Combined inhibition of the phosphatidylinositol 3-kinase/Akt and Ras/mitogen-activated protein kinase pathways results in synergistic effects in glioblastoma cells

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Abstract

The present study uses cell-based screening assays to assess the anticancer effects of targeting phosphatidylinositol 3-kinase–regulated integrin-linked kinase (ILK) in combination with small-molecule inhibitors of Raf-1 or mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase (MEK). The objective was to determine if synergistic interactions are achievable through the use of agents targeting two key cell signaling pathways involved in regulating glioblastoma cancer. The phosphatidylinositol 3-kinase/protein kinase B (PKB)/Akt and the Ras/MAPK pathway were targeted for their involvement in cell survival and cell proliferation, respectively. The glioblastoma cell lines U87MG, SF-188, and U251MG were transiently transfected with an antisense oligonucleotide targeting ILK (ILKAS) alone or in combination with the Raf-1 inhibitor GW5074 or with the MEK inhibitor U0126. Dose and combination effects were analyzed by the Chou and Talalay median-effect method and indicated that combinations targeting ILK with either Raf-1 or MEK resulted in a synergistic interaction. Glioblastoma cells transfected with ILKAS exhibited reduced levels of ILK and phosphorylated PKB/Akt on Ser473 but not PKB/Akt on Thr308 as shown by immunoblot analysis. These results were confirmed using glioblastoma cells transfected with ILK small interfering RNA, which also suggested enhanced gene silencing when used in combination with U0126. U87MG glioblastoma cells showed a 90% (P < 0.05) reduction in colony formation in soft agar with exposure to ILKAS in combination with GW5074 compared with control colonies. A substantial increase in Annexin V–positive cells as determined by using fluorescence-activated cell sorting methods were seen in combinations that included ILKAS. Combinations targeting ILK and components of the Ras/MAPK pathway result in synergy and could potentially be more effective against glioblastoma cancer than monotherapy. [Mol Cancer Ther 2006;5(3):645–54]

Introduction

Two key cell signaling molecules have been implicated in glioblastoma multiforme pathogenesis (due to their mutation, gene amplification, or overexpression): the epidermal growth factor receptor (EGFR; ref. 1) and the gene for phosphatase and tensin homologue deleted on chromosome ten-PTEN (2). EGFR mutation can result in increased levels of p21-Ras, leading to constitutive activation of the Ras/mitogen-activated protein kinase (MAPK) pathway, which is functionally important in glioma proliferation. Mutations of phosphatase and tensin homologue in glioblastomas have been observed in as few as 15% to as many as 70% of cases (3–5), and loss of this tumor suppressor gene has been shown to result in high levels of protein kinase B (PKB)/Akt activation in glioblastoma multiforme (6). Integrin-linked kinase (ILK) is a serine/threonine kinase that is regulated in a phosphatidylinositol 3-kinase (PI3K)–dependent manner and can phosphorylate PKB/Akt in vitro (7). Conditional knockout of ILK with the Cre-Lox system indicated ILK is vital in PKB/Akt activation (8). For these reasons, our research team has been evaluating treatment strategies involving ILK as a therapeutic target. In the studies reported here, we have assessed the effects alone and in combination with other targeted agents.

3-(3,5-Dibromo-4-hydroxy-benzylidene)-5-iodo-1,3-dihydro-indol-2-one (GW5074) is an inhibitor of Raf-1, such that Raf-1 cannot phosphorylate and thereby activate MAPK/extracellular signal-regulated kinase kinase (MEK) (9). Similarly, U0126 inhibits MEK preventing MAPK phosphorylation (10, 11). This activity halts further signal transduction along the Ras/MAPK pathway. ILK antisense (ILKAS) can inhibit ILK protein production and should therefore reduce ILK activity. This will in turn reduce phosphorylation and hence activation of PKB/Akt and further signaling along the PI3K/Akt pathway. Because the Ras/MAPK and PI3K/Akt pathways have both been implicated in glioblastoma progression (12), it would be anticipated that therapies that target both of these cell signaling pathways may lead to an effective treatment option.
The use of drug combinations for treatment of glioblastoma multiforme is not particularly novel, particularly in the context that almost all chemotherapy regimens involve two or more drugs. The challenges faced by those interested in the development of effective combinations are daunting, particularly when evaluating novel targeted agents that affect pathways that are important, but not essential, for tumor cell survival. When used as single agents, these compounds may have target specific effects, but as a result of redundancy in cellular pathways these agents may have little single-agent effects as judged by in vitro measures of tumor cell cytotoxicity or in vivo assessments of tumor growth inhibition. It is particularly important to define strategies to select drug combinations that exhibit promise in the context of achieving improved therapeutic effects and the first strategy typically considered is cell-based in vitro assessments. These assays, particularly when developed using multiple therapeutic end points, can help select drug combinations that yield specific drug effects (i.e., synergism, additivity, or potentiation). In these studies, the use of small-molecule inhibitors with gene silencing agents [antisense oligonucleotides or small interfering RNA (siRNA)] were assessed for the treatment of glioblastoma multiforme. To our knowledge, this approach has not been studied extensively and the studies summarized here sought to investigate such drug interactions with ILK as a primary target for combination therapy of glioblastoma multiforme.

