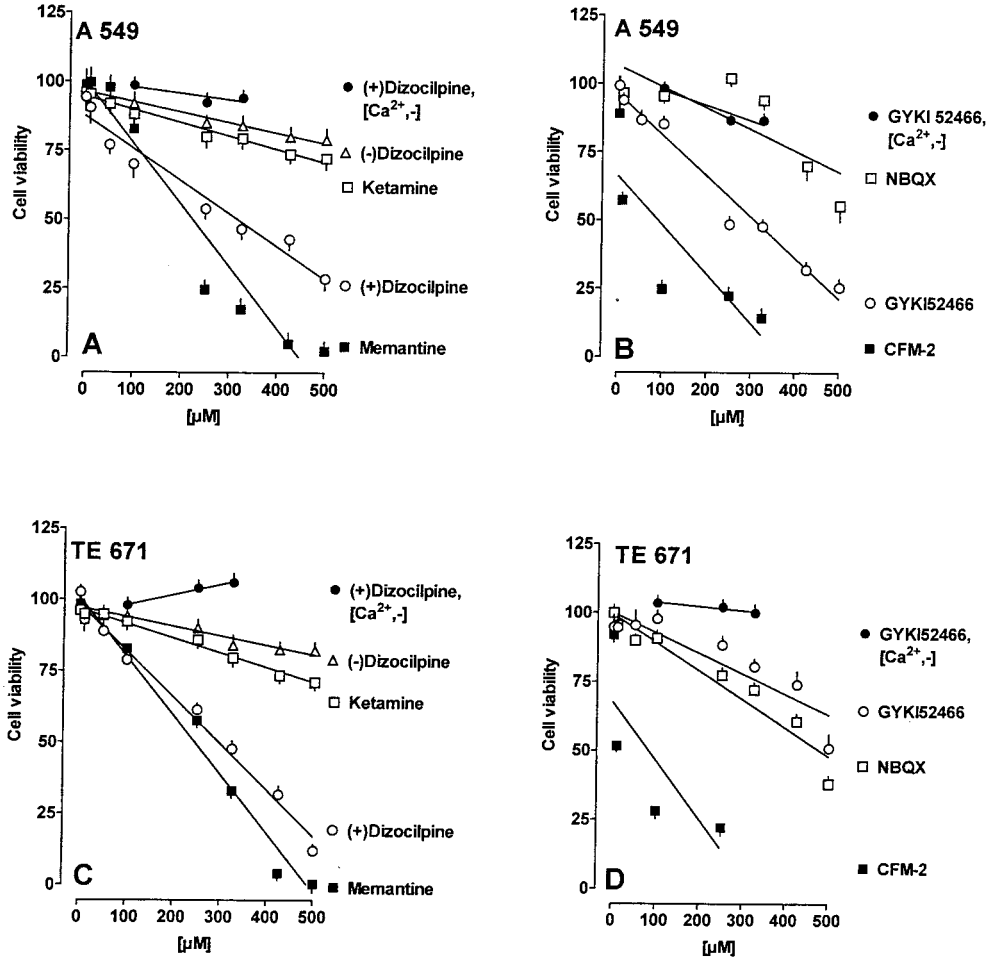

Fig. 3. The antiproliferative effect of dizocilpine and GYKI52466 can be reproduced by other NMDA and AMPA antagonists in human lung carcinoma (A549) and human rhabdomyosarcoma/medulloblastoma (TE671) cells and is reversed by Ca^{2+} deprivation. A549 (*A*) and TE671 (*C*) cells were exposed to either culture medium alone (control), or the NMDA antagonists (+)dizocilpine, (−)dizocilpine, ketamine, or memantine in concentrations ranging from 1 to 500 μM for 96 h, and viability was measured photometrically by means of the MTT assay. Data represent mean normalized optical densities ± SEM of 4–6 trials and were analyzed by means of analysis of variance. The antiproliferative effect of dizocilpine was abolished in Ca^{2+}-free medium in lung carcinoma (*A*) [$F_{A549}(1,37) = 77.10$, $P < 0.001$] and rhabdomyosarcoma/medulloblastoma cells (*C*) [$F_{TE671}(1,50) = 42.11$, $P < 0.001$]. The antiproliferative effect of (−)dizocilpine was less pronounced in both tumor cell lines compared to the effect of (+)dizocilpine [$F_{A549}(1,80) = 128.52$, $P < 0.001$; $F_{TE671}(1,80) = 268.60$, $P < 0.001$]. (*B* and *D*) A549 and TE671 cells were exposed to either culture medium alone (control) or the AMPA antagonists CFM-2 and NBQX in concentrations ranging from 1 to 500 μM for 96 h, and viability was measured photometrically by means of the MTT assay. Data represent mean normalized optical densities ± SEM of 4–6 trials and were analyzed by means of ANOVA. The antiproliferative effect of GYKI52466 was significantly reduced in Ca^{2+}-free medium in lung carcinoma (*B*) [$F_{A549}(1,39) = 51.27$, $P < 0.001$] and abolished in rhabdomyosarcoma/medulloblastoma cells (*D*) [$F_{TE671}(1,45) = 17.34$, $P < 0.001$].

