

A Novel Low-Molecular Weight Inhibitor of Focal Adhesion Kinase, TAE226, Inhibits Glioma Growth

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Glioblastomas are highly lethal cancers that resist current therapies. Novel therapies under development target molecular mechanisms that promote glioblastoma growth. In glioblastoma patient specimens, the non-receptor tyrosine kinase focal adhesion kinase (FAK) is overexpressed. Upon growth factor receptor stimulation or integrin engagement, FAK is activated by phosphorylation on critical tyrosine residues. Activated FAK initiates a signal transduction cascade which promotes glioma growth and invasion by increasing cellular adhesion, migration, invasion, and proliferation. We find that human glioma cell lines express different levels of total FAK protein and activating phosphorylation of tyrosine residues Tyr397, Tyr861, and Tyr925. As all glioma cell lines examined expressed phosphorylated FAK, we examined the efficacy of a novel low-molecular weight inhibitor of FAK, TAE226, against human glioma cell lines. TAE226 inhibited the phosphorylation of FAK as well as the downstream effectors AKT, extracellular signal-related kinase, and S6 ribosomal protein in multiple glioma cell lines. TAE226 induced a concentration-dependent decrease in cellular proliferation with an associated G₂ cell cycle arrest in every cell line and an increase in apoptosis in a cell-line-specific manner. TAE226 also decreased glioma cell adhesion, migration, and invasion through an artificial extracellular matrix. Together, these data demonstrate the potential benefit of TAE226 for glioma therapy. © 2007 Wiley-Liss, Inc.

Key words: FAK; glioblastoma; small molecule inhibitor

INTRODUCTION

Glioblastomas represent one of the most lethal human malignancies, with a historical mean life expectancy of less than 12 mo and a 5-yr survival rate of less than 5% [1]. Current treatment of these cancers includes surgery followed by radiation and chemotherapy with highly toxic, non-specific agents that damage DNA or target the mitotic machinery. Temozolomide, an oral methylator, has provided an important increase in patient survival, but that increase is still only less than 3 mo [2]. The low rate of long-term survival for glioblastoma patients reflects the inability of current therapies to effectively prevent tumor growth and spread. Tumor invasion of normal brain represents a key contributor to treatment failure because invasive tumor cannot be surgically resected and displays resistance to cytotoxic therapies [3]. Therefore, treatment of glioblastoma patients may be improved by novel therapeutics that target glioma invasion.

One signal transduction pathway, that contributes to glioma growth and invasion involves the non-receptor tyrosine kinase, focal adhesion kinase (FAK, p125^{FAK}). FAK integrates signals from activated growth factor receptors and integrins to regulate cell motility, invasion, proliferation, apoptosis, and angiogenesis [4]. Growth factors and integrins lead to the autophosphorylation of FAK on tyrosine 397 (Tyr397), which creates a SH2

Abbreviations: FAK, focal adhesion kinase; ERK, extracellular signal-related kinase; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

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Received 31 July 2006; Revised 20 October 2006; Accepted 15 November 2006

DOI 10.1002/mc.20297

binding site for the Src family of tyrosine kinases [5,6]. Src is then thought to lead to the phosphorylation of several other tyrosine residues, including Tyr861 and Tyr925 [7]. However, recent evidence indicates that Src kinase is required for the phosphorylation of Tyr925 but not Tyr861 [8]. Phosphorylated Tyr925 can bind to the adaptor protein Grb2 [6], which may lead to the regulation of adhesion turnover [8]. Phosphorylated Tyr861 can regulate p130^{CAS} binding to control ras-mediated transformation [9]. Therefore, differences in the site of FAK tyrosine phosphorylation may contribute to the formation of distinct signaling complexes which promote different biological effects.

FAK can promote tumor growth, and a role for FAK in glioma pathogenesis is suggested by its expression and localization. FAK is overexpressed in glioma specimens compared to normal human brain [10–12]. Increased regional expression of FAK is found at the invasive tumor edge, implicating FAK in tumor invasion [11]. Additionally, FAK is expressed in the microvascular endothelial cells of human glioma tumor biopsies and U251MG glioma xenografts, which links FAK to glioma angiogenesis [13].

FAK overexpression in human glioma cell lines contributes to several malignant phenotypes, including increased cellular proliferation and survival. Overexpression of wild-type FAK in SF767 or G112P glioma cells increased cell proliferation [14,15], while antisense oligonucleotides targeting FAK decreased the proliferation of U251MG cells [16]. The focal adhesion targeting (FAT) domain, a C-terminal truncated form of FAK, prevents FAK localization to focal adhesions and increases apoptosis with serum starvation or cisplatin treatment, as determined by increased caspase-3 activity in LN-229 and LN-410 glioma cells [17]. Furthermore, overexpression of the Tyr397F FAK phosphorylation mutant in T98G gliomas cells increased apoptosis due to elevated caspase 6 activity [18], and antisense oligonucleotides targeting FAK expression induced apoptosis in U251MG cells [16]. Thus, FAK has the ability to regulate tumor growth through control of both cell proliferation and cell death.