Materials and Methods

Tumor Cell Lines and Transfections

Phosphatase and tensin homologue (PTEN)–negative U251MG and U87MG glioblastoma cells and PTEN–positive SF-188 glioblastoma cells were obtained from American Type Culture Collection Repository (Manassas, VA). Cells were cultured in DMEM containing 10% fetal bovine serum with 1% l-glutathione and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO2. For all experiments, cells were used in exponential growth phase. The cells were transiently transfected with ILK siRNA, reverse ILK siRNA, nonsilencing control siRNA, ILKAS, or the reverse ILK antisense (RILK) using either LipofectAMINE 2000 reagent (Invitrogen, Frederick, MD) or the Nucleofector technology (Amaxa Biosystems, Gaithersburg, MD). LipofectAMINE 2000 was used according to the manufacturer’s guidelines using 1 to 4 μg antisense or siRNA and 6 or 12 μL LipofectAMINE 2000 reagent in six-well plates. Untreated control cells were incubated overnight or to a maximum of 36 hours without LipofectAMINE 2000 reagent in serum-free medium. The Nucleofector technology was used according to the manufacturer’s guidelines using 0.125 or 1 μg siRNA and 100 μL Solution T (Amaxa Biosystems) in combination with Nucleofector device program U24. Transfections were carried out and the cells were harvested 24 to 48 hours later or 12 to 14 hours after transfection depending on the experiment.

Drugs and Antisense Sequences

GW5074 was purchased from Sigma-Aldrich (St. Louis, MO). The Raf-1 inhibitor GW5074 was prepared fresh by dissolving in sterile 100% DMSO at a stock concentration of 1,038 μmol/L and protected from light. The drug was diluted with serum-free DMEM before use. MEK inhibitor U0126 (Promega, Madison, WI) was prepared by dissolving in sterile 100% DMSO to a stock concentration 10 mmol/L. We have determined that a DMSO concentration of 1% can affect glioblastoma cell viability. In all of the experiments, the final DMSO concentration did not exceed 0.3%. A patent library of >80 antisense sequences affecting ILK was obtained from ISIS Pharmaceuticals, Inc. (Carlsbad, CA) in which antisense sequence ID no. 37 (5'-GAGATTCTGGCCCATCTTCT-3') was used and is designated here as ILKAS. ILKAS is a 20-mer antisense oligonucleotide with a phosphothioate backbone, 5'-methylycytidines with the first five nucleotides (at the 5'-end) 2'-O-methyls and the last five nucleotides (at the 3'-end) 2'-O-methyls. The ILKAS affects the 635 ‘coding region’ or open reading frame, which lies between the translation initiation codon and the translation termination codon of human ILK. As a control, the reverse of ILKAS was also obtained, designated RILK, with the same modifications as those of ILKAS. Antisense sequences were generated at the University of British Columbia Nucleic Acid Protein Service Unit (Vancouver, British Columbia, Canada). Antisense sequences were ammonia-butanol purified. siRNA sequences (23 bp) against the human ILK gene (Genbank accession no. gi 3150001) were generated by Qiagen, Inc. (Mississauga, Ontario, Canada) and have been described previously (8). Briefly, ILK siRNAs that targeted the kinase domain (ILK-FSF, sense UGUCAGAUUCUCUUUCACAUGdTdT and antisense CAUUGGAAGAACACUGACAdTT) or the pH domain (ILK-H, sense CCUCGACGAGCCUACAGGAGAdTT and antisense UUCUCUGAGCUUUCUGACGAdTT) were used, and a reverse ILK siRNA sequence to the pH domain (sense AAGACCAAUCGAGCAGCGCAdTT and antisense GGACUCUUGAGCUUGCUUCUdTdT) and a 21-bp nonsilencing control sequence (sense UUUCGAGCAGCUUUCUGACGAddTdT and antisense ACGUGACAGCUUUCUGACGAdTd) were also included.

