Inhibition of Human Glioblastoma Cell Adhesion and Invasion by 4-(4’-Hydroxylphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P131) and 4-(3’-Bromo-4’-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P154)

Rama Krishna Narla, Xing-Ping Liu, Daniel Klis, and Fatih M. Uckun


ABSTRACT

Glioblastoma multiforme is a highly invasive primary brain tumor with a disappointingly high local recurrence rate and mortality despite intensive multimodality treatment programs. Therefore, new agents that are capable of inhibiting the infiltration of normal brain parenchyma by glioblastoma cells are urgently needed. Here, we show that the novel quinazoline derivatives 4-(4’hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P131) and 4-(3’-bromo-4’hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P154) are potent inhibitors of glioblastoma cell adhesion and migration. Specifically, both compounds inhibited at micromolar concentrations: (a) integrin-mediated glioblastoma cell adhesion to the extracellular matrix proteins laminin, type IV collagen, and fibronectin; (b) integrin-independent epidermal growth factor-induced adhesion of glioblastoma cells to poly-L-lysine-coated tissue culture plates; (c) fetal bovine serum-induced polymerization of actin and actin stress fiber formation as well epidermal growth factor-stimulated formation of focal adhesion plaques in serum-starved glioblastoma cells; and most importantly, (d) glioblastoma cell migration in in vitro assays of tumor cell invasiveness using tumor cell spheroids and/or Matrigel-coated Boyden chambers. Further preclinical development of WHI-P131 and WHI-P154 may provide the basis for the design of more effective adjuvant chemotherapy programs for glioblastoma multiforme.

INTRODUCTION

GM is a highly invasive primary tumor of the human central nervous system (1). Total surgical resection of GM is rarely possible because of a diffuse infiltration of the surrounding normal brain parenchyma by glioblastoma cells and the lack of a distinct tumor-free margin (2-4). The ability of glioblastoma cells to migrate within the context of the ECM is thought to play a major role in local recurrence and progression of GM after multimodality treatment programs using surgery in combination with radiochemotherapy (5-11). Therefore, new agents that can inhibit the infiltration of normal brain parenchyma by glioblastoma cells may provide the basis for more effective multimodality treatment programs for GM.

In a recent study, we discovered that the novel quinazoline derivatives, WHI-P131 and WHI-P154, exhibit significant cytotoxicity against human glioblastoma cell lines causing apoptotic cell death at 100–200 μM concentrations (12). In a systematic effort to discover new agents with antiapoptotic and anti-invasive activities against tumor cells, we examined these and other dimethoxyquinazoline derivatives for their ability to prevent both integrin-dependent and integrin-independent adhesion of human glioblastoma cells to ECM proteins. Here, we provide experimental evidence that compounds WHI-P131 and WHI-P154 are potent inhibitors of glioblastoma cell adhesion and migration in the context of ECM. At noncytotoxic concentrations, both compounds inhibited: (a) integrin-mediated glioblastoma cell adhesion to the ECM proteins laminin, type IV collagen, and fibronectin; (b) integrin-independent EGF-induced adhesion of glioblastoma cells to poly-L-lysine-coated tissue culture plates; (c) fetal bovine serum-induced polymerization of actin and actin stress fiber formation as well EGF-stimulated formation of focal adhesion plaques in serum-starved glioblastoma cells; and (d) glioblastoma cell migration in in vitro assays of invasiveness using tumor cell spheroids and/or Matrigel-coated Boyden chambers.

MATERIALS AND METHODS

Analysis of Quinazoline Derivatives. Proton (1H) and carbon (13C) nuclear magnetic resonance spectra were recorded