Studies in malignant glioma cell lines indicate additional roles for FAK in cell migration, invasion, and angiogenesis. The role of FAK in tumor invasion is complex and may demonstrate contradictory results in different models of tumor invasion/motility. Expression of the FAT domain decreased invasion of LN-229 and LN-410 cells in a Boyden chamber Matrigel assay [17], which suggests a positive role of FAK in tumor invasion. In contrast, overexpression of wild-type FAK decreased cell migration in other models [14,15]. The Tyr397F FAK phosphorylation mutant in G112P cells did not regulate cell migration [15], consistent with the requirement for phosphorylation of tyrosine residues (mainly at sites 397, 576/577, 861, and 925)

for FAK activation [4–8]. A naturally occurring dominant negative form of FAK, FAK-related non-kinase (FRNK), has also been used to demonstrate a role for FAK in angiogenesis [13]. Overexpression of FRNK in brain endothelial cells reduced endothelial capillary tube formation, tube length, and migration, which supports the conclusion that FAK promotes glioma angiogenesis by increasing endothelial cell migration and tube formation [13]. Together, these data suggest that the overexpression of FAK in glioblastomas may increase tumor proliferation, invasion, survival, and angiogenesis.

As FAK is overexpressed in gliomas and has many potential pro-tumorigenic functions, targeting FAK activity may be an effective brain tumor therapy. We now report the effects of a novel ATP-competitive tyrosine kinase small-molecule inhibitor designed to target FAK, TAE226, on glioma growth and invasion. TAE226 effectively prevented FAK phosphorylation and downstream signal transduction, as determined by decreased AKT, extracellular signal-related kinase (ERK), and S6 ribosomal protein phosphorylation. Furthermore, TAE226 treatment potentially disrupted glioma cell proliferation, attachment, migration, and invasion.

MATERIALS AND METHODS

Cell Lines and Culture

The human malignant glioma cell lines U87MG and U373MG were purchased from American Type Culture Collection (Manassas, VA). U251MG was provided by Dr. Jan Poten (University of Uppsala, Sweden). We confirmed that these lines were from different sources using comparative genomic hybridization (data not shown). D54MG is the Duke University subline of A-172 [19]. D54MG, U87MG, U251MG, and U373MG human glioma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and glutamine (Gibco).

TAE226

TAE226 was generously provided by Novartis Pharma AG, Switzerland. TAE226 in 10 mM stock solutions was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and stored at -80°C until use. The 10 mM stock solutions were diluted in DMSO to 1000 \times of indicated final concentrations.

Antibodies and Western Blotting

Cells were serum starved overnight and then treated with the indicated concentrations of TAE226 with 1 $\mu\text{L}/\text{mL}$ of 1000 \times TAE226 or with DMSO control added directly to the media of the serum-starved cells. After 45 min, 100 μL serum (final 1%) or 100 μL of 1 g/mL bovine serum albumin (BSA) in media without

serum was added to the media of the cells for 15 min. Cells were then lysed in buffer (62.5 mM Tris-HCl, 2% w/v sodium dodecyl sulfate [SDS], 10% glycerol, 40 mM dithiothreitol and protease inhibitors), sonicated for 5 s, and centrifuged at 12,000g for 10 min at 4°C. Protein content was determined by with Protein Assay Reagent (Biorad Laboratories, Hercules, CA). Equal protein amounts were run on SDS polyacrylamide gels (Invitrogen, Carlsbad, CA), transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA), and probed with phospho-FAK Tyr397 (Invitrogen), phospho-FAK Tyr861, phospho-FAK Tyr925 (Cell Signaling Technology, Beverly, MA), total FAK (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-AKT Ser473, phospho-AKT Thr308, total AKT, phospho-S6 Ser240/244, total S6 (Cell Signaling Technology), phospho-(ERK) 1/2 Tyr204, ERK1, ERK2 (Santa Cruz Biotechnology), and alpha-tubulin (Sigma-Aldrich). Proteins were detected by using an enhanced chemiluminescence system (Pierce Biotechnology, Rockford, IL).

Flow Cytometric and Annexin V Analysis

For cell cycle analysis, 150,000 cells were plated in each well of a 6-well tissue culture plate. Cells were grown in 10% serum overnight and then treated for 16 h with DMSO control or 1 $\mu\text{L}/\text{mL}$ of 1000 \times TAE226 to produce the indicated final concentration of TAE226 in the presence of 10% serum. Because cells undergoing cell death become detached, conditioned media was collected. Treated cells were trypsinized and added to the conditioned media. Cells were collected by centrifugation, washed with Dulbecco's phosphate-buffered saline (PBS, Gibco), fixed in ethanol, and stained with propidium iodide. For Annexin V assays to indicate apoptotic cells, both cells and corresponding conditioned media were collected for analysis, washed with PBS, and labeled with propidium iodide and Annexin V (BD Pharmingen, San Diego, CA) according to manufacturer's instructions. For both cell cycle and apoptosis, analysis was performed on a FACScan instrument (BD Biosciences, San Jose, CA) gated to exclude cellular debris and collecting 10^4 events. Calculations were performed with BD software.

Cell Adhesion Assay

Cells cultured in media containing 10% serum were collected by trypsinization, washed, diluted to a concentration of 100,000 cells/mL in media containing 10% serum, and then treated for 30 min with DMSO control or 1 $\mu\text{L}/\text{mL}$ of 1000 \times TAE226 to produce the indicated final concentration of TAE226. Next, 100 μL of the treated cells with inhibitor (10,000 cells) was added to a 96-well plate and incubated for 1 h to allow attachment. Cells were fixed by 4% paraformaldehyde (PFA) in PBS for 1 h and then stained by 0.1% Toluidine Blue O (Sigma-Aldrich) in 4% PFA for 30 min. After washing with

PBS, attached cells were imaged with a digital camera mounted to a light microscope (Olympus CK40, Melville, NY). The number of adherent cells was quantified by using ImageJ software (<http://rsb.info.nih.gov/ij>).

