4-(3’-Bromo-4’hydroxylphenyl)-amino-6,7-dimethoxyquinazoline: A Novel Quinazoline Derivative with Potent Cytotoxic Activity against Human Glioblastoma Cells

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ABSTRACT

The novel quinazoline derivative 4-(3’-bromo-4’-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P154) exhibited significant cytotoxicity against U373 and U87 human glioblastoma cell lines, causing apoptotic cell death at micromolar concentrations. The in vitro antiglialbloma activity of WHI-P154 was amplified >200-fold and rendered selective by conjugation to recombinant human epidermal growth factor (EGF). The EGF-P154 conjugate was able to bind to and enter target glioblastoma cells within 10–30 min via receptor (R)-mediated endocytosis by inducing internalization of the EGF-R molecules. In vitro treatment with EGF-P154 resulted in killing of glioblastoma cells at nanomolar concentrations with an IC50 of 813 ± 139 mM, whereas no cytotoxicity against EGF-R-negative leukemia cells was observed, even at concentrations as high as 100 μM. The in vivo administration of EGF-P154 resulted in delayed tumor progression and improved tumor-free survival in a severe combined immunodeficient mouse glioblastoma xenograft model. Whereas none of the control mice remained alive tumor-free beyond 33 days (median tumor-free survival, 19 days) and all control mice had tumors that rapidly progressed to reach an average size of >500 mm3 by 58 days, 40% of mice treated for 10 consecutive days with 1 mg/kg/day EGF-P154 remained alive and free of detectable tumors for more than 58 days with a median tumor-free survival of 40 days. The tumors developing in the remaining 60% of the mice never reached a size >50 mm3. Thus, targeting WHI-P154 to the EGF-R may be useful in the treatment of glioblastoma multiforme.

INTRODUCTION

As the most malignant primary central nervous system tumors, high-grade anaplastic astrocytoma and glioblastoma multiforme respond poorly to contemporary multimodality treatment programs using surgical resection, radiation therapy, and chemotherapy with a median survival of <1 year after initial diagnosis (1–4). Consequently, the development of effective new agents and novel treatment modalities against these very poor prognosis brain tumors remains a major focal point in translational oncology research.

In a systematic effort to identify a cytotoxic agent with potent antitumor activity against glioblastoma cells, we synthesized several dimethoxy-substituted quinazoline derivatives and examined their in vitro and in vivo effects on human glioblastoma cells. Here, we provide experimental evidence that the novel quinazoline derivative WHI-P154 exhibits potent cytotoxic activity against human glioblastoma cells. Notably, targeting of WHI-P154 to the surface EGF-R further enhanced its cytotoxic activity, resulting in rapid apoptotic death of glioblastoma cells at nanomolar concentrations in vitro and significantly improved tumor-free survival in an in vivo SCID mouse glioblastoma xenograft model.

MATERIALS AND METHODS

Synthesis and Analysis of Quinazoline Derivatives. All chemicals were purchased from the Aldrich Chemical Company (Milwaukee, WI) and were used directly for synthesis. Anhydrous solvents such as acetonitrile, methanol, ethanol, ethyl acetate, tetrahydrofuran, chloroform, and methylene chloride were obtained from Aldrich as Sure Seal bottles under nitrogen and were transferred to reaction vessels by cannulation. All reactions were carried out under a nitrogen atmosphere.