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2 The abbreviations used are: GM, glioblastoma multiforme; ECM, extracellular matrix; WHI-P79, 4-(3’-bromophenyl)-amino-6,7-dimethoxyquinazoline; WHI-P97, 4-(3’-5'-dibromo-4’-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline; WHI-P131, 4-(4’-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline; WHI-P154, 4-(3’-bromo-4’-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline; EGF, epidermal growth factor; FBS, fetal bovine serum; FAK, focal adhesion kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide.
on a Mercury 2000 Varian spectrometer operating at 300 and 75 MHz, respectively, using an automatic broad band probe. Unless otherwise noted, all nuclear magnetic resonance spectra were recorded in CDCl3 at room temperature. 1H chemical shifts are quoted in parts per million (δ in ppm) downfield from tetramethylsilane, which was used as an internal standard at 0 ppm, and s, d, t, q, m designate singlet, doublet, triplet, quartet, and multiplet, respectively. Melting points were determined using a Fisher-Johns melting apparatus and are uncorrected. UV spectra were recorded using a Beckmann Model DU 7400 UV/Vis spectrometer with a cell path length of 1 cm. Methanol was used as the solvent for the UV spectra. Fourier Transform Infra Red spectra were recorded using an FT-Nicolet model Protege 460 instrument. The IR spectra of the liquid samples were run as neat liquids using KBr discs. The KBr pellet method was used for all solid samples. The GC/mass spectrum analysis was conducted using a Hewlett-Packard GC/mass spectrometer model 6890 equipped with a mass ion detector and ChemStation software. The temperature of the oven was steadily increased from 70°C to 250°C, and the carrier gas was helium.

**General Procedure for Synthesis of 6,7-Dimethoxyquinazoline Derivatives.** The 6,7-dimethoxyquinazoline derivatives for this study were prepared by the condensation of 4-chloro-6,7-dimethoxyquinazoline and the substituted anilines as described recently in detail (12).

In brief, a mixture of 4-chloro-6,7-dimethoxyquinazoline (448 mg, 2 mmol) and the substituted aniline (2.5 mmol) in ethanol (20 ml) was heated to reflux. Heating was continued for 4–24 h, excess amount of Et3N was added to basify the solution, and the solvent was concentrated to give the crude product, which was recrystallized from dimethylformamide. The key starting material, 4-chloro-6,7-dimethoxy quinazoline, was prepared using published procedures (13, 14). Detailed analytical data for the 6,7-dimethoxy quinazoline derivatives and their precursors have been reported previously (12).

**Other Reagents.** DMEM, FBS, antibiotics, laminin, fibronectin, and collagen were obtained from Life Technologies, Inc. (Gaithersburg, MD). Tissue culture plates were from Falcon (Oxnard, CA). Matrigel matrix was obtained from Becton Dickinson (Bedford, MA). The mouse monoclonal anti-FAK antibody was purchased from Chemicon International, Inc. (Temecula, CA). Rhodamine phalloidin was obtained from Molecular Probes (Eugene, OR). BSA and soybean trypsin inhibitor were purchased from Sigma Chemical Co. (St. Louis, MO). Hema-3 staining solutions were obtained from Curtin Matheson (Houston, TX).

**Cell Lines.** Human brain tumor cell lines derived from adult patients with glioblastoma, U-87 MG, U-118 MG, U-138 MG, U-373 MG, T98-G, and medulloblastoma Daoy were obtained from American Type Culture Collection (Rockville, MD) and maintained in liquid culture using DMEM supplemented with 10% FBS and antibiotics. Fibroblast conditioned medium was prepared and used as a source of chemoattractants in *in vitro* invasion assays. For the preparation of this conditioned medium, NIH/3T3 embryonic fibroblasts (American Type Culture Collection) were grown to 80% confluency in DMEM supplemented with 10% FBS and cultured for 24 h in serum-free medium containing 0.5 μg/ml BSA. The culture supernatants were collected, centrifuged at 1000 × g for 15 min to remove cellular debris, and used as conditioned medium.