Cell Migration and Invasion Assay

Cells were serum starved for 24 h, trypsinized, centrifuged, washed with PBS, resuspended to a concentration of 50,000 cells/mL in serum-free medium, and then treated for 30 min with DMSO control or 1 $\mu\text{L}/\text{mL}$ of 1000 \times TAE226 to produce the indicated final concentration of TAE226. The serum-starved, inhibitor-treated cell mixture (500 μL , containing 25,000 cells) was added to the upper Transwell chambers (Corning Life Sciences, Acton, MA) coated with (invasion) or without (migration) Matrigel. Media containing 1% serum was added to the bottom well of the Transwell chambers as chemoattractant. After 24 h, cells were fixed and stained with Diff-Quik Fixative Solutions (Dade Behring, Inc., Newark DE). Attached cells were imaged with a digital camera mounted to a light microscope (Olympus CK40). Cell numbers were then counted by using ImageJ software.

Image Quantification

Adhesion, migration, and invasion data were obtained from microscopic images analyzed by ImageJ software. Cells were imaged with a digital camera mounted to a light microscope (Olympus CK40). Cell numbers were then assessed with ImageJ software by converting RGB color images to eight-bit black and white images, using the threshold function to designate the black pixels (cells) to be counted, and then using the analyze particles function to count the number of groups of pixels in each image.

Statistical Analysis

Descriptive statistics were generated for all quantitative data, with presentation of means \pm standard error of the means. Significance was tested through the Student's *t*-test by using the GraphPad InStat3 program (GraphPad Software, San Diego, CA).

RESULTS

TAE226 Inhibits FAK Phosphorylation and Phosphorylation of Signal Transduction Targets Downstream of FAK

TAE226 is a novel ATP-competitive tyrosine kinase inhibitor designed to target FAK activity by binding to ATP-binding sites to prevent FAK autophosphorylation induced by growth factor receptor activation. TAE226 is a potential anti-neoplastic agent based on its ability to potently and selectively inhibit *in vitro*

FAK kinase activity (IC_{50} for FAK inhibition is 5.5 nM with IC_{50} eightfold higher for the insulin receptor and 25-fold higher for the insulin-like growth factor receptor (Novartis, data not shown). To determine if TAE226 inhibits FAK signal transduction in human glioma cells, we initially assessed the ability of TAE226 to inhibit FAK phosphorylation upon the addition of serum to serum-starved D54MG, U87MG, U251MG, and U373MG cells. TAE226 did not alter total FAK protein levels in any of the cell lines tested, but dramatically inhibited the phosphorylation of FAK at critical tyrosine residues depending upon the cell type (Figure 1). Specifically, 0.5 μ M of TAE226 reduced Tyr861 phosphorylation in all of the glioma lines evaluated (Figure 1). In distinction, 0.1 μ M of TAE226 was sufficient to decrease the phosphorylation of FAK at Tyr925 in D54MG, U87MG, and U251MG cells, but minimal effect was observed in U373MG cells (Figure 1). Also, 0.1 μ M of TAE226 was sufficient to prevent serum-induced Tyr397 phosphorylation in U87MG and U251MG cells, whereas no change was observed in D54MG or U373MG cells (Figure 1). While autophosphorylation of Tyr397 is generally regarded as a prerequisite for Src tyrosine kinase binding and Tyr925 phosphorylation [5–7], our results demonstrate FAK phosphorylation at Tyr861 and Tyr925 can be targeted by TAE226 in the absence of changes in Tyr397 phosphorylation. Together, these data demonstrate that 0.5–0.1 μ M of TAE226 decreased serum-induced FAK phosphorylation at multiple activation sites in multiple glioma cell lines, but the concentration of TAE226 necessary to target

phosphorylation on any one tyrosine residue varies with the glioma cell line investigated. The reduction in phosphorylation by TAE226 appears to be specific for FAK signaling, as concentrations of TAE226 which inhibited FAK phosphorylation (0.1 μ M) did not inhibit epidermal growth factor-induced epidermal growth factor receptor phosphorylation or serum-induced platelet-derived growth factor receptor phosphorylation (data not shown). However, we cannot entirely rule out the possibility that TAE226 may affect the phosphorylation of other tyrosine kinases in addition to FAK.

The reduction in FAK phosphorylation achieved by treatment with TAE226 correlated with the inhibition of several downstream targets of FAK signaling. In every cell line tested, TAE226 inhibited serum-induced activation of AKT in serum-starved glioma cells in a concentration-dependent manner, as shown by the decrease in AKT phosphorylation at both serine residue 473 (Ser473) and threonine residue 308 (Thr308), without altering total AKT protein levels (Figure 1). Serum-induced AKT phosphorylation was substantially reduced with 5 μ M of TAE226, as was the phosphorylation of ERK1, ERK2, and S6 ribosomal protein (Figure 1). This reduction of effector protein phosphorylation by TAE226 did not reflect unequal protein loading, as demonstrated by tubulin controls, nor did TAE226 decrease total ERK1, ERK2, or S6 ribosomal protein levels (Figure 1). Thus, TAE226 inhibits the activating phosphorylation of FAK and downstream targets of FAK, including AKT, ERK1, ERK2, and S6 ribosomal protein.