dium. In the serum-replacement medium, glutamate stimulated the proliferation of lung carcinoma cells in a concentration-dependent manner (0.5–10 mM) with a maximum of 21 ± 3% at 10 mM ($n = 4$). These observations indicate that glutamate may have a trophic effect on lung carcinoma cells. Similarly, NMDA (10–250 μM), serine (100–500 μM), or α-amino-3-hydroxy-5-tert-butyl-4-isoxazole-propionate (ATPA; 0.1–10 μM) did not affect proliferation of lung carcinoma or rhabdomyosarcoma/medulloblastoma cells in medium containing 10% serum. NMDA and serine, but not ATPA, stimulated the proliferation of lung carcinoma cells in serum-deprived or serum-replacement medium.

Immunocytochemistry. To verify that tumor cells express subunits forming NMDA or AMPA receptor/ion channel complexes, we applied immunocytochemistry to colon adenocarcinoma (LS180), astrocytoma (MOGGCCM), lung (A549) and breast (T47D) carcinoma, neuroblastoma (SKNAS), rhabdomyosarcoma/medulloblastoma (TE671), and thyroid carcinoma (FTC238) cells as well as to human skin fibroblasts by using antibodies against either the NR1 subunit of the NMDA receptor/ion channels or the GluR2/3 subunits of the AMPA receptor/ion channels. A positive immunostaining for both subunits was detected in all tumor cell lines, whereas no immunoreactivity was present in human skin fibroblasts and tumor cells not exposed to primary antibodies.

Glutamate Antagonists Alter Morphology and Limit Migration of Tumor Cells. To evaluate the effect of glutamate antagonists on tumor cell morphology and migration, lung carcinoma (A549), rhabdomyosarcoma/medulloblastoma (TE671), and thyroid carcinoma (FTC238) cells were exposed to the NMDA antagonist dizocilpine (100 μM) or the AMPA antagonist GYKI52466 (100 μM) for 96 h, and their morphology was examined by light and scanning electron microscopy. Light microscopy revealed that dizocilpine induced rounded cell appearance with prominent vacuoles in the cytoplasm, whereas exposure to GYKI52466 produced less prominent vacuoles and shrinkage of the cells.