**Adhesion Assays.** *In vitro* adhesion assays were performed to: (a) study the baseline adhesive properties of various glioblastoma cell lines; and (b) evaluate the effects of quinazoline derivatives on the adhesive properties of glioblastoma cells. The plates for the adhesion assays were precoated with the ECM proteins laminin, fibronectin, or type IV collagen (each at a final concentration of 1 μg/ml in PBS) overnight at 4°C and dried. On the day of the experiment, the wells were rehydrated and blocked with 10% BSA in PBS for 1 h at room temperature and used for the adhesion assays, as described below. In order to study the effects of quinazoline derivatives on glioblastoma cell adhesion, exponentially growing cells in DMEM were incubated with the compounds WHI-P79, WHI-P97, WHI-P131, WHI-P154, WHI-P258, or genistein at concentrations ranging from 1 μM to 100 μM in 0.1% DMSO for 16 h in a humidified 5% CO2 atmosphere. DMSO (0.1%) was included as a vehicle control. After treatment, cells were detached from the flasks with 0.05% trypsin (Life Technologies, Inc.) resuspended in DMEM, transferred to 50-ml centrifuge tubes, incubated at 37°C for 2 h to allow them to recover from the trypsinization stress, then transferred to 96-well plates and examined for their ability to adhere to plates precoated with ECM proteins after 1 h of culture. During the 2-h recovery time after trypsinization, cell adhesion to the centrifuge tubes was prevented with gentle rocking.

**Molecular Probes (Eugene, OR). BSA and soybean trypsin inhibitor were purchased from Sigma Chemical Co. (St. Louis, MO). Hema-3 staining solutions were obtained from Curtin Matheson (Houston, TX).**

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<table>
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<th>Fraction of Cells Adhered (%)</th>
<th>Plastic</th>
<th>POLY-L-LYSINE</th>
<th>LAMININ</th>
<th>COLLAGEN</th>
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<td>25.1%</td>
<td>23.6%</td>
<td>24.0%</td>
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<td>25.2%</td>
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**Fig. 1** Adhesive properties of brain tumor cells to ECM proteins. Trypsinized cells were incubated for 1 h in 96-well plates coated with laminin, collagen, fibronectin, or poly-l-lysine; uncoated plates were used as controls. The nonadherent cells were removed by washing the wells with PBS, and the adherent cells were detected using MTT assays, as described in "Materials and Methods." The data points represent the means from three independent experiments; bars, SE. As compared with poly-l-lysine and plastic, all of the ECM proteins substantially promoted the adhesion of brain tumor cells.
order to study the effects of quinazoline derivatives on EGF-stimulated cell adhesion, the trypsinized and recovered cells were incubated with varying concentrations of quinazolines ranging from 1 to 50 µM for 4 h at 37°C and then stimulated with 250 ng/ml of EGF and examined for their ability to adhere to poly-L-lysine-coated plates. In adhesion assays, cells were centrifuged, washed twice with serum-free DMEM, counted, and resuspended in serum-free DMEM to a final concentration of 2.5 × 10^5 cells/ml. One hundred µl of the cell suspension containing 2.5 × 10^4 cells were added to each well, and cells were allowed to adhere for 1 h at 37°C in a humidified 5% CO₂ atmosphere. In EGF stimulation experiments, the cells were plated in the presence of 250 ng/ml (note: similar results were obtained in other studies performed by our group using 50 ng/ml EGF) of EGF and allowed to adhere for 1 h. The nonadherent cells were removed by gently washing the cells with PBS, and then the adherent fraction was quantitated using MTT assays. In brief, after washing the wells, 10 µl of MTT (final concentration, 0.5 mg/ml; Boehringer Mannheim Corp., Indianapolis, IN) were added to each well, and the plates were incubated at 37°C for 4 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized overnight at 37°C in a solution containing 10% SDS in 0.01 N HCl. The absorbance of each well was measured in a microplate reader (Labsystems) at 540 nm and a reference wavelength of 690 nm. To translate the A₅₄₀ values into the number of cells in each well, the A₅₄₀ values were compared with those on standard A₅₄₀ versus cell number curves generated for each cell line. The adherent fractions of cells treated with quinazoline derivatives were compared with those of DMSO-treated control cells, and the percentage of inhibition of adhesion was determined using the formula:

\[
\text{% Inhibition} = \frac{1 - \text{Adherent fraction of drug-treated cells}}{\text{Adherent fraction of control cells}} \times 100
\]

Each treatment condition was evaluated in duplicate in three independent experiments. The IC₅₀ values were calculated by nonlinear regression analysis using an Graphpad Prism software version 2.0 (Graphpad Software, Inc., San Diego, CA).