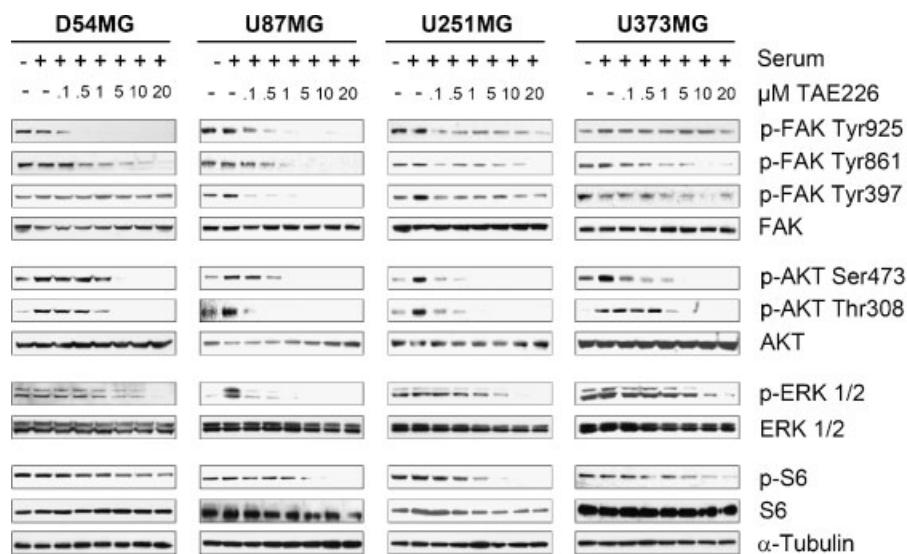


Figure 1. TAE226 suppresses FAK-mediated signal transduction. D54MG, U87MG, U251MG, and U373MG human glioma cell lines exhibited differential activation of FAK, and TAE226 inhibited FAK phosphorylation at different residues depending on the cell line. In all cell lines tested, TAE226 dose dependently inhibited phosphorylation of FAK to prevent FAK signaling. The indicated glioma cells were

serum starved overnight and treated with DMSO or increasing concentrations of TAE226 (0.1–20 μ M) for 45 min before incubation with 1% serum or BSA control for 15 min. Equal amounts of protein from each sample were resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with the indicated antibodies.

Treatment With TAE226 Reduces Glioma Proliferation by Inducing G₂ Cell Cycle Arrest

To determine the effects of TAE226 on pro-tumorigenic glioma cell behavior, we evaluated the ability of TAE226 to inhibit cell proliferation. Thymidine incorporation into glioma cells was reduced by TAE226 in a concentration-dependent manner in every cell line tested (Figure 2). Treatment

with 0.5 μ M of TAE226 was sufficient to significantly inhibit the proliferation of D54MG (Figure 2A), U87MG (Figure 2B), U373MG (Figure 2C), and U251MG (Figure 2D) human glioma cells.

To determine the mechanism by which TAE226 inhibits cellular proliferation, FACS analyses of a panel of propidium iodide-stained glioma cell lines in the presence and absence of TAE226 were completed (Figure 3). Although cell-line-specific differences were apparent, TAE226 induced G₂ cell cycle arrest in all glioma lines evaluated (Figure 3). D54MG cell proliferation was most potently repressed by TAE226. Treatment with 1 μ M of TAE226 caused 80% of D54MG cells to arrest in the G₂ phase of the cell cycle and resulted in a fourfold increase in the fraction of cells arrested at G₂ (Figure 3A–D). Approximately 40% of U87MG cells, 30% of U251MG cells, and 70% of U373MG cells arrested at G₂ when treated with 1 μ M of TAE226 (Figure 3A–D). Due to cell-line-specific differences in the original percentage of cells arrested at G₂ during the cell cycle, the percentage of cells in G₂ arrest increased by 2.5-fold in U87MG cells, 1.5-fold in U251MG cells, and twofold in U373MG cells (Figure 3A–D). In all cell lines tested, 1 μ M of TAE226 was sufficient to significantly increase the percentage of G₂ arrested cells (Figure 3D). Thus, TAE226 inhibits the growth of multiple glioma cell lines through a mechanism associated with preventing progression through the G₂ phase of the cell cycle.

TAE226 Potently Induces Apoptosis in D54MG Glioma Cells

In addition to cell cycle arrest, TAE226 treatment also increased the fraction of sub-G₀ cells in all glioma cell lines tested (Figure 3C). The percentage of cells in the sub-G₀ phase of the cell cycle increased greater than twofold in D54MG, U251MG, and U373MG and greater than fourfold in U87MG (Figure 3C and data not shown). As these data indicated a possible induction of apoptosis by TAE226, we measured apoptosis through Annexin V staining with FACS analysis in the presence and absence of TAE226 (Figure 4). Although there was no change in apoptosis in U251MG cells, 1 μ M of TAE226 significantly increased apoptosis in U87MG, U373MG, and D54MG cells (Figure 4B). However, the total change in the percentage of apoptotic cells in U373MG and U87MG was only 1.3-fold and 1.5-fold, respectively. D54MG cells were very sensitive to TAE226-induced apoptosis, with 1 μ M of TAE226 being sufficient to produce a greater than threefold increase in apoptosis (Figure 4A and B). These data, in combination with the previous results demonstrating increased G₂ arrest with TAE226 treatment, demonstrate that TAE226 reduces the proliferation of multiple glioma cell lines by increasing apoptosis in a cell-line-specific manner and by broadly increasing cell cycle arrest.