Western Blot Analysis

The following antibodies were used in this study: anti-ILK (affinity-purified rabbit polyclonal; Upstate Biotechnology, Charlottesville, VA) and anti-phosphorylated Akt-Ser473, anti-phosphorylated Akt-Thr308, anti-MAPK, anti-phosphorylated MAPKp44/p42, anti-Akt, anti-MEK1/2, anti-phosphorylated MEK1/2-Ser217/Ser221 (rabbit polyclonal; New England Biolabs, Pickering, Ontario, Canada). The secondary antibody used was horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG (Promega). Proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and visualized after exposure to Kodak (Guelph, Ontario, Canada) autoradiography film. Scanning densitometry (Molecular Dynamics,
Sunnyvale, CA) was done to quantify band intensities by volume/area integration. Equivalent amounts of protein (30 μg/lane determined by Bradford assay) were resolved by 12% SDS-polyacrylamide gels or 4% to 15% gradient SDS-polyacrylamide premade gels (Bio-Rad, Philadelphia, PA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay and Drug Combination Effects

Growth inhibition of U87MG, U251MG, and SF-188 cells was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were plated at 3,000 per 100 μL in 96-well plates and allowed to adhere overnight before exposure to antisense and/or inhibitors and incubated for 24 or 48 hours at 37°C [ILKAS (1–16 μmol/L), GW5074 (2–32 μmol/L), and U0126 (10–80 μmol/L)]. Plates were read using the microtitrter plate reader (Dynex Technologies, Inc., Chantilly, VA) at a wavelength of 570 nm. The percentage of viable cells following treatment was normalized to untreated controls. All assays were done in triplicate at least twice. The MTT data obtained following treatment of cells with the indicated agents alone or in combination were combined so that the number of samples analyzed for each drug or drug ratio concentration was ≥6. Effective concentrations were analyzed using the CalcuSyn software (Biosoft, Ferguson, MO). CalcuSyn program provides a measure of the combined drug interaction by the generation of a combination index (CI) value. The CI value is based on the multiple drug-effect equation of Chou and Talalay (13) and defines the drug interactions as synergistic (which is more than the expected activity effect or greater than an additive interaction) or antagonistic (which is less than the expected activity effect or less than an additive interaction). Chou and Talalay defined the CI value as <1 for synergism, 1 for additive, and >1 for antagonism.

Clonogenic Assay

Colony formation was evaluated using a soft agar colony-forming assay. Briefly, cells were exposed to drug for 5 hours. Cells were washed with serum-free DMEM. Subsequently, 2,000 cells per well were mixed with DMEM containing 15% fetal bovine serum and 0.5% agar and plated on six-well plates (three wells per condition). The plates were then transferred to a 37°C incubator with 5% CO2. After 12 to 14 days of incubation, colonies were scored in two colony grids (Epicentre, Madison, WI) per well using a Zeiss ID02 microscope (Don Mills, Ontario, Canada). Colony formation for each condition was calculated in relation to values obtained for untreated control cells.

Nuclear Morphology

Untreated and treated cells were incubated a minimum of 12 hours in medium at 37°C without additional drug treatment. Cells were then harvested and stained with 0.10 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) for 30 minutes at room temperature. Cells were cytopsunt onto a glass slide and viewed with a Leica (Wetzlar, Germany) microscope with a ×40 objective lens under UV fluorescent illumination. Images were captured using DC100 digital camera and Image database version 4.01 Software (Leica).

Flow Cytometric Assay of Apoptosis

Cells (5 × 10⁴) were cultured with or without drug and later incubated for a minimum of 12 hours in culture medium at 37°C without additional drug treatment. Camptothecin (32 μmol/L) was used as a positive control for induction of apoptosis, which was measured by Annexin V-FITC/propidium iodide (PI) staining. Cells were treated with ILKAS, GW5074, or the combination of ILKAS and GW5074. Control cells were untreated or treated with RILK in combination with GW5074. A liposomal vector control was also included in the experiment. Cells were then harvested and fixed with cold 70% ethanol, stored overnight at −20°C, and stained with PI staining buffer (1 mg/mL RNase A, 0.1% Triton X-100, 50 μg/mL PI in PBS) to determine the apoptotic/necrotic cell population (the sub-G1-G0 cell fraction). To evaluate the early stages of apoptosis, treated cells were stained with Annexin V-FITC (Caltag, Burlingame, CA) in Annexin V staining buffer for 15 minutes at room temperature and counterstained with 50 μg/mL PI in phenol red–free HBSS (Stem Cell Technologies, Vancouver, British Columbia, Canada) and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) for the induction of apoptosis.

