Research Paper

AMPA Antagonists Inhibit the Extracellular Signal Regulated Kinase Pathway and Suppress Lung Cancer Growth

Andrzej Stepulak 1,2
Marco Sifringer 3
Wojciech Rzeski 4,5
Katja Brocke 1
Alexander Gratopp 3
Elena E. Pohl 6
Lechoslaw Turski 7
Chrysanthi Ikonomidou 1, *

1 Department of Pediatric Neurology, Children’s Hospital, Medical Faculty Carl Gustav Carus, Technical University Dresden; Dresden, Germany
2 Department of Biochemistry and Molecular Biology, Medical University of Lublin; Lublin, Poland
3 Department of Anatomy, Institute of Cell Biology and Neurobiology, Charité, Berlin, Germany
4 Department of Toxicology, Institute of Agricultural Medicine, Lublin, Poland
5 Department of Virology and Immunology, Institute of Microbiology and Biotechnology, Maria Curie-Sklodowska University, Lublin, Poland
6 Solvay Pharmaceuticals Research Laboratories; Weesp, The Netherlands
7 Correspondence to: C. Ikonomidou; Department of Pediatric Neurology, Children’s Hospital, Medical Faculty Carl Gustav Carus, Technical University Dresden; Fetscherstrasse 74; Dresden 01307 Germany; Tel.: +49.351.4582230; Fax: +49.351.4584355; Email: irissanthi.ikonomidou@uniklinikum-dresden.de

Original manuscript submitted: 06/08/07
Revised manuscript submitted: 08/27/07
Manuscript accepted: 09/01/07

Previously published as a Cancer Biology & Therapy E-publication: http://www.landesbioscience.com/journals/cbt/article/4965

KEY WORDS
AMPA receptor antagonists, lung cancer, ERK1/2 pathway, growth factors, cell cycle

ACKNOWLEDGEMENTS
This study was supported in part by DFG grant IK-2/6-1.

ABSTRACT
Antagonists at α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-type glutamate receptors limit growth of human cancers in vitro. However, the mechanism of anticancer action of AMPA antagonists is not known. Here we report that the AMPA antagonists GYKI 52466 and CFM-2 inhibit the extracellular signal regulated kinase (ERK1/2) pathway, an intracellular signaling cascade which is activated by growth factors and controls proliferation of lung adenocarcinoma cells. AMPA antagonists reduced phosphorylation of cAMP-responsive element binding protein (CREB), suppressed expression of cyclin D1, upregulated the cell cycle regulators and tumor suppressor proteins p21 and p53 and decreased number of lung adenocarcinoma cells in G2 and S phases of the cell cycle. These findings reveal potential mechanism of antiproliferative action of AMPA antagonists and indicate that this class of compounds may be useful in the therapy of human cancers.

ABBREVIATIONS
1-(4-Aminophenyl)-4-methyl-7,8-methyleneoxy-5H-2,3-benzodiazepine hydrochloride (GYKI 52466); 1-(4’-Aminophenyl)-3,5-dihydroxy-7,8-dimethoxy-4H-2,3-benzodiazepine-4-one (CMF-2); α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA); 5-bromo-2′-deoxyuridine (BrdUrd); 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT); calmodulin-dependent kinase II (CaMKII); CAMK II; CAMK II; cAMP-responsive element binding protein (CREB); dithiothreitol (DTT); extracellular signal regulated kinase 1 and 2 (ERK1/2); glutamate receptor 2 (GluR2); glutamate receptor-interacting protein (GRIP); mitogen-activated protein kinase (MAPK); N-methyl-D-aspartate (NMDA); polyacrylamide gel electrophoresis (PAGE); protein interacting with C kinase (PICK); phorbol 12-myristate 13-acetate (PMA); phenylmethanesulfonyl fluoride (PMSF); radioimmuno-precipitation buffer (RIPA); reverse-transcriptase polymerase chain reaction (RT-PCR); sodium dodecyl sulphate (SDS).