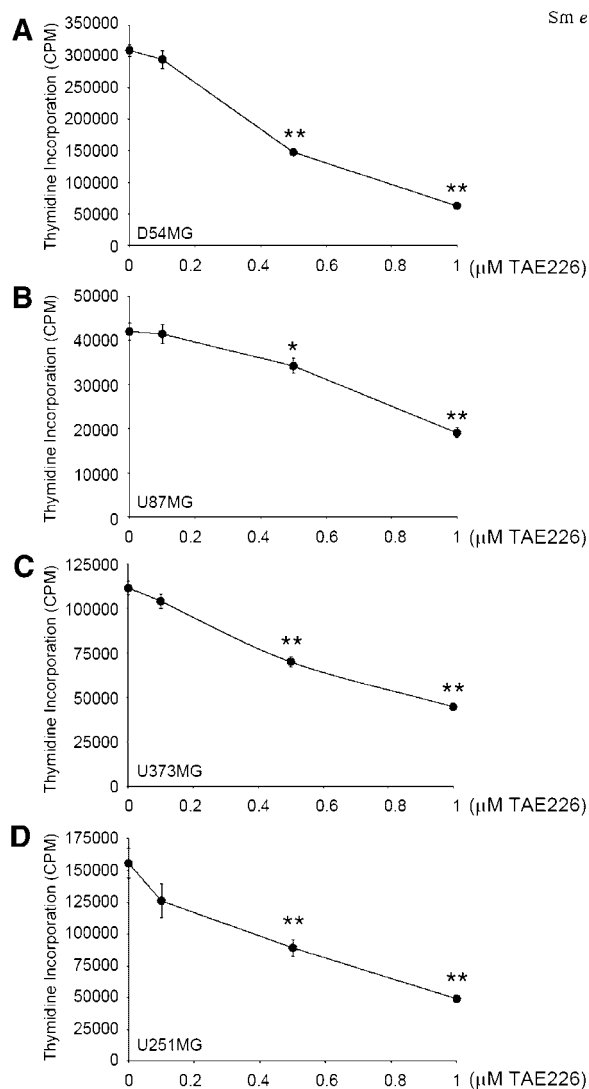


Figure 2. TAE226 inhibits the proliferation of human glioma cell lines. (A) TAE226 inhibited thymidine incorporation of D54MG cells with concentration dependence. D54MG cells ($n = 20,000$) were plated and allowed to recover for 24 h before cells were serum starved overnight. After serum starvation, cells were stimulated with 10% serum in the presence of DMSO control or increasing concentrations of TAE226 (0.1–1 μ M) and allowed to grow for 48 h. 3H-thymidine was added for the last 4 h of cell culture. Cells were fixed in 10% trichloroacetic acid, lysed in 0.1 N NaOH, and assessed by scintillation counting. (B) TAE226 decreased the proliferation of U87MG cells. U87MG cells ($n = 20,000$) were plated, and treatment was similar to that for D54MG cells. (C) TAE226 decreased the proliferation of U251MG cells. U251MG cells ($n = 10,000$) were plated and treated as indicated above. (D) TAE226 decreased the proliferation of U373MG cells. U373MG cells ($n = 20,000$) were plated and treated as indicated above (* $P < 0.05$ or ** $P < 0.001$ with *t*-test comparison to DMSO-treated control).