Statistical Analysis

All of the statistical analyses were done using the Statistica software program. Data analysis for multiple comparisons of treatment and control groups was done using the one-way ANOVA Tukey test. Data were considered significant with a P < 0.05.

Results

Disruption of the PI3K/Akt pathway was achieved by using an ILK targeted antisense oligonucleotide sequence (ILKAS), transiently transfected into SF-188 cells. Results in Fig. 1A indicate that ILKAS transfection resulted in knockdown of ILK protein and decreased PKB/Akt phosphorylation on Ser473 but not Thr308 (Fig. 1A, lanes 3 and 4). Transfection with the RILK did not decrease ILK or phosphorylated PKB/Akt protein levels (Fig. 1A, lane 3), nor did the vector control affect ILK or phosphorylated PKB/Akt levels (Fig. 1A, lane 2). These data show in vitro that the selected ILK targeted antisense can suppress ILK protein levels and decrease phosphorylated PKB/Akt activity. Treatment of SF-188 and U87MG cells with ILKAS, at doses ranging from 1 to 10 μmol/L, decreased cell viability (Fig. 1B). At the highest concentrations tested (8 and 10 μmol/L), the data suggested >80% decrease in cell viability. To determine whether the cytotoxic/cytostatic effects of ILKAS against glioblastoma cells could be enhanced when used in combination with GW5074, the effects of which have been described previously (14), a fixed ratio of this
combination (ILKAS/GW5074 = 1:2 mol/mol) was analyzed at and above the IC_{50}s of these individual agents. The Chou and Talalay median-effect method (15) was used to determine drug efficacy and the nature of the drug interaction. The dose-effect plot for U87MG cells in Fig. 1C showed that the combination of ILKAS (1–8 μmol/L) with GW5074 (2–16 μmol/L) exhibited an enhanced drug combination effect (decreased cell viability labeled “effect” on the Y axis) relative to ILKAS and GW5074 used alone. The interactions were judged to be synergistic, with CI < 0.25 (Fig. 1D) for effect levels (fa) ranging from 0.82 to 0.98. Similarly, the dose-effect plot and CI values for SF-188 cells (Fig. 1E and F, respectively) indicate that the combination of ILKAS (1–8 μmol/L) and GW5074 (2–16 μmol/L) also resulted in synergy, with CI < 0.4 (Fig. 1F) for fa values ranging from 0.75 to 0.95.

The data summarized thus far suggest a greater than additive effect on U87MG and SF-188 glioblastoma cells treated with ILKAS in combination with GW5074. These
results were assessed based on a MTT assay end point. It was important to confirm that synergistic interactions are also observed when different assay end points are used. A clonogenic assay was, therefore, used to determine the effect of the drugs alone and in combination against U87MG cells. U87MG cells were treated with ILKAS, GW5074, or the combination of the two and subsequent examination of clonogenic survival was analyzed (Fig. 1G). In these studies, the ILKAS added at a dose of 5 μmol/L resulted in a 22% reduction in colony formation (Fig. 1G, column 3). GW5074, when added alone, resulted in ~25% colony formation inhibition when added at 15 μmol/L (Fig. 1G, column 2). Addition of both ILKAS and GW5074 at a fixed drug ratio (1 μmol/L ILKAS/2 μmol/L GW5074) that was shown to be synergistic when measured using the Chou and Talalay median-effect method (16) engendered a 92% reduction in colony formation (Fig. 1G, column 4). These results are consistent with the dose reduction effects associated when using agents that interact synergistically to achieve a defined effect level.

To further evaluate the effect of this combination, apoptosis induction was assayed by measuring Annexin V labeling of externalized phosphatidylserine. The combination added at synergistic fixed ratio doses was used and the effects achieved were compared with those measured with the single agents added at higher concentrations (Fig. 2). SF-188 glioblastoma cells were treated with the single-agent ILKAS at 5 μmol/L or the single-agent GW5074 at 15 μmol/L in comparison with the drug combination of ILKAS at 1 μmol/L and GW5074 at 2 μmol/L. Results for SF-188 are summarized in Fig. 2, which summarizes Annexin V-FITC staining (Fig. 2A), PI profile (Fig. 2B), and DAPI staining data (Fig. 2C). Camptothecin, a known inducer of apoptosis, was used as a positive control and untreated cells served as the negative control (Fig. 2A). Results of studies assessing SF-188 cells treated with ILKAS and/or GW5074 indicated that there were very few PI-negative and Annexin V–positive cells in the control cultures (Fig. 2A). Camptothecin-treated cells exhibited 87.0% Annexin V labeling. Cells treated with ILKAS in combination with GW5074 showed a substantial amount of apoptosis observed by Annexin V labeling. Cells treated with the combined drugs exhibited 75.5% Annexin V labeling versus 51.2% and 33.7% measured for cells treated with the single agents ILKAS or GW5074, respectively. To further characterize the effects of ILKAS and GW5074 on glioblastoma cells, PI was used to stain nuclear DNA. Cells undergoing apoptosis and necrosis were identified as a population with reduced DNA content (the sub-G1-G0 population). The sub-G1-G0