INTRODUCTION
Glutamate is a neurotransmitter in the mammalian central nervous system which also influences neuronal proliferation, migration and survival during development. In the last decade evidence has emerged implicating a role for glutamate as signal mediator in non-neuronal tissues, in autocrine or paracrine systems, as well as in tumors. It has been reported that gliomas derived from human biopsy specimen and many human tumor cell lines can release glutamate. It has also been reported that glutamate stimulates tumor cell proliferation and motility via activation of glutamate receptors. Blockade of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-type glutamate receptors inhibits migration, induces apoptosis in human glioblastoma cells and decreases proliferation in several non-neuronal cancer cell lines. AMPA receptors are homo- or hetero-tetramers composed of subunits known as GluR1-GluR4. They mediate fast excitatory synaptic signalling in the brain and are involved in activity-dependent modulation of synaptic plasticity. AMPA receptors gate Na+/Ca2+ in response to ligand binding. Conductance and kinetic properties of the channel depend upon subunit-composition. Influx of ions causes a fast excitatory postsynaptic response in neurons, and Ca2+ can also activate second messenger pathways. The GluR2 subunit plays a critical role in determination of the permeability of AMPARs...
to Ca^{2+} receptors containing GluR2 are impermeable for calcium. However, AMPARs have been reported to also have metabotropic properties.\textsuperscript{11,12}

Stimulation of AMPA receptors in neurons has been linked to activation of the extracellular-signal-regulated kinases ERK1/2, members of the mitogen-activated protein kinase (MAPK) family.\textsuperscript{13,14} ERK1/2 has been shown to respond to a wide variety of extracellular stimuli, such as growth factors and mitogens, resulting in increased cell proliferation and migration.\textsuperscript{15,16}

In cancer cells the ERK1/2 signalling pathway often undergoes abnormal and sustained activation due to frequent mutation of the \textit{ras} and \textit{raf} genes and constitutive activation of MEK1/2 and ERK1/2 kinases, contributing to the uncontrolled growth of tumors.\textsuperscript{17,18}

ERKs can phosphorylate and activate the downstream kinase target p90RSK, which in turn leads to subsequent phosphorylation and activation of the CREB transcription factor.\textsuperscript{19} CREB activation results in increased expression of \textit{c-fos}\textsuperscript{20} and genes involved in cell cycle progression such as \textit{cyclin D1}\textsuperscript{21} or inhibition of apoptosis such as \textit{bcl-2}.\textsuperscript{22} C-Fos and c-Jun proteins are components of AP1 transcription factor, whose activation is associated with cell proliferation, differentiation or apoptosis.\textsuperscript{23} Expression of genes such as \textit{cyclin D1, cyclin A, p53, p21, p16} are under AP1 control.\textsuperscript{17} Regulation of c-Fos and c-Jun activity occurs mainly at the transcriptional level, and their induction is ERK1/2 dependent.\textsuperscript{17,24} We recently demonstrated that dizzinone, a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors, is able to suppress this pathway, resulting in decrease of cancer cell proliferation in vitro and in vivo.\textsuperscript{25}

Here we demonstrate that the non-competitive AMPA receptor antagonists GYKI 52466 [1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride] and CFM-2 [1-(4’-Aminophenyl)-3,5-dihydro-7,8-dimethoxy-4H-2,3-benzodiazepin-4-one] inhibit activation of ERK1/2 kinases cascade in A549 lung cancer cell line in vitro, decrease CREB phosphorylation and expression of \textit{c-fos, c-jun, cyclin D1} genes and increase expression of cell cycle inhibitory genes \textit{p21, waf1} and \textit{p53}. These effects may be responsible for decreased proliferation of cancer cells exposed to AMPA antagonists.

\section*{MATERIAL AND METHODS}

\textbf{Cell culture.} Human Caucasian lung carcinoma A549 cells were obtained from the Institute of Immunology and Experimental Therapy (Polish Academy of Sciences, Wroclaw, Poland) and were grown in 2:1 mixture of Dulbecco’s modified Eagle Medium (Gibco, Paisley, U.K.) and Ham’s F12 Medium (Bio Whittaker Europe, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 \text{\mu g/ml streptomycin} and 2 \text{mM L-glutamine}. Cells were maintained in a humidified incubator with 5% CO\textsubscript{2} in air at 37°C. To determine the effect of GYKI 52466 (Sigma, St. Louis, MO) on kinase activity, A549 cells were incubated with different concentrations (10–250 \text{M}) of GYKI 52466 and CFM-2 for indicated time periods. Cells were also exposed for three hours to 10 \text{M} U0126 and 200 \text{nM PMA} or for six hours to 200 \text{nM Wortmanin, 50 \text{M} KN93} (Calbiochem, La Jolla, CA) and for six hours and 24 hours to 250 \text{M CFM-2} (Tocris, Ellisville, MO). A549 cells were also incubated with 0.1–10 \text{M} concentration of AMPA, insulin growth factor (IGF, 100 \text{ng/ml}) and epidermal growth factor (EGF, 10 \text{ng/ml}), in media supplemented with serum-free replacement medium and without fetal calf serum. When indicated, cells were pre-treated with increased concentration of GYKI 52466.