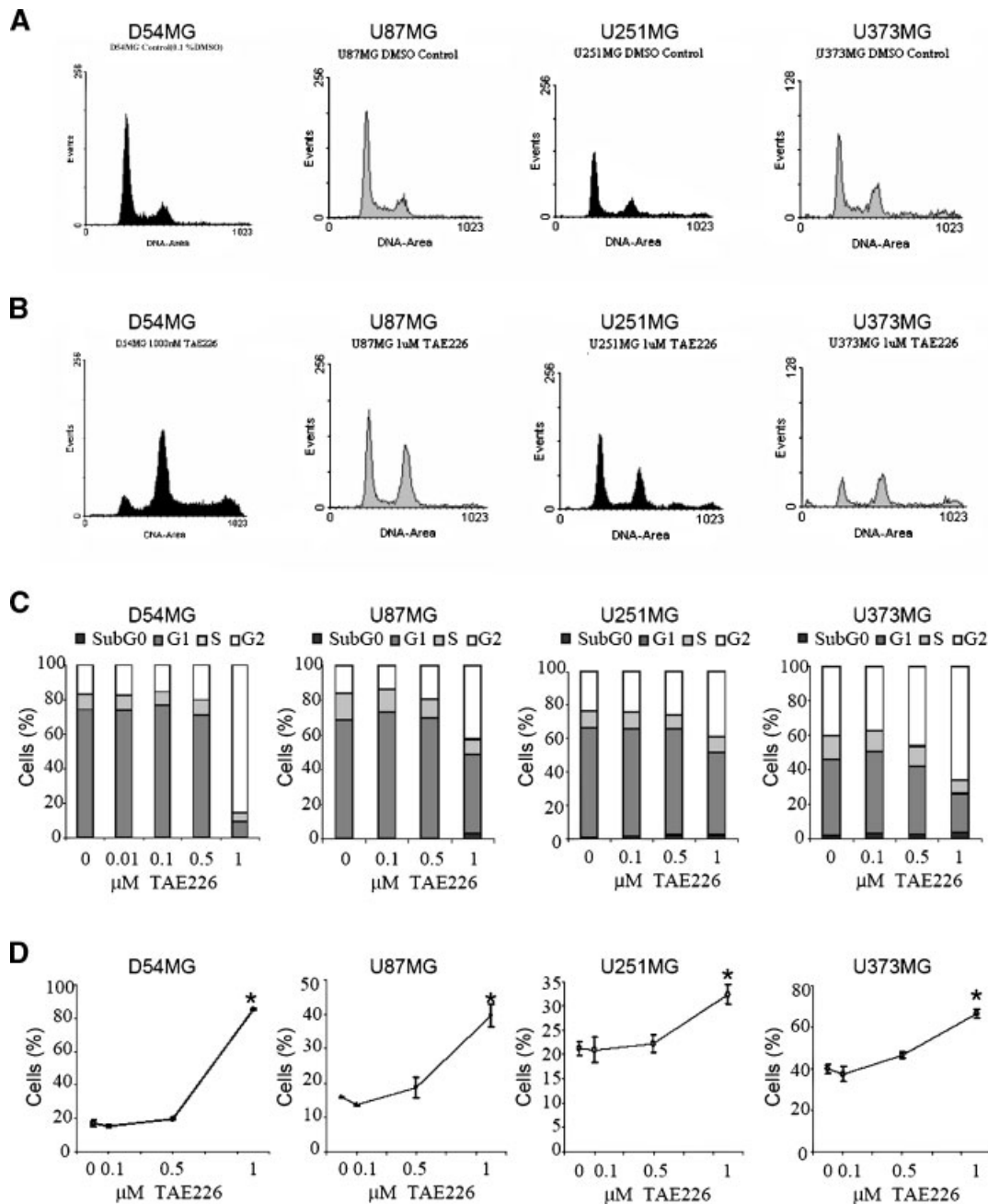


Figure 3. TAE226 causes G₂ cell cycle arrest in glioma cells. Glioma cell lines D54MG, U87MG, U251MG, and U373MG (from left to right by column) were treated with DMSO control or increasing concentrations of TAE226 (0.1, 0.5, or 1 μM) for 16 h in 10% serum. After incubation, cells were collected from the media and plate and stained with propidium iodide for cell cycle analysis. (A) Representative histograms of DMSO-treated controls demonstrate that the majority of glioma cells were in the G₁ cell cycle

fraction. (B) Representative histograms of 1 μM of TAE226 treated glioma cells demonstrate an increased G₂ cell cycle fraction. (C) Increasing concentrations of TAE226 increased the proportion of the cell population in the G₂ cell cycle fraction. (D) The percentage of cells in the G₂ phases of the cell cycle significantly increased with TAE226 treatment (**P* < 0.001 with *t*-test comparison to DMSO-treated control).

Reduction of D54MG Adhesion, Migration, and Invasion by TAE226 Treatment

As FAK is critical for cell attachment and invasion, we addressed the migratory potential of D54MG cells in the presence and absence of TAE226. Increasing concentrations of TAE226 prevented the attach-

ment, migration, and invasion of D54MG cells (Figure 5). Treatment with 0.5 μM of TAE226 significantly inhibited (by >twofold) the number of cells capable of adhering to tissue culture plates within one hour in 10% serum (Figure 5A and B). Similarly, 1 μM of TAE226 significantly decreased the number of cells migrating through uncoated Boyden