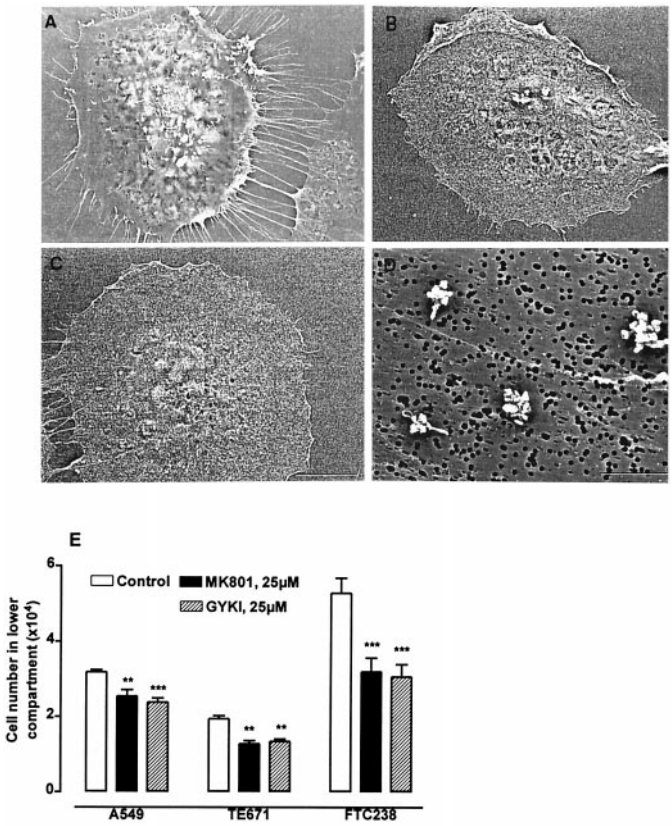

Fig. 4. Glutamate antagonists alter tumor cell morphology and decrease tumor cell motility. Scanning electron micrographs of thyroid carcinoma cells (FTC238) under control conditions (*A*) and after exposure to dizocilpine (*B*, 100 μM) or GYKI52466 (*C*, 100 μM). Tumor cells display numerous pseudopodia (*A*), which are far less prominent after exposure to glutamate antagonists (*B* and *C*). (*D*) Protrusion of tumor cell pseudopodia of FTC238 cells through the 0.4-μm pores of a polycarbonate filter are shown under control conditions. After exposure to dizocilpine (100 μM) or GYKI52466 (100 μM), protrusion of cell pseudopodia was nearly absent. (*E*) Glutamate antagonists decrease migration of A549, TE671, and FTC238 cells. The 1×10^5 cells were placed on the upper chambers of 12-well transwells (polycarbonate filter with 3-μm pore size) and allowed to grow for 96 h alone or in the presence of dizocilpine (25 μM) or GYKI52466 (25 μM). The cells, which migrated into the lower chamber through the 3-μm pores of the polycarbonate filter, were counted. Results represent mean ± SEM of six measurements. **, $P < 0.01$; ***, $P < 0.001$ vs. control, Student's *t* test. (Scale bars = 10 μm in *A–C* and 5 μm in *D*.)

Electron microscopy revealed that tumor cells displayed an invasive phenotype with marked membrane ruffling and numerous pseudopodia (Fig. 4 *A* and *D*). In contrast, tumor cells exposed to glutamate antagonists displayed a noninvasive phenotype with fewer pseudopodial protrusions (Fig. 4 *B* and *C*).

To test the hypothesis that glutamate antagonists may decrease tumor cell locomotion and invasiveness, we examined motility of lung carcinoma (A549), rhabdomyosarcoma/medulloblastoma (TE671), and thyroid carcinoma (FTC238) cells exposed to NMDA or AMPA antagonists. For that purpose, cells were plated on polycarbonate membrane filters with 3-μm pore size in the presence and absence of dizocilpine or GYKI52466. In cultures exposed to dizocilpine or GYKI52466, fewer cells migrated through the filters than in control cultures (Fig. 4*E*).