\textbf{Immunofluorescence and cell staining.} A549 cells were grown on cover slips, then fixed with 100% methanol (-20°C, 5 min), and probed with rabbit anti-GluR2 antibody (2.0 \text{Mg/ml in 1% BSA/PBS, Chemicon, Temecula, CA}) and anti-phospho ERK1/2 antibody (1:150, 5% BSA/TTBS, Cell Signalling), followed by incubation with chicken anti-rabbit Alexa Fluor 594 secondary antibody (5 \text{Mg/ml in 1% BSA/TBS, Molecular Probes, Eugene, OR}). Stained cells were analyzed using an upright confocal laser-scanning microscope (Leica, Heidelberg, Germany) equipped with 40X or 63X oil immersion objectives. Alexa Fluor 594 was excited by the 543nm line of the GreenNe laser. Emitted fluorescence was measured in the range 600–680 nm. Images were processed off-line using Image J (W. Rasband, NIH) and Volocity (Improvision) software.

\textbf{Flow cytometric analysis.} Experiments were performed using a FACScan\textsuperscript{TM} flow cytometer (Becton Dickinson, Heidelberg, Germany), equipped with a 488 nm argon laser. For cell cycle analyses, cells were stained with propidium iodide utilizing the CycleTEST PLUS DNA Reagent kit (BD Pharmingen, Heidelberg, Germany) according to the manufacturer’s instructions. Acquisition rate was at least 60 events per second in low acquisition mode and at least 20,000 events were measured. Cell cycle analysis was performed by using noncommercial flow cytometry analyzing software (Cylched Version 1.0.2 for Windows; source: www.uwcm.ac.uk/study/medicine/haematology/cytonetuk/documents/software.htm) and WinMDI 2.8 for Windows (source: facs.scripps.edu/software.html). The cells were acquired and gated by using dot plot FL-2 Width (X) versus FL-2 Area (Y)-gate to exclude aggregates and analyzed in histograms displaying fluorescence 2-area (yellow-orange fluorescence: 585 nm).

\textbf{Cell viability/growth assay.} Cells were plated on 96-well microplates (NUNC, Roskilde, Denmark) at a density of 10\textsuperscript{4} A549 cells/ml. On the following day the culture medium was removed and cells were exposed to 10–250 \text{M} of GYKI 52466 or CFM-2, and 0.1–10 \text{M} of AMPA. Cell proliferation was assessed after 48-96 h by using the MTT method in which the yellow tetrazolium salt 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) is metabolized by viable cells to purple formazan crystals. Tumor cells were incubated for 3 h with MTT solution (5 mg/ml). Formazan crystals were solubilized overnight in SDS buffer (10% SDS in 0.01 N HCl) and the product was quantified spectrophotometrically by measuring absorbance at 570 nm wavelength using the Victor Multilabel Counter (PerkinElmer-Wallac, Freiburg, Germany).

\textbf{Cell proliferation assay.} Tumor cells were incubated with 10 \text{M} of 5-bromo-2-deoxyuridine (BrdUrd; BrdUrd labeling and detection kit II; Roche Diagnostics GmbH, Mannheim, Germany) over 18 h. Cells were subsequently fixed with 0.5 M ethanol/HCl and were then incubated with nucleases to partially digest DNA. Monoclonal anti-BrdUrd antibodies conjugated to peroxidase were subsequently added and detected using 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate. Quantitation was performed colorimetrically at 405–490 nm wavelength.