Figure 2. Apoptosis assessment in ILKAS and combination-treated cells. A, SF-188 glioblastoma cells were isolated and exposed to 2 μmol/L GW5074 ± 1 μmol/L ILKAS or left untreated for a minimum of 8 h, where camptothecin was used as a positive control for Annexin V-FITC and PI staining. The induction of apoptosis was determined by flow cytometric analysis of Annexin V-FITC and PI-staining. Bottom right quadrant, Annexin V–positive, early apoptotic cells; top right quadrant, Annexin V–positive/PI-positive, late apoptotic cells. Y axis, PI fluorescence intensity; X axis, Annexin V-FITC intensity. B, SF-188 cells were treated with 2 μmol/L GW5074 ± 1 μmol/L ILKAS or left untreated and harvested and the percentage of cells with sub-G1-G0 (apoptotic/necrotic) DNA content was measured by flow cytometric analysis of PI staining for 20,000 events. Y axis, number of events; X axis, PI fluorescence. C, nuclear morphology of SF-188 cells treated with 2 μmol/L GW5074 ± 1 μmol/L ILKAS or left untreated were stained with DAPI and cytospin preparations. Cells were viewed under fluorescence microscopy at ×40 magnification.
cell population of SF-188 cells treated with the ILKAS/GW5074 (1:2 μmol/L) combination was significantly higher than that observed when treating cells with ILKAS (5 μmol/L) or GW5074 (15 μmol/L) alone. This effect was achieved at a lower combined concentration than the single agents (Fig. 2B), a result that is again consistent with data suggesting that this is a synergistic combination. To confirm whether transfection of glioblastoma cells with ILKAS alone and in combination with GW5074 resulted in apoptotic cell death, we examined the morphologic changes of SF-188 cells by DAPI staining (Fig. 2C). No nuclear fragmentation or chromatin condensation in SF-188 control cultures was observed. Significantly more cells with fragmented nuclei and condensed chromatin, indicative of late apoptosis, were observed in cultures treated with ILKAS and the ILKAS/GW5074 combination (Fig. 2C, arrows).

Having assessed the effects of targeting ILK in combination with the Raf-1 inhibitor GW5074, a relatively infrequently used inhibitor, we wanted to determine if a synergistic interaction could be achieved using a drug that acted further downstream within the Ras/MAPK pathway. The well-known small-molecule inhibitor U0126, which targets MEK, was selected for these studies. Synergistic interactions were seen with ILKAS in combination with U0126 in U251MG and SF-188 glioblastoma cells (see Fig. 3). The dose-effect plot of U251MG cells in Fig. 3A showed that the combination of ILKAS (1–4 μmol/L) with U0126 (10–40 μmol/L) exhibited an enhanced drug combination effect (decreased cell viability labeled “effect” on the Y axis) relative to ILKAS and U0126 used alone. Data analysis indicated CI values ranging from 0.5 to <0.1 (Fig. 3B) over fa values as high as 0.95. The dose-effect plot for SF-188 cells treated with ILKAS in combination with U0126 (Fig. 3C) in the same fixed ratio as U251MG glioblastoma cells also resulted in strong synergy (CI < 0.4; Fig. 3D). As a control, a combination of RILK and GW5074 and a combination of RILK and U0126 were used.
at the same concentrations as that of the ILKAS and GW5074 or U0126 in combination. For these combinations, the CI values exceeded 10, suggesting that the combination with the control antisense sequences actually inhibited any measured therapeutic effects due to GW5074 or U0126 (data not shown).