**Transfilter Cell Invasion Assays.** The *in vitro* invasiveness of glioblastoma cells was assayed using a method published previously, which uses Matrigel-coated Costar 24-well transwell culture chambers (“Boyden chambers”) with 8.0-µm pore polycarbonate filter inserts that have been demonstrated to permit the migration of human glioblastoma cells, including the U87 cell line analyzed in our experiments (15). The diameters of the glioblastoma cells analyzed in the present study were 20.2 ± 4.8 µm for U87 cells, 21.6 ± 7.8 µm for U373 cells, 22.4 ± 6.7 µm for T98 cells, and 19.1 ± 7.1 µm for U138 cells, as determined by confocal imaging of 20–50 cells/cell line (data not shown). The chamber filters were coated with 50 µg/ml of Matrigel matrix, incubated overnight at room temperature under a laminar flow hood, and stored at 4°C. On the day of the experiment, the coated inserts were rehydrated with 0.5 ml of serum-free DMEM containing 0.1% BSA for 1–2 h. To study the effects of quinazoline derivatives on invasiveness of glioblastoma cells, exponentially growing cells were incubated with WHI-P97, WHI-P131, and WHI-P154 at various

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**Fig. 2** Effect of quinazolines on U373 glioblastoma cell-adhesion to ECM proteins. The cells were preincubated with 10, 25, or 50 µM concentrations of WHI-P79, WHI-P97, WHI-P131, WHI-P154, or WHI-P258, and then processed for adhesion assays, as described in “Materials and Methods.” All of the quinazoline derivatives inhibited the U373 cell adhesion to ECM proteins. The highest inhibition levels were achieved with WHI-P154, followed by WHI-P97. The data points represent the means from three independent experiments; bars, SE. The mean IC₅₀ (±SE) of WHI-P154 were: 29.8 ± 3.1 µM for fibronectin; 36.1 ± 3.5 µM for laminin; and 42.7 ± 2.5 µM for collagen. The IC₅₀ of WHI-P79, WHI-P97, and WHI-P131 were >50 µM for each of the ECM proteins.
concentrations ranging from 1 to 50 μM in 0.1% DMSO overnight. The cells were trypsinized, washed twice with serum-free DMEM containing BSA, counted, and resuspended at 1 × 10⁶ cells/ml. Cell suspension (0.5 ml) containing 5 × 10⁵ cells in a serum-free DMEM containing quinazoline compounds or vehicle was added to the Matrigel-coated and rehydrated filter inserts. Next, 750 μl of NIH/3T3 fibroblast-conditioned medium were placed as a chemoattractant in 24-well plates, and the inserts were placed in wells and incubated at 37°C for 48 h. After the incubation period, the filter inserts were removed, the medium was decanted off, and the cells on the top side of the filter that did not migrate were scraped off with a cotton-tipped applicator. The invasive cells that migrated to the lower side of the filter were fixed, stained with Hema-3 solutions, and counted under the microscope. No cells were detected at the bottom of the Boyden chambers. Therefore, the number of cells on the lower side of the filters accounted for all cells that have migrated through the filter. Five to 10 random fields per filter were counted to determine the mean (±SE) values for the invasive fraction. The invasive fractions of cells treated with quinazoline derivatives were compared with those of DMSO (0.1%)-treated control cells, and the percentage of inhibition of invasiveness was determined using the formula:

\[
\%\text{Inhibition} = \frac{1 - \text{Invasive fraction of drug-treated cells}}{\text{Invasive fraction of control cells}} \times 100
\]

Each treatment condition was evaluated in duplicate in three independent experiments. The IC₅₀ values were calculated by nonlinear regression analysis using Prism software version 2.0 (Graph pad Software, Inc.).