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2 The abbreviations used are: WHI-P154, 4-(3’-bromo-4’hydroxylphenyl)-amino-6,7-dimethoxyquinazoline; WHI-P79, 4-(3’-bromophenyl)-amino-6,7-dimethoxyquinazoline; WHI-P97, 4-(3’,5’-dibromo-4’-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline; WHI-P111, 4-(4’-bromo-4’-methylphenyl)-amino-6,7-dimethoxyquinazoline; WHI-P131, 4-(4’-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline; WHI-P132, 4-(2’-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline; WHI-P180, 4-(3’-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline; WHI-P197, 4-(3’-chloro-4’-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline; WHI-P258, 4-(phenyl)-amino-6,7-dimethoxyquinazoline; SCID, severe combined immunodeficient; EGF, epidermal growth factor; rEGF, recombinant human EGF; EGF-R, EGF receptor; NMR, nuclear magnetic resonance; s, d, t, q, m, and br, singlet, doublet, triplet, quartet. multiplet, and broad, respectively; IR, infrared; GC/MS, gas chromatography/mass spectrometry; m.p., melting point; HPLC, high-performance liquid chromatography; Sulfo-SANPAH, sulfosuccinimidyl 6-(4’azido-2’-nitrophenylamino)hexanoate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; GEN, genistein; TdT, terminal deoxynucleotidyl transferase; PTK, protein tyrosine kinase.
Proton and carbon NMR (1H and 13C NMR) spectra were recorded on a Mercury 2000 Varian spectrometer operating at 300 and 75 MHz, respectively, using an automatic broad-band probe. Unless otherwise noted, all NMR 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, and m. Melting points were determined using a Fisher-Johns melting apparatus and are uncorrected. UV spectra were recorded using a Beckman 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 IR spectra were recorded using an FT-Nicolet model Protege 460 instrument. The IR spectra of the liquid samples were run as undiluted liquids using KBr discs. The KBr pellet method was used for all solid samples. The GC/MS spectrum analysis was conducted using a Hewlett-Packard GC/mass spectrometer model 6890 equipped with a mass ion detector and Chem Station 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 outlined in Scheme 1:

![Scheme 1]

R = substituent; n = number

Specifically, 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, an excess amount of Et3N was added to 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-dimethoxyquinazoline (compound 2), was prepared using published procedures (5, 6) as outlined in Scheme 2:

![Scheme 2]

Specifically, 4,5-dimethoxy-2-nitrobenzoic acid (compound 1) was treated with thionyl chloride, which was directly reduced with ammonia to yield 4,5-dimethoxy-2-nitrobenzamide (compound 2). Compound 2 was reduced with sodium borohydride in the presence of catalytic amounts of copper sulfate to give 4,5-dimethoxy-2-aminobenzamide (compound 3), which was directly refluxed with formic acid to yield 6,7-dimethoxyquinazoline-4(3H)-one (compound 4). Compound 4 was refluxed with phosphorus oxychloride to give 4-chloro-6,7-dimethoxyquinazoline (compound 5) in good yield.

**Quinazoline Derivatives and Their Physical Data.** Table 1 lists the quinazoline derivatives synthesized for the present study. Selected analytical data for these compounds and their precursors are as follows:

4,5-Dimethoxy-2-nitrobenzamide (precursor compound 2). Yield 88.50%, m.p. 197.0-200.0°C. 1H NMR (DMSO-d6): 8 7.60 (s, 2H, NH2), 7.57 (s, 1H, 6-H), 7.12 (s, 1H, 3-H), 3.90, 3.87 (s, 3H, OCH3). UV (methanol) λmax (nm): 206.0, 244.0, 338.0 nm. IR (KBr) νmax (cm⁻¹): 3545, 2840, 1648, 1504, 1261, 1070 cm⁻¹. GC/MS m/z 226 (M⁺, 10.0), 178 (98.5), 163 (100.0), 135 (51.0).

6,7-Dimethoxyquinazoline-4(3H)-one (precursor compound 4). Yield 81.50%, m.p. 197.0-200.0°C. 1H NMR (DMSO-d6): 8 12.03 (br, s, 1H, NH), 7.99 (s, 1H, 2-H), 7.42 (s, 1H, 5-H), 7.11 (s, 1H, 8-H), 3.88, 3.85 (s, 3H, OCH3). UV (methanol) λmax (nm): 212.0, 241.0 nm. IR (KBr) νmax (cm⁻¹): 3015, 2840, 1648, 1504, 1261, 1070 cm⁻¹. GC/MS m/z 206 (M⁺, 100), 191 (M⁺ -CH3, 31.5), 163 (16.7), 120 (15.2).