\textbf{Western blot analysis.} After treatment cells were harvested and lysed in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 20 mM NaF,
0.5 mM DTT, 1 mM PMSF and protease inhibitor cocktail in PBS pH 7.4), then centrifuged at 3,000 x g for 10 min. For Western blot analysis supernatants of RIPA cell lysates were solubilized in 3x Laemmli sample buffer (30% glycerol, 3% SDS, 0.19 M Tris-HCl, pH 6.8, 0.015% bromophenol blue, 3% β-mercaptoethanol), then boiled for 5 min at 100°C. Equal amounts of cellular protein extract were electrohoresed on 10% SDS-PAGE under reducing conditions and transferred by electroblotting onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). After blocking for one hour at room temperature (RT) with 5% non-fat dry milk/ TBS/0.1% Tween, membranes were probed overnight at 4°C with primary antibody as follows: anti-phospho-ERK1/2, anti-phospho-MEK, anti-phospho-p90RSK, anti-phospho-CREB (Ser133), anti-phospho-Akt (Ser473), anti-Akt (1:1000 in 1% BSA/TBS/0.1% Tween, Cell Signaling), anti-ERK2, anti-phospho-CaMKII (Thr286) and anti-CaMKII (1:500 in 1% BSA/1% non-fat dry milk/ TBS/0.1% Tween, Santa Cruz Biotechnology), then incubated with the secondary antibody coupled to horseradish peroxidase (1:5000 in 1% BSA/TBS/0.1% Tween, Amersham Pharmacia Biosciences, Buckinghamshire, UK) and serial exposures were made on autoradiographic film (Hyperfilm ECL, Amersham Pharmacia Biosciences). For stripping, membranes were incubated with stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50°C for 20 min, then washed, blocked and probed with the relevant antibody, as described above.

RNA isolation. Total RNA from A549 cells was isolated by acidic phenol/chloroform extraction and DNaseI treated. The concentration of RNA was determined spectrophotometrically, and the integrity of all samples was confirmed by electrophoresis in ethidium bromide-stained 1.0% agarose gels.

Semiquantitative RT-PCR. 500 ng of RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and oligo d(T)16 primer (Promega) in 25 µl of reaction mixture. The resulting cDNA (1-2 µl) was amplified by polymerase chain reaction for bcl-2, c-fos, c-jun, cyclin D1, p21, p53, GluR2. Primer sequences and cycle conditions for PCR amplification are available from the authors on request. Amplified cDNA was subjected to polyacrylamide gel electrophoresis, subsequent silver staining and densitometric analysis with the image analysis program BioDocAnalyze (Whatman Biometra, Göttingen, Germany). The housekeeping gene β-actin was co-amplified as an internal control.

Statistical analysis. ANOVA with Bonferroni test for multiple comparisons and Student’s t-test were used. Data are expressed as the mean ± SEM. (*p < 0.05, **p < 0.01, ***p < 0.001).

RESULTS

A549 human lung cancer cells express AMPA receptor. There is accumulating evidence on the expression of glutamate receptors outside the central nervous system. We analysed expression of AMPA receptor subunits in A549 human lung cancer cells. RT-PCR analysis revealed presence of mRNA for GluR2 receptor subunit and lack of other AMPA receptor subunits in A549 cells (Fig. 1A). These findings were confirmed by immunocytochemistry, showing expression of GluR2 protein in A549 cells. Confocal microscopy revealed cytoplasmic and cell membrane staining with GluR2 subunit specific antibody (Fig. 1B).

AMPA receptor antagonists GYKI 52466 and CFM-2 decrease proliferation of lung cancer cells. To determine whether lung cancer cells respond to AMPA antagonists by growth inhibition, human non-small lung cancer cell line A549 was treated with increasing concentrations of GYKI 52466 or CFM-2 over a period of 96 hours. Cell growth was measured by means of MTT assay. Incubation with GYKI 52466 or CFM-2 over 96 hours reduced cell viability compared with non-exposed cells in a concentration dependent manner (Fig. 2A and C). The effect of GYKI 52466 and CFM-2 on cancer cell proliferation was attributed to decreased cell division, as determined by decreased incorporation of BrdUrd. Incubation of A549 cells with 200