**Migration Assay.** Migration of brain tumor cells was monitored using U373 glioblastoma cell spheroids. Glioblastoma cell spheroids were cultured in 100-mm² tissue culture plates (Falcon) precoated with 0.75% agar prepared in DMEM supplemented with 10% FBS. Cells (3 × 10⁶) were suspended in the medium, seeded onto agar-coated plates, and cultured for 5–7 days at 37°C. Spheroids of 200–400 μm diameter were selected for use in additional experiments. For migration experiments, the selected spheroids were incubated for 2 h at 37°C in DMEM containing various concentrations of WHI-P154 ranging from 1 to 25 μM. The drug-treated and vehicle control (DMSO, 0.1%) spheroids were transferred onto fibronectin-coated coverslips (Becton Dickinson, Bedford, MA) or fibronectin-coated coverslips (Becton Dickinson, Bedford, MA) and placed in six-well plates containing the same concentration of drugs in DMEM. Spheroids were then kept in a humidified 5% CO₂ incubator at 37°C for 48 h. A total of 4–6 spheroids were used for each concentration. After the 48-h incubation period, the spheroids were fixed and stained with Hema-3 solutions on glass slides. The distance migrated from spheroids was measured using an ocular micrometer and a transmitted light microscope (Olympus).

**Confocal Laser Scanning Microscopy.** Immunofluorescence was used to study the effects of quinazoline derivatives on the formation of focal adhesion plaques and polymerization of actin. Cells were plated on poly-L-lysine-coated, glass-bottomed, 35-mm Petri dishes (Mattek Corp., Ashland, MA) or fibronectin-coated coverslips and maintained in DMEM supplemented with 10% FBS for 24 h. The medium was removed, and the cells were washed twice with serum-free DMEM and incubated in the same medium for 16 h. After this serum starvation, cells were incubated with varying concentrations of WHI-P131, WHI-P154, or vehicle (0.1% DMSO) for 4–16 h at 37°C and then stimulated either with 250 ng/ml of human recombinant EGF or 10% FBS for 15, 30, 60, 120, or 180 min at 37°C. At the end of the EGF stimulation, cells were washed twice with PBS, fixed in 2% paraformaldehyde in PBS (pH 7.2), and permeabilized, and nonspecific binding sites were blocked with 2.5% BSA and
RESULTS AND DISCUSSION

Effects of the Novel Quinazoline Derivative WHI-P154 on Glioblastoma Cell Adhesion to ECM Proteins. During the multistep process of tissue invasion, tumor cells initially adhere to the ECM proteins via cell surface integrin receptors and then gain migratory capacity to enter the surrounding tissue. ECM proteins such as laminin, fibronectin, and type IV collagen are thought to play an important role in tumor cell attachment and migration. Laminin, fibronectin, and collagen have been found in the basal lamina of blood vessels and in the glial limitans externa in the brain that promote the adhesion and invasion of tumor cells in situ (16–18). The effects of these ECM proteins on integrin-mediated glioblastoma cell adhesion was examined using four different human glioblastoma cell lines (U87, U373, T98, and U138) and one medulloblastoma (Daoy) cell line. As shown in Fig. 1, a significantly greater fraction of glioblastoma and medulloblastoma cells adhered to plates precoated with laminin, type IV collagen, or fibronectin than to uncoated or poly-L-lysine-coated control plates. Of the five tumor cell lines examined, U373 cells were the most adhesive. Therefore, U373 cells were used in subsequent experiments that were designed to examine the effects of various quinazoline derivatives on integrin-mediated glioblastoma cell adhesion.

As shown in Fig. 2, the novel quinazoline derivative WHI-P154 (but not the unsubstituted parent compound WHI-P258) inhibited the adhesion of U373 cells to laminin-, fibronectin-, and collagen-coated plates in a dose-dependent fashion with mean IC50 values of 29.8 ± 3.1 μM (n = 3) for adhesion to fibronectin-coated plates, 36.1 ± 3.5 μM (n = 3) for adhesion to laminin-coated plates, and 42.7 ± 2.5 μM (n = 3) for adhesion to collagen-coated plates. The 3′-bromo substitution on the phenyl ring likely contributes to the activity of WHI-P154 because WHI-P131 lacking this bromo substituent was less potent than WHI-P154 (all IC50 values: >50 μM). The 4′-hydroxyl substituent on the phenyl ring also contributes to the inhibitory activity of WHI-P154 because WHI-P79, which differs from WHI-P154 only by the lack of the 4′-hydroxyl group on the phenyl ring, was less potent (all IC50 values: >50 μM; Fig. 2). Introduction of a second bromo group at the 5′ position of the phenyl ring did not result in improved inhibitory activity; WHI-P97 was not more potent than WHI-P154.