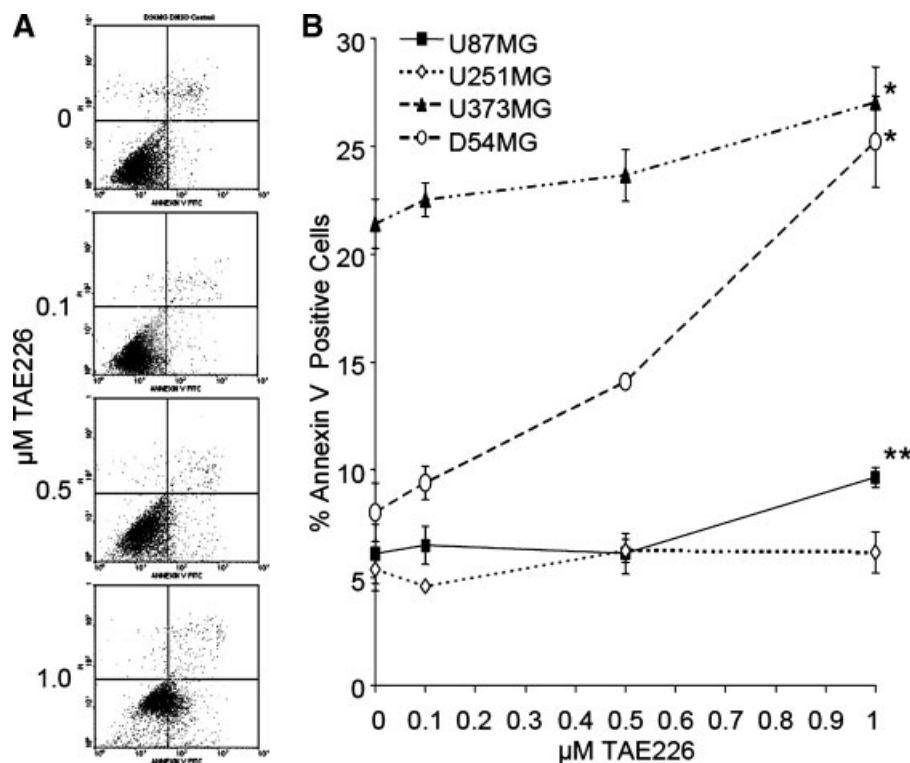


Figure 4. TAE226 induces apoptosis in glioma cells. Glioma cell lines were treated with increasing concentrations of TAE226 (0.1, 0.5, and 1 μ M) for 16 h in 10% serum. After incubation, cells were harvested from the media and trypsinized from the culture dish. Cells were stained with both Annexin V and propidium iodide followed by FACS analyses. (A) Representative FACS plots of populations from D54MG DMSO control or TAE226-treated cells are displayed. (B) TAE226 treatment increases the percentage of apoptotic D54MG, U87MG, U251MG, and U373MG cells (* P < 0.001; ** P < 0.01 with t -test comparison to DMSO treated control).

chambers (Figure 5A and C) after 24 h and significantly decreased the number of cells migrating through Matrigel-coated inserts (Figure 5A and D) after 24 h. The ratio of migrating cells to invading cells (Figure 5E) reveals that TAE226 has a greater effect on invasion of D54MG cells than on their migration. Therefore, TAE226 can prevent cell adhesion, migration, and invasion of human glioma cells in vitro.

DISCUSSION

We have now demonstrated that TAE226, a novel low-molecular-weight inhibitor of FAK, inhibits the phosphorylation of FAK and downstream oncogenic signals. As FAK regulates several tumor cellular behaviors—including cellular proliferation, resistance to apoptosis, adhesion, and invasion—we investigated the effects of TAE226 on these phenotypes. TAE226 demonstrated a concentration-dependent inhibition of proliferation associated with an induction of G_2 cell cycle arrest in all four human glioma cell lines investigated. Three of these cell lines also demonstrated significantly induced apoptosis in response to TAE226 treatment, although the induction of apoptosis was greater than twofold in D54MG only. These data demon-

strate that TAE226 has the potential to decrease glioma growth by increasing cell cycle arrest and programmed cell death. Furthermore, the ability of TAE226 to inhibit glioma cell attachment, migration, and invasion suggests that TAE226 could be beneficial for glioma control by decreasing the ability of glioma cells to invade normal brain.

The differences in survival responses to TAE226 treatment observed in the human glioma cell lines suggest that patient responsiveness to TAE226 may vary. These variations in tumor response are probably to be due to genetic differences occurring during cancer development and progression. However, we did not detect correlations between cell-line-specific responses to TAE226 and total FAK levels, FAK phosphorylation, or the status of major tumor suppressors commonly altered in gliomas. D54MG and U87MG both express wild-type p53, and all of the tested lines have mutated or deleted PTEN and p16INK4A. Thus, p53, PTEN, and p16INK4A status do not account for the cell-line-specific differences in TAE226 apoptotic response.

The anti-proliferative, pro-apoptotic, and anti-invasive effects of TAE226 suggest that TAE226 monotherapy could be beneficial for glioma patients. However, the greatest therapeutic advantage will