To determine if ILKAS and U0126 were inhibiting their specific targets in the PI3K/Akt and the Ras/MAPK pathways at the same concentrations at which synergistic combination effects were seen, ILK, PKB/Akt, and MAPK levels/activity were assessed by Western blot analysis in SF-188 and U251MG glioblastoma cells. These studies were completed using cells cultured in the presence of EGF added exogenously to determine if the effects of this mitogen could be abrogated by drug treatment. The results of these studies have been summarized in Fig. 3E and F. SF-188 cells transfected with ILKAS alone resulted in loss of ILK protein (Fig. 3E, lane 4). When the ILKAS was used in combination with the MEK inhibitor U0126, U0126 did not interfere with ILKAS targeting as judged by ILK protein inhibition measured in the presence of U0126 (Fig. 3E, lane 5). Transfection of ILKAS alone or in combination with U0126 also resulted in decreased phosphorylation of PKB/Akt on Ser473 (Fig. 3E, lanes 4 and 5). Interestingly, ILKAS also resulted in decreased MAPKp44/p42 phosphorylation (Fig. 3E, lane 4), although the ILKAS targets the PI3K/Akt pathway. SF-188 cells exposed to the MEK inhibitor U0126 in the presence and absence of ILKAS resulted in loss of MAPKp44/p42 phosphorylation (Fig. 3E, lanes 3 and 5). Total Akt and MAPK levels were unaffected by the ILKAS/U0126 combination as judged by comparisons with actin as a loading control.

Similar results were seen for the glioblastoma cell line U251MG (Fig. 3F). However, the results provided in Fig. 3F suggest that the ILKAS-mediated down-regulation of ILK in the U251MG cell line is not as efficient under the conditions used. We chose to use one set of transfection conditions rather than optimizing ILKAS transfection for the different cell lines used and conditions that resulted in >90% down-regulation in the SF-188 cell line engendered a decrease of ~50% in the U251MG cell line. Consistent with the reduced activity, the downstream effects of ILKAS transfection as measured by phosphorylated Akt (Ser473) was less robust. The fact that down-regulation of PKB/Akt on Ser473 is not as effective in U251MG cells with ILKAS highlights the relationship between ILK protein level and subsequent loss of phosphorylated PKB/Akt on Ser473 (Fig. 3F, lane 5), which was also reduced by ~50%. Although the activity was less in the U251MG cells, this provided an opportunity to better assess the effect of combinations with U0126 on ILK suppression. Loss of ILK and decreased phosphorylation of PKB/Akt seen following ILKAS transfection was more pronounced when the ILKAS was used in combination with U0126 (Fig. 3F, lanes 4 and 5). This surprising result could be explained based on U0126-mediated increases in ILKAS transfection efficiency or could be a consequence of drug combination effects that enhance down-regulation of ILK. Regardless, the in vitro results suggest that there is an interaction between ILKAS and U0126 that is consistent with the data shown in Fig. 3A and B. In addition, it is worth noting that ILKAS resulted in loss of MAPKp44/p42 phosphorylation (Fig. 3F, lane 3). Treatment of U251MG cells with U0126 resulted in MAPKp44/p42 loss. As with the SF-188 cell line, total Akt and MAPK levels were unaffected as measured relative to actin levels as a loading control.

Given the surprising results summarized in Fig. 3F, it was suggested following review of this article that an alternative approach should be used to validate this combination effect on gene silencing. For this reason, U251MG cells were transfected using LipofectAMINE 2000 with two different ILK specific siRNAs. ILK siRNA targeting the pH domain of ILK (ILK-H) and ILK siRNA targeting the kinase domain of ILK (ILK-FSF) resulted in significant suppression of ILK expression (Fig. 4A, lanes 4 and 5, respectively). No effect on ILK expression was seen with cells left untreated (Fig. 4, lane 1), cells transfected with a nonsilencing siRNA (Fig. 4A, lane 2), or cells transfected with a reverse ILK siRNA sequence (Fig. 4A, lane 3). In addition, loss of PKB/Akt Ser473 was only observed for U251MG cells transfected with ILK-H and ILK-FSF (Fig. 4A, lanes 4 and 5). Total Akt levels were unaffected and actin was used as a loading control. These data suggested that the siRNA sequences selected were providing gene silencing effects comparable with those obtained using the ILK targeted antisense oligonucleotide (Fig. 1A).