4-Chloro-6,7-dimethoxyquinazoline (precursor compound 5). Yield 75.00%, m.p. 259.0-263.0°C. 1H NMR (DMSO-d6): 8 8.75 (s, 1H, 2-H), 7.53 (s, 1H, 5-H), 7.25 (s, 1H, 8-H), 3.91 (s, 3H, OCH3), 3.89 (s, 3H, OCH3). UV (methanol) λmax (nm): 207.0, 212.0, 241.0 nm. IR (KBr) νmax (cm⁻¹): 2963, 2834, 1880, 1612, 1555, 1503, 1315, 962 cm⁻¹. GC/MS m/z 224 (M⁺, 100), 209 (M⁺ -CH3, 9.4), 189 (19.39), 169 (10.55). Anal. (C19H12ClNO2) C, H, N.

(3'-Bromophenyl)-amino-6,7-dimethoxyquinazoline (WHI-P197). Yield 85.17%, m.p. 246.0-249.0°C. 1H NMR (DMSO-d6): 8 10.42 (br, s, 1H, NH), 8.68 (s, 1H, 2-H), 8.07-7.36 (m, 5H, 5', 6', 7', 8'). 7.24 (s, 1H, 8H), 3.98 (s, 3H,
4-(3′,5′-Dibromo-4′-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P97). Yield 72.80%, m.p. >300.0°C. 1H NMR (DMSO-d6): δ 8.71 (s, 1H, -NH), 8.39 (s, 1H, -OH), 8.38 (s, 1H, -H), 8.07 (s, 2H, 2'-H, 6'-H), 7.76 (s, 1H, 5-H), 7.17 (s, 1H, 8-H), 3.94 (s, 3H, -OCH3), 3.91 (s, 3H, -OCH3). UV (methanol) λmax(e): 204.0, 210.0, 245.0, 320.0 nm; IR (KBr) vmax: 3500 (br), 3494, 3419, 2868, 1627, 1512, 1443, 1243, 1068 cm⁻¹. GC/MS m/z 361 (M+ 1, 61.83), 360 (M⁺, 100.00), 359 (M⁺-1, 63.52), 344 (11.34), 222 (10.87), 140 (13.65). Anal. (C₁₅H₁₂BrN₂O₂) C, H, N.

4-(3′-Chloro-4′-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P197). Yield 84.14%, m.p. 245.0°C (dec.). 1H NMR (DMSO-d6): δ 10.00 (s, 1H, -NH), 9.37 (s, 1H, -OH), 8.41 (s, 1H, 2-H), 7.78 (s, 1H, 5-H), 7.49 (d, 1H, J₂₋₆₂ = 2.7 Hz, 2-H), 7.55 (dd, 1H, J₂₋₆₂ = 9.0 Hz, J₂₋₆₂ = 2.7 Hz, 6'-H), 7.16 (s, 1H, 8-H), 6.97 (d, 1H, J₂₋₆₂ = 9.0 Hz, 5'-H), 3.93 (s, 3H, -OCH3), 3.91 (s, 3H, -OCH3). UV (methanol) λmax(e): 209.0, 224.0, 249.0, 330.0 nm. IR (KBr) vmax: 3448, 2842, 1630, 1526, 1423, 1241 cm⁻¹. GC/MS m/z 282 (M+ 1, 10.50), 281 (M⁺, 55.00), 280 (M⁺-1, 100.00), 264 (16.00), 207 (8.50). Anal. (C₁₅H₁₂ClN₂O₂) C, H, N.