Glutamate Antagonists Enhance Tumoricidal Effects of Cytostatic Drugs. Lung carcinoma (A549), astrocytoma (MOGGCCM), neuroblastoma (SKNAS), and rhabdomyosarcoma/medulloblastoma (TE671) cells were subjected to treatment with cyclophosphamide, cisplatin, thiotepa, or vinblastin and either dizocilpine (100 μM) or GYKI52466 (100 μM). The cytostatic drugs decreased tumor cell viability in a concentration-dependent manner. This effect was enhanced in all cell lines tested by both (+)dizocilpine and GYKI52466. Fig. 5 shows enhancement of antiproliferative effect of cyclophosphamide and thiotepa by (+)dizocilpine and GYKI52466 in rhabdomyosarcoma/medulloblastoma and neuroblastoma cells. Comparable effects were observed in lung carcinoma and astrocytoma cells subjected to (+)dizocilpine or GYKI52466 and cyclophosphamide or thiotepa. Enhancement of antiproliferative effects of cytostatic agents by glutamate antagonists was due to enhanced tumor cell death as demonstrated by means of the LDH assay and to decreased cell division as demonstrated by means of the BrdUrd incorporation assay. With the use of trypan blue staining, both (+)dizocilpine and GYKI52466 were found to enhance toxicity of cytostatic drugs at concentrations as low as 10 μM.

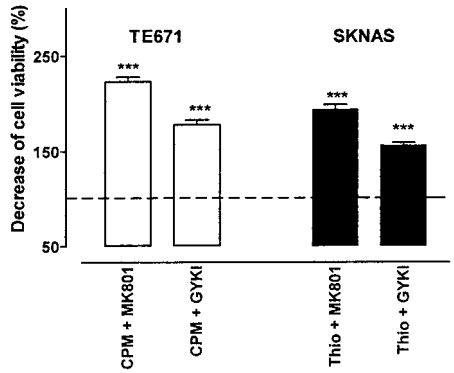

Fig. 5. Glutamate antagonists enhance antiproliferative effect of cytostatic drugs. Cyclophosphamide (CPM; 1.5 mM) and thiotepa (100 μM) decreased viability of TE671 and SKNAS cells as measured by means of the MTT assay. The effect of cyclophosphamide and thiotepa on viability of TE671 and SKNAS cells was enhanced by dizocilpine (100 μM) and GYKI52466 (100 μM). Tumor cells were incubated for 96 h in culture medium (control) and with either cytostatic drug in the absence or presence of glutamate antagonists. Decrease of cell viability by the cytostatic drug in control cultures was set as 100% (dotted line). Enhancement of cytostatic effect by glutamate antagonists is expressed as % of the cell viability decrease achieved with the cytostatic drug alone. Bars represent mean increase of cytostatic effect expressed in percentage ± SEM ($n = 6$). ***, $P < 0.001$ vs. cytostatic drug alone, Student's *t* test.

Discussion

Glutamate antagonists inhibited division and migration, enhanced death and altered morphology of tumor cells *in vitro* resembling cytostatic drugs used in therapy of cancer. Antiproliferative effect of the NMDA antagonist dizocilpine could be reproduced by two other NMDA antagonists memantine and ketamine suggesting receptor/ion channel specificity. It was also stereoselective confirming involvement of NMDA receptor/ion channel complexes. Antiproliferative effect of GYKI52466 could be reproduced by two other AMPA antagonists CFM-2 and NBQX implicating involvement of AMPA receptor/ion channel complexes. Furthermore, tumor cells displayed immunoreactivity for subunits NR1 and GluR2/3 indicating that they express membrane proteins similar to those that form NMDA or AMPA receptor/ion channel complexes (19). Glutamate stimulated the proliferation of tumor cells in serum-deprived culture media. The stimulatory effect of glutamate on tumor growth under serum deprivation suggests that glutamate itself can elicit trophic effect on tumor cells.