To confirm what was observed with the combination of antisense oligonucleotide and U0126 (i.e., enhanced gene silencing effects when the MEK inhibitor U0126 was used), we repeated the study transfecting ILK siRNA in U251MG cells. For this set of experiments, we used a more robust transfection technique, Nucleofector technology, which resulted in increased transfection efficiency at a lower concentration of siRNA than what was used with LipofectAMINE 2000. It is also notable that enhanced transfection was achieved under conditions that were better tolerated by the cells. Using this methodology, ~80% of ILK expression was observed at the lowest concentration of ILK-H used (3 nmol/L), and a complete knockdown of ILK was observed at the highest concentration (21 nmol/L; Fig. 4B, lanes 4 and 5). Using two concentrations of U0126 (3,125 and 25 μmol/L) in the absence of ILK targeted siRNA had no effect on ILK expression in U251MG cells (Fig. 4C, lanes 2 and 3). Importantly and consistent with the results in Fig. 3F, the combination of ILK-H transfection and U0126 treatment resulted in complete ILK knockdown at the lowest concentration of ILK-H (3 nmol/L) used (Fig. 4D, lane 4). As indicated above, reduced gene silencing effects observed with the U251MG cell line actually helped to illustrate the fact that the combination of ILKAS with U0126 achieved enhanced suppression of ILK. This result may help to explain the synergistic interactions identified in the in vitro screening assays.
Treatment of U251MG cells with U0126 and ILKAS alone and in combination promoted apoptosis (Fig. 5). The percentage of PI-negative and Annexin V–positive cells in single agent–treated cultures was 98.3% and 57.8% for U0126 and ILKAS, respectively. There was a high proportion (98.0%) of cells expressing phosphatidylserine in cells treated with the combination of ILKAS and U0126 (Fig. 5A). Note that this effect level was achieved at a drug concentration of the agents that were 1.75- and 2.5-fold lower than that used for the single agents ILKAS and U0126, respectively. Note that in U251MG cultures treated with ILKAS and U0126 in combination (Fig. 5A) there were more double-positive (Annexin V-FITC, PI-stained) cells than in either of the single agent–treated U251MG cells. This is consistent with faster progression to late-stage cell death with the combination. Despite using lower drug doses, similar levels of Annexin V–positive labeling were obtained. In addition, PI analysis indicates that there was an increase in the number of cells with sub-G1-G0 DNA content when the cells were treated with the drugs in combination (Fig. 5B). Analysis of DAPI-stained U251MG cultures confirm the presence of cells with fragmented nuclei and condensed chromatin in single agent–treated samples and the combination-treated samples (Fig. 5C).

Discussion

The present study examines the role of drug combinations selected to affect distinct aberrant cell signaling pathways known to play a role in glioblastoma cell proliferation and survival. These in vitro studies provide a rationale for the preclinical development of targeted drug combinations in animal models of glioblastoma multiforme and hopefully clinical testing of an effective combination of drugs affecting these pathways. We have shown that treatment of U87MG, U251MG, and SF-188 glioblastoma cells with ILKAS (Figs. 1 and 3) or selected siRNA (Fig. 4) can decrease ILK protein levels and downstream phosphorylation of the cell survival protein PKB/Akt on Ser473, the site specifically phosphorylated by ILK. This ILKAS specific effect and the associated decrease in the PKB/Akt cell survival activity likely led to increased cell death in this cell population treated with the selected ILKAS. Furthermore, when ILKAS or ILK siRNA were combined with the MEK inhibitor U0126, the measured therapeutic effects were judged to be synergistic. Synergistic interactions, in this context, means that the therapeutic effects can be achieved at a much lower concentration of ILKAS and U0126 when used in combination compared with doses required to achieve the same effect level when used as single agents.

We acknowledge the fact that ILKAS transfection is not as robust in U251MG cells shown in Fig. 3F; however, this highlights the effect of the combination in which greater knockdown of ILK and phosphorylated PKB/Akt is achieved in the combination setting compared with the use of ILKAS alone. These results were confirmed using ILK silencing methods based on transfection with ILK.

Figure 4. Effects of specific ILK targeting. A, U251MG cells were transiently transfected using LipofectAMINE 2000 (see Materials and Methods) with ILK specific siRNA (120 nmol/L) targeting the ILK pH domain (ILK-H) or the ILK kinase domain (ILK-FSF) and harvested 48 h later. Untreated cells or a nonsilencing siRNA (Cont-NS) or an inverted ILK-H siRNA (Cont-HC) were included as controls. Knocking down ILK expression by siRNA substantially decreased phosphorylation of PKB/Akt on Ser473. B, U251MG cells were transiently transfected using Nucleofector technology (see Materials and Methods) with 3 or 21 nmol/L ILK-H. Nonsilencing siRNA or an inverted ILK-H siRNA were included as controls. C, U251MG cells were treated with either 3.125 or 25 μmol/L U0126 MEK inhibitor and harvested 48 h later. D, U251MG cells were transiently transfected with ILK-H alone and in combination with U0126 at a molar ratio of 1:1:190. Equivalent amounts of protein were loaded (30 μg/lane determined by Bradford assay).
targeted siRNA. The reason for this effect is not well understood at this time; however, one could anticipate that knocking down two key cell signaling pathways involved in regulating glioblastoma cancer through cell survival via PI3K/PKB/Akt and cell proliferation via the Ras/MAPK pathway could lead to unexpected effects as measured by ILK protein suppression. A less interesting explanation is the possibility under these in vitro conditions that U0126 enhance ILKAS or siRNA transfection efficiencies, but this seems unlikely given the fact that remarkably different transfection methods were used (LipofectAMINE 2000 versus Nucleofector technology) and the effect was observed using both antisense oligonucleotide and siRNA-based gene silencing methods.