Preparation of the EGF-P154 Conjugate. rhEGF was produced in *Escherichia coli* harboring a genetically engineered plasmid that contains a synthetic gene for human EGF fused at the NH₂ terminus to a hexapeptide leader sequence for optimal protein expression and folding. rhEGF fusion protein precipitated in the form of inclusion bodies, and the mature protein was recovered by trypsin cleavage, followed by purification using ion exchange chromatography and HPLC. rhEGF was 99% pure by reverse-phase HPLC and SDS-PAGE with an isoelectric point of 4.6 ± 0.2. The endotoxin level was 0.172 EU/mg. The rhEGF fusion protein precipitated at 4°C with gentle mixing for 10 min with UV light at wavelengths 285-366 nm with a multiband UV light emitter (model UVGL-15 Mineralight; UVP, San Gabriel, CA). Photolytic generation of a reactive singlet nitrene on the other terminus of EGF-P154 (50 inst solution in DMSO) and then irradiated with gentle mixing for 10 min with UV light at wavelengths 254-366 nm with a multiband UV light emitter (model UVGL-15 Mineralight; UVP, San Gabriel, CA). Photolytic generation of a reactive singlet nitrene on the other terminus of EGF-SANPAH in the presence of a 10-fold molar excess of UVGL-15 Mineralight; UVP, San Gabriel, CA). The endotoxin level was 0.172 EU/mg. The rhEGF fusion protein precipitated at 4°C with gentle mixing for 10 min with UV light at wavelengths 285-366 nm with a multiband UV light emitter (model UVGL-15 Mineralight; UVP, San Gabriel, CA).

Post-translational modification of the EGF-P154 conjugate. rhEGF was produced in *Escherichia coli* harboring a genetically engineered plasmid harboring a genetically engineered plasmid that contains a synthetic gene for human EGF fused at the NH₂ terminus to a hexapeptide leader sequence for optimal protein expression and folding. rhEGF fusion protein precipitated in the form of inclusion bodies, and the mature protein was recovered by trypsin cleavage, followed by purification using ion exchange chromatography and HPLC. rhEGF was 99% pure by reverse-phase HPLC and SDS-PAGE with an isoelectric point of 4.6 ± 0.2. The endotoxin level was 0.172 EU/mg. The rhEGF fusion protein precipitated at 4°C with gentle mixing for 10 min with UV light at wavelengths 285-366 nm with a multiband UV light emitter (model UVGL-15 Mineralight; UVP, San Gabriel, CA)
ascertain the Amax for EGF-P154, EGF-SANPAH, and unmodified EGF. Each HPLC chromatogram was subsequently run at wavelengths of 214, 265, and 480 nm using the multiple wavelength detector option supplied with the instrument to ensure optimal detection of the individual peaks in the chromatogram. Analysis was achieved using a gradient flow consisting of 0–100% eluent in a time interval of 0–30 min. Five-μl samples applied to the above column were run using the following gradient program: 0–5 min, 0–20% eluent; 5–20 min, 20–100% eluent; 25–30 min, 100% eluent; and 30–35 min, 100–0% eluent. The eluent was a mixture of 80% acetonitrile (CH3CN), 20% H2O, and 0.1% trifluoroacetic acid. Electrospray ionization mass spectrometry (8, 9) was performed using a triple quadruple mass spectrometer (PE SCX, API; Finnigan, Norwalk, CT) to determine the stoichiometry of P154 and EGF in EGF-P154.

**Cell Lines.** Human glioblastoma cell lines U87 and U373 were used as targets for various cytotoxic agents, including EGF-P154. These brain tumor cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained as continuous cell lines in DMEM supplemented with 10% fetal bovine serum and antibiotics. The B-lineage acute lymphoblastic leukemia cell line Nalm-6 was used as a negative control.