Ca^{2+} was identified as a key mediator of antiproliferative action of glutamate antagonists because tumor cells grown under Ca^{2+} deprivation were resistant to the antiproliferative effects of

both NMDA and AMPA antagonists. Elevation of extracellular Ca^{2+} as well as stimulation of Ca^{2+} entry into tumor cells have stimulatory effects on tumor growth (20, 21). Furthermore, Ca^{2+} is necessary for cell division, homeostasis, and survival (22–24) and regulates protein trafficking through the nuclear membrane (25). Ca^{2+} is also essential for formation of cell processes; it regulates axon extension and pathfinding, controls formation of pseudopodia, and affects migration (26–29). Glutamate receptor/ion channel complexes, including the embryonic ones, are permeable to Ca^{2+} (30–32).

Little is known about functional properties of receptor/ion channel complexes on tumor cells responding to glutamate. Glutamate can depolarize membranes and may lead to elevation of intracellular Ca^{2+} in astrocytoma or neuroblastoma cells (33, 34). The resting membrane potential ranges in tumor cells between -30 and -50 mV (35, 36). Patch clamp recordings in neurons demonstrate that at such membrane potentials Mg^{2+} block of NMDA channels is reduced and that AMPA channels become permeable to cations (37, 38). Should this apply to tumor cells, then the glutamate-regulated ion channels in tumor cell membranes should become more permeable to ions regardless of whether or not the glutamate-binding site is occupied by agonists. This assumption implies that glutamate-dependent receptor/ion channel complexes on tumor cells could contribute to regulation of proliferation and migration of tumor cells via modulation of Ca^{2+} homeostasis, as they do with neuronal progenitors during embryogenesis (39).

Tumor cell lines demonstrated different preferential susceptibility to antiproliferative action of glutamate antagonists *in vitro*. Tumors of peripheral origin responded favorably to either NMDA or AMPA antagonists, whereas those derived from neuronal and glial tissues were less sensitive to glutamate antagonists. Such susceptibility differences could aid the design of optimal chemotherapeutic regimens taking into account the tissue of tumor origin.

NMDA and AMPA antagonists enhanced tumoricidal effects of cytostatic drugs *in vitro* by inhibiting tumor cell proliferation and enhancing tumor cell death. These observations suggest that glutamate antagonists possess anticancer potential, which could add to existing therapies of cancer. Because glutamate NMDA and AMPA receptor/ion channel complexes on tumor cells may differ from those of mature receptor/ion channel complexes, the design of novel drugs with fewer side effects seems feasible. In addition, NMDA and AMPA antagonists, which do not penetrate the blood–brain barrier and therefore do not cause neurological side effects, may be suitable for therapy of peripheral cancers.