Figure 1. AMPA receptor subunits are present in A549 lung adenocarcinoma cells. (A) Polyacrylamid gel electrophoresis illustrating results of RT-PCR analysis with primers specific for human GluR1, GluR2, GluR3 and GluR4 subunits in brain samples [brain] and A549 cells. There is expression of mRNA specific for GluR2 (line 4) but no other subunits in A549 cells. The RNA for the housekeeping gene β-actin was coamplified as an internal control. (B) Confocal image demonstrating positive immunostaining for GluR2 subunits in A549 cells, as revealed by immunocytochemistry. In photographs (C-E) immunostaining for phosphorylated ERK1/2 is shown. A549 cells were grown on coverslips, they were further incubated in the absence-control (C) or presence of 250 µM of GYKI 52466 (D) or CFM-2 (E) for six hours. Cells were then fixed with methanol (20°C, 5 min.) and stained using anti-phospho-ERK1/2 antibody and anti GluR2 antibody, followed by incubation with Alexa Fluor 594 secondary antibody and analyzed by confocal microscopy. Under control conditions (C) phospho-ERK1/2 is present within the nucleus of A549 cells. Note reduced nuclear staining for phospho-ERK1/2 in GYKI 52466 (D) and CFM-2 (E) treated cells.
AMPA Antagonists Inhibit ERK1/2 Pathway

Figure 2. The AMPA antagonists GYKI 52466 and CFM-2 suppress growth of lung adenocarcinoma cells. A549 cells were treated with 10–250 μM GYKI 52466 or CFM-2. (A) Normalized cell viability, measured by the MTT assay at 96 h, is presented as mean ± SEM at each concentration. Analysis of variance (ANOVA) and Student’s t-test revealed highly significant effect of treatment with GYKI 52466 on cell viability compared to vehicle treated cultures. There is clear concentration-dependency of anticancer action of GYKI 52466 (***p < 0.001 compared to control, n = 6 at each concentration).

(B) Decreased levels of incorporated BrdUrd in A549 cells following 24 h treatment with GYKI 52466 or CFM-2. (C) Normalized cell viability, measured by the MTT assay at 96 h, is presented as mean ± SEM at each concentration. Analysis of variance (ANOVA) and Student’s t-test revealed highly significant effect of treatment with CFM-2 on cell viability compared to vehicle treated cultures. There is clear concentration-dependency of anticancer action of CFM-2 (**p < 0.01, ***p < 0.001 compared to control, n = 6 at each concentration).

(D) Decreased levels of incorporated BrdUrd in A549 cells following 24 h treatment with CFM-2, 10–250 μM (normalized means ± SEM, n = 8 per column; ***p < 0.001, Student’s t-test). (E) AMPA stimulates proliferation of A549 lung cancer cells in a concentration-dependent manner. A549 cells were exposed for 96 hours to increasing concentrations of AMPA (0.1–10 μM). F) IGF and EGF stimulate lung cancer cell growth. This stimulatory effect is blocked when 10 μM GYKI 52466 or CFM-2 are coadministered. Cell viability was assessed by the MTT assay. Columns represent mean cell viability ± SEM of 6 measurements. Statistical comparisons between control and drug-treatment groups (A–C) or as indicated (F) were performed by means of Student’s t-test (**p < 0.01).

Similar findings were obtained when cells were incubated with CMF-2 (Fig. 2C and D).

Next, we attempted to determine whether AMPA, a specific agonist at AMPA receptors, would increase proliferation of A549 lung cancer cells. AMPA stimulated proliferation of A549 cancer cells in a concentration-dependent manner (Fig. 2E). The effect of AMPA was blocked by GYKI 52466.

A549 cells were also subjected to insulin growth factor (IGF, 100 ng/ml), and epidermal growth factor (EGF, 10 ng/ml) over a period of 96 hrs. Growth factors stimulated tumor cell proliferation, as revealed by MTT assay. When tumor cells were subjected to growth factors in the presence of the AMPA antagonist GYKI 52466 (10 μM), no such stimulatory effect was evident (Fig. 2F). Of note is that this concentration of the AMPA antagonist did not influence tumor cell proliferation when given in the absence of growth factors (Fig. 2A).