The best evidence of synergy, from our perspective, is achieved with data showing comparable or enhanced therapeutic effects can be achieved at significantly lower drug doses when the drugs are used in a combination setting. Using apoptosis as an end point, for example, concentrations of 4 \( \mu \text{mol/L} \) ILKAS and 40 \( \mu \text{mol/L} \) U0126 achieved equal or greater effects when compared with that achieved using 7 \( \mu \text{mol/L} \) ILKAS or 100 \( \mu \text{mol/L} \) U0126 singly. This dose reduction, due to drug combination synergism, has potential advantages clinically as optimal therapy may be achieved at lower dose levels and perhaps with reduced toxicity (13). These cell-based screening assays are, however, difficult to interpret in light of potential in vivo factors effecting drug effects. It has been reported that there is low expression of EGFR associated with glioma cells in vitro (17), which may result in a lower activity of the Ras/MAPK pathway. To combat this problem, we stimulated treated and untreated cells with EGF to increase EGFR expression and/or activation before assessing the effects of the targeted agents. It has also been reported that EGFR is up-regulated in malignant glioma (18) and this could be due to the three-dimensional arrangement of the tumor in vivo, which may engender EGFR expression due to poor tumor perfusion and associated stress effects of starvation and hypoxia. This would not be seen in a monolayer culture. The monolayer culture condition may therefore limit the ability of GW5074 to act effectively due to artificially reduced EGFR expression and subsequent low Ras/MAPK pathway activity. An indirect method to address this relied on plating the glioblastoma cells in soft agar (Fig. 1G), in anticipation that EGFR expression would be enhanced.

Figure 5. A, apoptosis assessment in ILKAS and combination-treated cells. U251MG glioblastoma cells were isolated and exposed to 40 \( \mu \text{mol/L} \) U0126 \( \pm 4 \mu \text{mol/L} \) ILKAS or left untreated for a minimum of 8 h, where camptothecin was used as a positive control for Annexin V-FITC and PI staining. The induction of apoptosis was determined by flow cytometric analysis of Annexin V-FITC and PI-staining. Bottom right quadrant, Annexin V–positive, early apoptotic cells. Y axis, PI fluorescence intensity; X axis, Annexin V-FITC intensity. Top right quadrant, Annexin V–positive/PI-positive, late apoptotic cells. B, U251MG cells were treated with 40 \( \mu \text{mol/L} \) U0126 \( \pm 4 \mu \text{mol/L} \) ILKAS or left untreated and harvested and the percentage of cells with sub-G1-G0 (apoptotic/necrotic) DNA content was measured by flow cytometric analysis of PI staining for 20,000 events. Y axis, number of events; X axis, PI fluorescence. C, nuclear morphology of U87MG cells treated with 40 \( \mu \text{mol/L} \) U0126 \( \pm 4 \mu \text{mol/L} \) ILKAS or left untreated were stained with DAPI and cytospun preparations were obtained. Cells were viewed under fluorescence microscopy at \( \times 40 \) magnification.
EGFR expression was not shown, our results suggest that the therapeutic effects of the drugs alone and in combination were comparable with results generated using the MTT assay.

The activity of ILKAS not only had an effect on the PI3K/Akt pathway but also on the Ras/MAPK pathway in which phosphorylated MAPK activity was decreased (see Fig. 5E and F). This was not unexpected as other groups have shown that inhibition of the PI3K pathway can result in a decrease in the activity of Ras/MAPK pathway (19). Regardless, the in vitro data suggest that the combination of ILKAS and U0126 or GW5074 can result in more than additive effects. The flow cytometry and DAPI staining results support this conclusion. There was equal or greater cell death when ILKAS and U0126 or GW5074 were used in combination and this effect was seen at lower concentrations than required to see the same effect when using either of the agents alone (Figs. 2A–C and 5A–C). It is trations than required to see the same effect when using the MTT assay.

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References


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