1. Sporn, M. B. (1996) *Lancet* **347,** 1377–1381.
2. Vijayakumar, S. & Hellman, S. (1997) *Lancet* **349,** S1–S3.
3. Vokes, E. V. (1997) *Lancet* **349,** S4–S6.
4. Scott, A. M. & Cebon, J. (1997) *Lancet* **349,** S19–S22.
5. Cavalheiro, E. A., Lehmann, J. & Turski, L. (1988) *Frontiers in Excitatory Amino Acid Research* (Liss, New York).
6. Lee, J.-M., Zipfel, G. J. & Choi, D. W. (1999) *Nature (London)* **399,** A7–A14.
7. McNamara, J. O. (1999) *Nature (London)* **399,** A15–A22.
8. Turski, L., Schoepp, D. D. & Cavalheiro, E. A. (2001) *Excitatory Amino Acids: Ten Years Later* (IOS, Amsterdam).
9. Kleinschmidt, A., Bear, M. F. & Singer, W. (1987) *Science* **238,** 355–358.
10. Komuro, H. & Rakic, P. (1993) *Science* **260,** 95–97.
11. Ikonomidou, C., Bosch, F., Miksa, M., Bittigau, P., Vöckler, J., Dikranian, K., Tenkova, T. I., Stefovska, V., Turski, L. & Olney, J. W. (1999) *Science* **283,** 70–74.
12. Behar T. N., Scott, C. A., Greene, C. J., Wen, X., Smith, S. V., Maric, D., Liu, Q.-Y., Colton, C. A. & Barker, J. L. (1999) *J. Neurosci.* **19,** 4449–4461.
13. Welch, D. R., Fabra, A. & Nakajima, M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 7678–7682.
14. Said, S. I., Berisha, H. I. & Pakbaz, H. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4688–4692.
15. Chenu, C., Serre, C. M., Raynal, C., Burt-Pichat, B. & Delmas, P. D. (1998) *Bone* **22,** 295–299.
16. Patton, A. J., Genever, P. G., Birch, M. A., Suva, L. J. & Skerry, T. M. (1998) *Bone* **22,** 645–649.
17. Peet, N. M., Grabowski, P. S., Laketic-Ljubojevic, I. & Skerry, T. M. (1999) *FASEB Lett.* **13,** 2179–2185.
18. Laketic-Ljubojevic, I., Suva, L. J., Maathuis, F. J. M., Sanders, D. & Skerry, T. M. (1999) *Bone* **25,** 631–637.
19. Seeburg, P. H., Bresink, I. & Turski, L. (1998) *Excitatory Amino Acids: From Genes to Therapies* (Springer, Berlin).
20. Meloni, F., Brochieri, A., Ballabio, P. C., Tua, A., Grignani, G. & Grassi, G. G. (1998) *Monaldi Arch. Chest Dis.* **53,** 405–409.
21. Celli, A., Treves, C., Nassi, P. & Stio, M. (1999) *Neurochem. Res.* **24,** 691–698.
22. Gleason, E. L. & Spitzer, N. C. (1998) *J. Neurophysiol.* **79,** 2986–2998.
23. Clapham, D. E. (1995) *Cell* **80,** 259–268.
24. Horner, P. J. & Gage, F. H. (2000) *Nature (London)* **407,** 963–970.
25. Stehno-Bittel, L., Perez-Terzic, C. & Clapham, D. E. (1995) *Science* **270,** 1835–1838.
26. Marks, P. W. & Maxfield, F. R. (1990) *J. Cell Biol.* **110,** 43–52.
27. Nakato, K., Furuno, T., Inagaki, K., Teshima, R., Terao, T. & Nakanishi, M. (1992) *Eur. J. Biochem.* **209,** 745–749.
28. Lawson, M. A.& Maxfield, F. R. (1995) *Nature (London)* **377,** 75–79.
29. Gomez, T. M. & Spitzer, N. C. (1999) *Nature (London)* **397,** 350–355.
30. Gallo, V., Pende, M., Cherer, S., Molne, M. & Wright, P. (1995) *Neurochem. Res.* **20,** 549–560.
31. Bardoul, M., Levallois, C. & Konig, N. (1998) *J. Chem. Neuroanat.* **14,** 79–85.
32. Scherer, S. E. & Gallo, V. (1998) *J. Neurosci. Res.* **52,** 356–368.
33. Van der Valk, J. B. & Vijverberg, H. P. (1990) *Eur. J. Pharmacol.* **185,** 99–102.
34. Casado, M., Lopez-Guajardo, A., Mellstrom, B., Naranjo, J. R. & Lerma, J. (1996) *J. Physiol. (London)* **490,** 391–404.
35. Iwata, M., Komori, S., Unno, T., Minamoto, N. & Ohashi, H. (1999) *Br. J. Pharmacol.* **126,** 1691–1698.
36. Sonnier, H., Kolomytkin, O. V. & Marino, A. A. (2000) *Cell. Mol. Life Sci.* **57,** 514–520.
37. Burnashev, N., Zhou, Z., Neher, E. & Sakmann, B. (1995) *J. Physiol. (London)* **485,** 403–418.
38. Jonas, P. & Burnashev, N. (1995) *Neuron* **15,** 987–990.
39. Komuro, H. & Rakic, P. (1996) *Neuron* **17,** 275–285.