AMPA antagonists inhibit the ERK1/2 signalling pathway. The ERK1/2 pathway is activated by several growth factors. Incubation of A549 cells with 10 μM AMPA for six hours activated ERK1/2 kinases (Fig. 3A, left panel). This activation was abolished when lung cancer cells were pre-treated with GYKI 52466. Increasing concentration of GYKI 52466 (up to 50 μM) reduced ERK1/2 phosphorylation to control levels whereas 100 μM GYKI 52466 decreased ERK1/2 phosphorylation below control levels. In addition, treatment of A549 cells with GYKI52466, in the absence of AMPA, also resulted in concentration-dependent decrease of ERK1/2 phosphorylation (Fig. 3A, right panel). Total protein level remained equal, as demonstrated by reblotting the same membrane with ERK2 antibody recognizing total - phosphorylated and non-phosphorylated - forms of the kinase.

Inhibition of ERK1/2 phosphorylation by GYKI52466 (250 μM) was time-dependent, already evident at 1 h after beginning of the exposure and persisting up to 24 h of treatment (Fig. 3B).

To prove that this effect is not compound-specific, we employed another noncompetitive AMPA receptor antagonist, CFM-2, and exposed A549 cells for six hours to CFM-2. We chose this exposure time, because we observed significant decrease of ERK1/2 kinase phosphorylation at six hours after

μM of GYKI 52466 resulted in 60% reduction of BrdUrd incorporation (Fig. 2B), which mirrored the inhibition of cell growth up to around 60%. Similar reduction of cell viability was measured by the MTT assay at the 250 μM GYKI 52466 concentration (Fig. 2A).
incubation with GYKI 52466 (Fig. 3B). Treatment with 250 μM of CFM-2 also resulted in decreased levels of phosphorylated ERK1/2 (Fig. 3C). Reduction of ERK1/2 kinase phosphorylation was accompanied by decreased phosphorylation of MEK1/2 kinases, which are upstream regulators of ERK1/2, as well as the downstream kinase p90RSK (Fig. 3B). Reblotting of the same nitrocellulose membranes demonstrated that the expression level of ERK2 protein was unaltered (Fig. 3B and C). Confocal microscopy revealed that treatment of A549 cells with GYKI 52466 or CFM-2 abolished translocation of pERK1/2 into the cell nucleus (Fig. 1C–E).

GYKI 52466 and CFM-2 treatment decreases CREB phosphorylation. CREB is a transcription factor that regulates expression of various target genes and is involved in growth factor signalling.26,27 Phosphorylation of CREB at Ser133 is critical for its transcriptional activation. CREB Ser133 phospho-acceptor site is identified as target for CaM kinases,28 AKT kinase,29 ERK1/2 and p90RSK kinases.30 In our experiments, the AMPA receptor antagonist GYKI 52466 reduced CREB phosphorylation in a time-dependent manner. This effect closely correlated with time-course of inhibition of ERK1/2 kinases (Fig. 3B), suggesting that decrease of CREB phosphorylation is due to ERK1/2 signalling inhibition. Inhibition of CREB phosphorylation was also observed when cancer cells were incubated with CFM-2 for six hours (Fig. 3C).

Treatment of A549 cells with GYKI 52466 influenced activation state of AKT and CaMKII kinases (Fig. 3D and E). We observed initial reduction of AKT kinase phosphorylation after treatment with 250 μM GYKI 52466, but this effect was short lasting and no more present at 3 h of treatment (Fig. 3D). We also observed a slight decrease of the phosphorylation of CaMKII kinase following incubation for one hour with 250 μM GYKI 52466. This effect was absent at later time points (Fig. 3E). Specific inhibitors of AKT and CaM kinases, Wortmannin and KN93, decreased phosphorylation of the kinases, showing functional assays. Amounts of both kinases were unchanged, as demonstrated by reblotting membranes with respective antibodies recognizing total, phosphorylated and non-phosphorylated forms of the kinases (Fig. 3D and E, lower panels). These results suggest that observed prolonged decrease of CREB phosphorylation and activation is mainly due to inhibition of ERK1/2 kinases.