Effects of WHI-P154 on EGF-induced Glioblastoma Cell Adhesion. In addition to binding of cell surface receptors to ECM proteins and integrin clustering, formation of focal adhesion plaques is also regulated by the activation of FAK by certain growth factors upon binding to their receptors (19–22). EGF is a potent mitogen for various brain tumor cells that certain growth factors upon binding to their receptors (19-22). Cell Adhesion. In addition to binding of cell surface receptors to ECM proteins and integrin clustering, formation of focal adhesion plaques is also regulated by the activation of FAK by certain growth factors upon binding to their receptors (19-22). EGF is a potent mitogen for various brain tumor cells that certain growth factors upon binding to their receptors (19-22).
potent, inhibitory effects were observed when cells were pre-treated with WHI-P131.

Effects of WHI-P154 on U373 Glioblastoma Cell Invasion through Matrigel Matrix. Matrigel matrix is made up of growth factors and several ECM components, including collagen, laminin, and proteoglycans. As shown in Fig. 4A, U373 glioblastoma cells were highly invasive in Matrigel-coated Boyden chambers. WHI-P154 inhibited the invasion of U373 cells through the Matrigel matrix in dose-dependent fashion, and it was more potent than WHI-P131 or WHI-P97 (Fig. 4B). The mean IC50 values obtained from three independent experiments were 10.6 ± 1.8 μM (range, 9.6–11.6 μM) for WHI-P97, 7.1 ± 1.8 μM (range, 5.1–8.6 μM) for WHI-P131, and 4.5 ± 0.8 μM (range, 3.5–5.0 μM) for WHI-P154. The IC50s derived from the average values of three experiments were 9.6 μM for WHI-P97, 7.9 μM for WHI-P131, and 5.2 μM for WHI-P154.

Effects of WHI-P154 on U373 Glioblastoma Cell Migration from Spheroids. Dissociation and migration are the initial steps in tumor cell infiltration into the surrounding tissue. As shown in Fig. 5A, U373 glioblastoma cells rapidly migrate to a distance 825 ± 29 μm away from the spheroid. Treatment of spheroids with WHI-P154 inhibited tumor cell migration in a dose-dependent fashion (Fig. 5, B–E) with an IC50 value of 3.2 ± 0.2 μM. Treatment with 10 μM WHI-P154 resulted in 96.1 ± 3.0% inhibition of the tumor cell migration from spheroids (migration distance, 32.1 ± 25.1 μm; Fig. 5E).

WHI-P154 Inhibits EGF-induced as well as Serum-induced Formation of Focal Adhesion Plaques and Serum-induced Actin Polymerization in Human Glioblastoma Cells. Cytoskeletal organization and cellular adhesion are two crucial determinants of cell motility, and these processes are controlled by the complex coordination of actin cytoskeletal rearrangement and changes in focal adhesions (27–30). Polymerization of actin filaments and formation of lamelipodia and filopodia at the leading edges are essential for the cell attachment and detachment from the ECM and play pivotal roles in cell motility and migration (31, 32). Formation of adhesion plaques is also important in this process because the polymerized actin fibers are tethered and linked to ECM at these junctions, and the cell movement is dependent on the strength of these focal adhesions. A moderate level of cellular adhesive strength is thought to be necessary for cell migration (31, 33, 34). Adhesions that are too strong may impair cell motility, whereas adhesions that are too weak may not provide sufficient momentum to move the cell forward.