**Uptake and Internalization of EGF-P154.** Immunofluorescence was used to: (a) examine the surface expression of EGF-R on brain tumor cells; (b) evaluate the uptake of EGF-P154 by brain tumor cells; and (c) examine the morphological features of EGF-P154-treated brain tumor cells. For analysis of EGF-R expression and cellular uptake of EGF-P154, U87 and U373 glioblastoma cells were plated on poly-L-lysine-coated, glass-bottomed, 35-mm Petri dishes (Mattek Corp., Ashland, MA) and maintained for 48 h. In uptake studies, the culture medium was replaced with fresh medium containing 5 μg/ml EGF, EGF-P154, or unconjugated WH1-P154, and cells were incubated at 37°C for 5, 10, 15, 30, and 60 min and 24 h. At the end of the incubation, cells were washed twice with PBS and fixed in 2% paraformaldehyde. The cells were permeabilized, and nonspecific binding sites were blocked with 2.5% BSA in PBS containing 0.1% Triton X-100 for 30 min. To detect the EGF-R/EGF-P154 complexes, cells were incubated with a mixture of a monoclonal antibody (1:10 dilution in PBS containing BSA and Triton X-100) directed to the extracellular domain of the human EGF-R (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a polyclonal rabbit anti-P154 antibody (dilution, 1:500) for 1 h at room temperature. After rinsing with PBS, cells were incubated for 1 h with a mixture of a goat anti-mouse IgG antibody conjugated to FITC (Amersham Corp., Arlington Heights, IL) and donkey anti-rabbit IgG conjugated to Texas Red (Amersham Corp.) at a dilution of 1:40 in PBS. Similarly, tubulin expression was examined by immunofluorescence using a monoclonal antibody against α-tubulin (Sigma Chemical Co., St. Louis, MO) at a dilution of 1:1000 and an anti-mouse IgG conjugated to FITC. Cells were washed in PBS and counterstained with TOTO-3 (Molecular Probes, Inc., Eugene, OR) for 10 min at a dilution of 1:1000. Cells were washed again with PBS, and the coverslips were mounted with Vectashield (Vector Labs, Burlingame, CA) and viewed with a confocal microscope (Bio-Rad MRC 1024) mounted in a Nikon Labophot upright microscope. Digital images were saved on a Jaz disc and processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA).

**Cytotoxicity Assay.** The cytotoxicity of various compounds against human brain tumor cell lines was performed using the MTT assay (Boehringer Mannheim Corp., Indianapolis, IN). Briefly, exponentially growing brain tumor cells were seeded into a 96-well plate at a density of 2.5 × 104 cells/well and incubated for 36 h at 37°C before drug exposure. On the day of treatment, culture medium was carefully aspirated from the wells and replaced with fresh medium containing the quinazoline compounds WHI-P79, WHI-P97, WHI-P131, and WHI-P154, unconjugated EGF, or EGF-P154 as well as the tyrosine kinase inhibitory isoflavone GEN, at concentrations ranging from 0.1 to 250 μM. Triplicate wells were used for each treatment. The cells were incubated with the various compounds for 24–36 h at 37°C in a humidified 5% CO2 atmosphere. To each well, 10 μl of MTT (final concentration, 0.5 mg/ml) was added, 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 M 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 A570 values into the number of live cells in each well, the A570 values were compared with those on standard A570 versus cell number curves generated for each cell line. The percentage of survival was calculated using the formula: % survival = Live cell number[test]/Live cell number[control] × 100. The IC50 was calculated by nonlinear regression analysis using Graphpad Prism software version 2.0 (Graphpad Software, Inc., San Diego, CA).