GYKI 52466 and CFM-2 alter expression of CREB-regulated genes. Activation of the CREB transcription factor by phosphorylation at Ser133 promotes the transcription of key target genes required for cell proliferation. Target genes of CREB include the immediate early genes c-fos and cyclin D1.31 To investigate whether the AMPA receptor antagonists GYKI 52466 and CFM-2 influence CREB-dependent gene expression, we analysed the expression of c-fos, c-jun and cyclin D1 in A549 cells. After 24 hours incubation of A549 lung cancer cells with 250 μM GYKI 52466 or CFM-2, RT-PCR revealed decreased expression of cyclin D1, c-fos and c-jun mRNAs (Fig. 4A and B). The expression of cyclin D1, c-fos and c-jun were reduced to 34.5%, 33.9% and 43% of control levels respectively following treatment with GYKI 52466 (Fig. 4A). CFM-2 also reduced the expression of cyclin D1, c-fos and c-jun genes (Fig. 4B). c-Fos and c-Jun proteins form AP1 transcription factor, which is involved in cell proliferation and transformation. Its activity is regulated by growth factors and neurotransmitters.23,32 Several genes implicated in cell proliferation are under control of AP1, including cell cycle regulators p21 and p53. Since GYKI 52466 and CFM-2 treatment resulted in decreased mRNA levels for both c-fos and c-jun, we postulated that antiproliferative agents GYKI 52466 and CFM-2 could affect the expression of p21 and p53 genes. Indeed, as shown in Figure 4B, CFM-2 treatment resulted in increased expression of p21 and p53 genes. Significant changes in the expression of both cell cycle regulators were found
In contrast to standard cytotoxic anti-cancer therapies which affect both cancer and normal cells, novel treatment strategies aim at inhibition of specific intracellular pathways that are deregulated in cancer cells. The ERK1/2 signalling pathway is fundamental for cancer cell proliferation and thereby a potential target for chemotherapeutic interventions.

In this study we have demonstrated that GYKI 52466 and CFM-2, two non-competitive AMPA receptor antagonists, inhibit proliferation of A549 lung adenocarcinoma cells. This effect is accompanied by decreased activity of ERK1/2 kinase and followed by decreased CREB phosphorylation. GYKI 52466 also influenced the activation of other CREB-phosphorylating kinases—CaMKII by decreasing its activity slightly and at an early time point only (one hour) and Akt by moderately increasing its activity. Observed Akt and CaMKII activity after AMPAR antagonists treatment does not correlate with the time-course of CREB hypophosphorylation. This is in agreement with the notion that calmodulin-dependent kinases are responsible for rapid and short-lived CREB phosphorylation, whereas ERK1/2 kinases are responsible for slow and sustained phase of CREB phosphorylation. Thus, ERK1/2 constitutes the predominant pathway mediating CREB phosphorylation in A549 cells.

It has been shown that inhibition of ERK1/2 and CREB phosphorylation inhibits growth of human fibrosarcoma cells. Transfection of dominant-negative CREB resulted in inhibition of growth of human melanoma and human adenocarcinoma A549 cells.

On the other hand, blast cells from patients with acute myeloid or lymphoid leukemias expressed higher levels of CREB compared to the levels found in control patients. Thereby, inhibition of CREB transcriptional activity in lung cancer cells after treatment with GYKI 52466 or CFM-2 could be responsible for decreased cell viability observed in our studies.

Activation of CREB is required for the induction of specific genes, including c-fos. It has been suggested that there is a correlation between higher expression of the fos oncogene and malignant progression of tumor cells. More recently, Lu et al. demonstrated using anti-sense strategy that c-fos is critical for breast cancer cell growth.

In our study we showed that inhibition of CREB phosphorylation by GYKI 52466 or CFM-2 correlated with marked inhibition of both c-fos and c-jun genes. This effect correlated with reduced cancer cell proliferation.

The growth-promoting activity of c-Jun is mediated by repression of tumour suppressors p21 and p53. Hence, AP1 can stimulate cell cycle progression through induction of cyclin D1 transcription and repression of p21 and p53 transcription. In our study, GYKI 52466 and CFM-2 treatment produced opposite effect—downregulation of cyclin D1 and upregulation of p53 and p21, which correlated with slowing of cell cycle progression.

Inhibition of a subset of AP1 target genes is a potential strategy for cancer chemoprevention and renders AMPA antagonists potential chemotherapeutic agents in cancer therapy. Recent studies have uncovered cyclin D1 as a chemopreventive target in lung cancers. Altered cyclin D1 expression could play a critical role in maintenance or progression of a precancerous bronchial lesion. Cyclin D1 is frequently overexpressed in lung cancers and serves as a negative prognostic marker.