EGF-induced cell adhesion is brought about by enhanced formation of FAK+/Actin+ focal adhesion plaques, which is triggered by redistribution of activated FAK. EGF-induced formation of focal adhesions in serum-starved U373 cells was examined by multicolor immunofluorescence and confocal laser scanning microscopy using a murine monoclonal anti-FAK antibody (green fluorescence) and rhodamine-labeled phalloidin that stains actin (red fluorescence). As shown in Fig. 6, a 2-h stimulation of serum-starved U373 cells with EGF resulted in the emergence of focal adhesion plaques with high intensity FAK staining (bright green fluorescence). These FAK+ adhesion plaques showed a strong phalloidin staining (bright red fluorescence), confirming the colocalization of actin. Notably, preincubation of U373 cells with WHI-P154 (Fig. 6B) or WHI-P131 (data not shown) at a 10 μM concentration prevented the formation of FAK+/Actin+ focal adhesion plaques after EGF stimulation.

To evaluate the actin polymerization process, cells plated on poly-L-lysine-coated plates were first serum-starved to depolymerize the actin stress fibers. Subsequently, cells were stimulated with FBS to induce de novo stress fiber formation. As shown in Fig. 7, A and B, a 2-h stimulation of serum-starved U373 cells with FBS (10% v/v) resulted in a marked increase of actin polymerization and formation of actin stress fibers. Pretreatment of serum-starved U373 cells with WHI-P154 inhibited serum-induced actin polymerization (Fig. 7C). Similar results
Fig. 6  WHI-P154 inhibits formation of focal adhesion plaques in U373 cells. U373 cells were grown to 70% confluency on poly-L-lysine-coated, glass-bottomed Petri dishes. Cells were serum-starved for 24 h and incubated with 10 μM WHI-P154. Subsequently, cells were stimulated with 250 ng/ml of EGF for 2 h and processed for immunofluorescence to detect FAK using a murine monoclonal anti-FAK antibody and actin using rhodamine-labeled phalloidin. Blue fluorescence represents the nuclei stained with TOTO-3. Left panel, 3-color confocal images of U373 cells depicting FAK (green fluorescence), actin (red fluorescence), and nuclei (blue fluorescence). The colocalization of actin and FAK in focal adhesion plaques (arrows) is reflected by the yellow fluorescence (i.e., superimposed red and green fluorescence). Right panel, redistribution of FAK (green fluorescence) after the stimulation of a serum-starved cells with EGF and the inhibition of this process by WHI-P154. A and A', serum-starved cells characterized by lack of actin stress fiber bundles and focal adhesion plaques. B and B', addition of EGF to serum-starved cells results in the redistribution of FAK from the perinuclear region to the periphery of the cell to form the focal adhesion plaques (arrows). C and C', preincubation of serum-starved cells with 10 μM of WHI-P154 and subsequent stimulation with EGF for 2 h results in near complete inhibition of focal adhesion plaque formation.

were obtained with WHI-P131 but not with the unsubstituted dimethoxy quinazoline compound WHI-P258 (data not shown).

A complex network of intracellular molecules, including receptor tyrosine kinases and Src family tyrosine kinases, in cooperation with several extracellular factors such as substrate, to which cells adhere, and external growth factors, such as EGF, regulates the tumor cell adhesion and motility (23–26, 35).

Activation of integrin family adhesion receptors upon binding to specific ECM proteins has been shown to enhance the phosphorylation of integrins and activation of several intracellular signaling proteins, including mitogen-activated protein kinase, FAK, Src tyrosine kinases, as well as p130Cas, talin, paxillin, and cortactin, which were identified as substrates for the Src tyrosine kinase (36–43). Subsequently, the adhesion of cells is strength-
Fig. 7 WHI-P154 inhibits formation of actin stress fibers in glioblastoma cells. Serum-starved U373 cells were incubated with 10 μM WHI-P154 for 24 h and then stimulated with 10% FBS for 2 h. Cells were then processed for immunofluorescence using rhodamine-labeled phalloidin to detect the polymerized actin stress fibers (red fluorescence). A, serum-starved cells with characteristic lack of actin stress fibers. B, addition of 10% FBS stimulates the actin polymerization and formation of actin stress fibers bundles. C, preincubation of cells with 10 μM WHI-P154 results in complete inhibition of FBS-induced actin polymerization and stress fiber formation.

REFERENCES


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