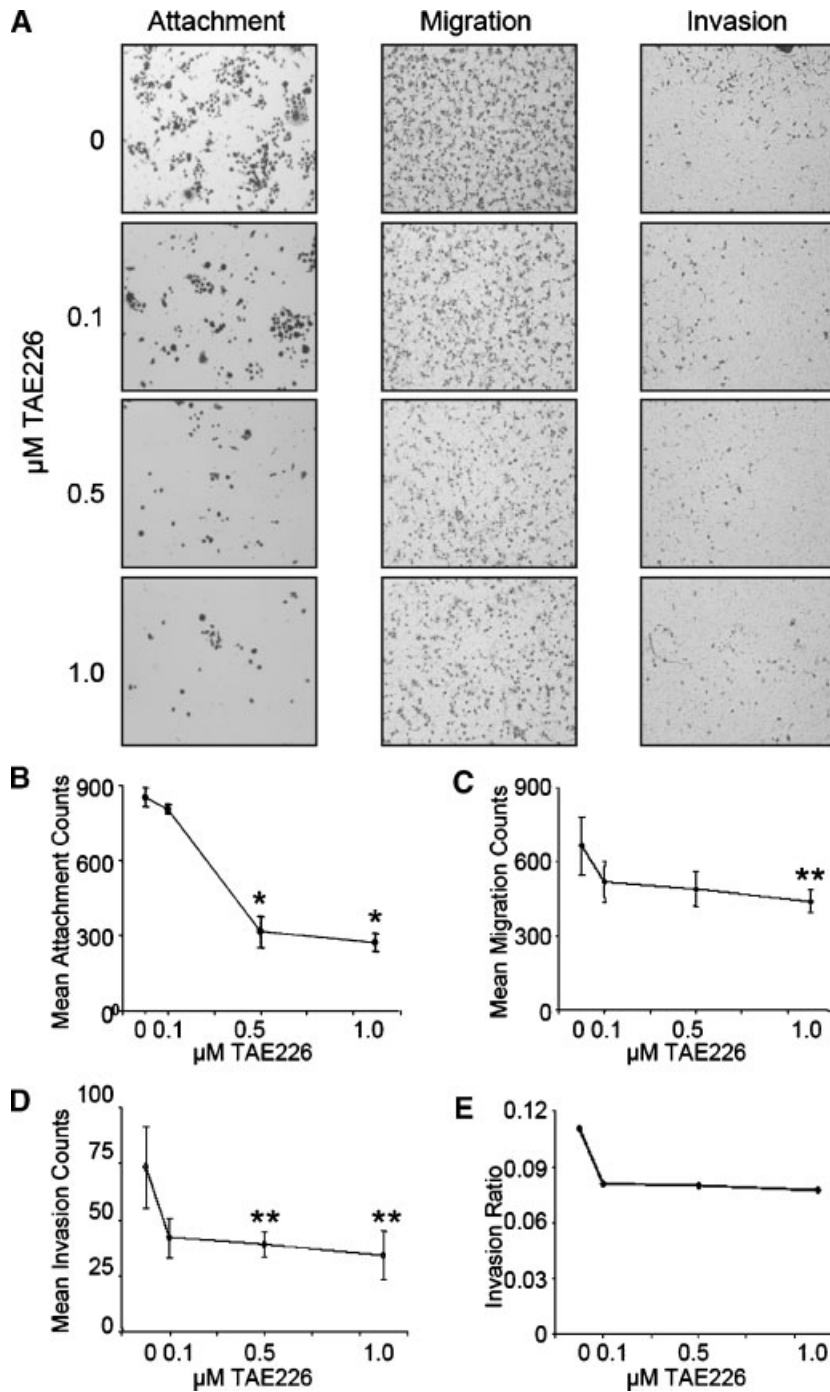


Figure 5. TAE226 reduces D54MG adhesion, migration, and invasion. (A) Representative images of attached, migrating, and invading D54MG cells in the presence of DMSO or TAE226 are displayed. (B) The average number of attached cells decreased with TAE226 treatment. D54MG cells were treated with DMSO control or increasing concentrations of TAE226 (0.1, 0.5, or 1 μ M) for 30 min in 10% serum and allowed to attach to tissue culture plates for 1 h. Randomly selected fields of fixed and stained attached cells were quantified with ImageJ software. (C) The average number of migrating cells decreased with TAE226 treatment. Serum-starved D54MG cells were treated with DMSO or increasing concentrations of TAE226 for 1 h before addition to the upper chamber of uncoated Biocoat Transwell chambers. Cells were allowed to migrate towards the bottom chamber, containing serum, for 24 h in the presence of DMSO or of the indicated concentrations of TAE226. Randomly

chosen fields of fixed and stained migrating cells were quantified with ImageJ software. (D) The average number of invading cells decreased with TAE226 treatment. Serum starved D54MG cells were treated with DMSO or increasing concentrations of TAE226 for 1 h before addition to the upper chamber of Matrigel-coated Biocoat Transwell chambers. Cells were allowed to invade towards the bottom chamber, containing serum, for 24 h in the presence of DMSO or of the indicated concentrations of TAE226. Randomly chosen fields of fixed and stained migrating cells were quantified with ImageJ software. (E) The invasion ratio decreased with TAE226 treatment. The ratio of the average counts from the invasion chambers to that of migration chambers indicate that TAE226 decreases invasion through an extracellular matrix. * $P < 0.001$ or ** $P < 0.05$ with *t*-test comparison to DMSO-treated control.

likely be derived by combining TAE226 with other targeted therapies. Monotherapies to date have failed to provide substantial benefit, but cytotoxic therapies—including ionizing radiation and chemotherapy—represent the mainstay of current post-surgical management of glioma patients. Therefore, the potential therapeutic effects of TAE226 may be best examined in combination with these agents. In particular, prior studies have suggested that targeting FAK expression may increase cellular sensitivity to chemotherapy [16]. As we have observed an induction of apoptosis in most glioma cell lines with TAE226, the combination of TAE226 with chemotherapy may result in decreased cell survival and added patient benefit.

ACKNOWLEDGMENTS

We thank Michael Cook for his technical assistance with flow cytometry. This work was supported in part by funds from the Pediatric Brain Tumor Foundation of the United States (J.N.R.), Accelerate Brain Cancer Cure (J.N.R.), Childhood Brain Tumor Foundation (J.N.R.), and Southeastern Brain Tumor Foundation (A.B.H.). This work was also supported by NIH grants NS047409, NS054276, CA108786, and CA116659 (J.N.R.). A.B.H. is a Paul Brazen/American Brain Tumor Association Fellow. J.N.R. is a Damon Runyon-Lilly Clinical Investigator supported by the Damon Runyon Cancer Research Foundation and a Kimmel Scholar supported by the Sidney Kimmel Foundation for Cancer Research.

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