It remains an open question, how the signal from the AMPAR is transmitted to the ERK1/2 kinase cascade. AMPA receptors in neurons are coupled to Ca2+/Na+-permeable ion channels. However,
AMP Antagonists Inhibit ERK1/2 Pathway

Figure 5. GYKI 52466 reduces numbers of A549 cells entering the cell cycle. Flow cytometric analysis of propidium iodide-stained cells revealed lower percentages of cells in G0/G1 phase of the cell cycle following exposure to GYKI 52466 (500 μM, green line; 250 μM, red line) compared to controls (grey area). The table shows quantitative results of the cell cycle analysis (% of total ± SEM from 4 measurements). One way ANOVA with Bonferroni multiple comparisons test revealed that the effects of 500 μM GYKI52466 were significant (*p < 0.05; ***p < 0.001 compared to control).

<table>
<thead>
<tr>
<th></th>
<th>Residual G1</th>
<th>S Phase</th>
<th>Residual G2</th>
<th>G2+S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>45.23 ± 2.47</td>
<td>41.9 ± 5.23</td>
<td>12.87 ± 4.32</td>
<td>54.77 ± 2.47</td>
</tr>
<tr>
<td>GYKI 250 μM</td>
<td>48.60 ± 1.58</td>
<td>40.47 ± 3.09</td>
<td>10.93 ± 2.96</td>
<td>51.40 ± 1.58</td>
</tr>
<tr>
<td>GYKI 500 μM</td>
<td>65.35 ± 5.73**</td>
<td>26.57 ± 7.65#</td>
<td>8.10 ± 3.03</td>
<td>34.67 ± 5.70***</td>
</tr>
</tbody>
</table>

AMPA Antagonists Inhibit ERK1/2 Pathway

presence of GluR2 subunits renders the channel impermeable for calcium,12 Since A549 lung cancer cells express GluR2 receptor subunit, it is rather unlikely that observed decrease of ERK1/2 phosphorylation after AMPA antagonist treatment is due to blockade of calcium influx. In addition, negative allosteric modulators such as GYKI 52466 block the AMPA receptor independent of membrane voltage.40 It is possible that the link between AMPA receptor and ERK1/2 cascade is mediated by scaffolding/adaptor protein interactions. It has been demonstrated that NMDA receptors are coupled to the ERK pathway by a direct interaction between NR2B subunit and RapGFR1.41 PDZ domain-containing scaffold proteins GRIP and PICK1 have been shown to interact with the C-terminus of GluR2.42 GRIP binding to GRIP-associated proteins links AMPAR to Ras signalling.43 Finally, Hayashi et al. demonstrated that homomeric GluR2 receptor ectopically expressed in HEK293T cells interacts with tyrosine kinase Lyn, resulting in Lyn kinase-dependent ERK1/2 kinase activation.44 Thus, it is possible that similar pathways are operant in A549 cells.

Inhibition of ERK1/2 kinase was observed already at relatively low (50 μM) concentration of GYKI 52466. Higher concentrations were required to affect cell cycle progression. However, we have demonstrated previously that concentrations up to 250 μM of GYKI 52466 did not affect proliferation of human skin fibroblasts and bone marrow stromal cells,9 and did not induce apoptosis in 14-day old neuronal cell cultures.45 It seems that anti-proliferative effects of AMPAR antagonists are much more prominent in cancer than in normal cells.

Our results further demonstrate that AMPA receptor antagonist GYKI 52466, at a low (10 μM) concentration, inhibits trophic influence of AMPA, EGF and IGF on lung cancer cells. Such actions render AMPAR antagonists possible chemopreventive agents. Importance of IGF signalling for proliferation of lung cancer cells is well documented.57-46 Moreover, it has been demonstrated that lung cancer cells secrete IGF in an autocrine fashion.47

Several compounds which block growth factor mediated stimulation of ERK1/2 pathway are currently under investigation in vitro and in vivo, in mouse xenograft models, and few of them are already in phase II/III clinical trials.18,48 In this study AMPA receptor antagonists, by inhibiting the ERK1/2 kinase pathway, reduce CREB phosphorylation, change the expression of genes involved in cell cycle progression, slow down cell cycle and inhibit proliferation of A549 lung adenocarcinoma cells. Thus, our findings outline therapeutic potential of AMPA antagonists in the treatment of some human cancers.

References

AMPA Antagonists Inhibit ERK1/2 Pathway