**In Situ Detection of Apoptosis.** The demonstration of apoptosis was performed by the in situ nick-end labeling method using the ApopTag in situ detection kit (Oncor, Gaithersburg, MD) according to the manufacturer’s recommendations. Exponentially growing cells were seeded in six-well tissue culture plates at a density of 50 × 10⁴ cells/well and cultured for 36 h at 37°C in a humidified 5% CO2 atmosphere. The supernatant culture medium was carefully aspirated and replaced with fresh medium containing unconjugated EGF or EGF-P154 at concentrations of 10, 25, or 50 μg/ml. After a 36-h incubation at 37°C in a humidified 5% CO2 incubator, the supernatants were carefully aspirated, and the cells were treated for 1–2 min with 0.1% trypsin. The detached cells were collected into a 15-ml centrifuge tube, washed with medium, and pelleted by centrifugation at 1000 rpm for 5 min. Cells were resuspended in 50 μl of PBS, transferred to poly-L-lysine-coated coverslips, and allowed to attach for 15 min. The cells were washed once with PBS and incubated with equilibration buffer for 10 min at room temperature. After removal of the equilibration buffer, cells were incubated for 1 h at 37°C with the reaction mixture containing TdT and digoxigenin-11-UTP for labeling of exposed 3’-hydroxyl ends of fragmented nuclear DNA. The cells were washed with PBS and incubated with antidigoxigenin antibody conjugated to FITC for 1 h at room temperature to detect the incorporated dUTP. After washing the cells with PBS, the coverslips were mounted onto slides with Vectashield containing propidium iodide (Vector Labs, Burlingame, CA) and viewed with a confocal laser scanning microscope. Nonapoptotic cells do not incorporate significant amounts of dUTP due to lack of exposed 3’-hydroxyl ends and consequently have much less fluorescence than apoptotic cells, which have an abundance of exposed
jugated EGF (1 mg/kg/dose), and unconjugated W1-ll-Pl54 (1 mg/kg/dose) were used as controls. Mice were monitored daily for food or water, or at the end of the 3-month observation period. However, to our surprise, WHI-P79, which is an equally potent inhibitor of EGF-R and Src family tyrosine kinases (14, 15), was found to be a potent inhibitor of the EGF-R tyrosine kinase as well as its activation of the Src family kinases. Therefore, we initially postulated that the cytotoxicity of WHI-P154 against glioblastoma cells was due to its tyrosine kinase inhibitory properties. However, to our surprise, WHI-P79, which is an equally potent inhibitor of EGF-R and Src family tyrosine kinases (14, 15), failed to cause any detectable cytotoxicity to U373 glioblastoma cells.

RESULTS AND DISCUSSION

**In Vitro Cytotoxicity of WHI-P154 against Human Glioblastoma Cells.** As shown in Fig. 1, WHI-P154, but not the unsubstituted parent quinazoline compound WHI-P258, exhibited significant cytotoxicity against the U373 human glioblastoma cell line in three of three independent experiments with a mean (± SE) IC50 of 167.4 ± 26.9 µM and a composite survival curve IC50 of 158.5 µM. The 4'-hydroxyl substituent on the phenyl ring was essential for the anti-brain tumor 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, failed to cause detectable cytotoxicity to U373 cells (Fig. 1). Replacement of the 4'-hydroxyl group with a 4'-methyl group (WHI-P111) resulted in substantial loss of activity (Fig. 1B). The 3'-bromo substitution on the phenyl ring likely contributes to the cytotoxicity of WHI-P154 because WHI-P311, lacking this bromo substituent, was less potent than WHI-P154 (IC50: >250 µM versus 167.4 ± 26.9 µM). Similarly, 3'-hydroxyphenyl (WHI-P132) and 3'-hydroxyphenyl (WHI-P258) derivatives were less active than WHI-P154. Notably, the replacement of the 3'-bromo substitution with a 3'-chloro substitution yielded a less active agent (WHI-P197). Introduction of a second bromo group at the 5' position of the phenyl ring did not result in altered cytotoxicity; the IC50 of WHI-P97 was 161.2 ± 22.2 µM, which is virtually identical to that of WHI-P154 (Fig. 1).

In experiments not shown in this report, WHI-P154 was found to be a potent inhibitor of the EGF-R tyrosine kinase as well as Src family tyrosine kinases. Therefore, we initially postulated that the cytotoxicity of WHI-P154 against glioblastoma cells was due to its tyrosine kinase inhibitory properties. However, to our surprise, WHI-P79, which is an equally potent inhibitor of EGF-R and Src family tyrosine kinases (14, 15), failed to cause any detectable cytotoxicity to U373 glioblastoma cells.

**SCID Mouse Xenograft Model of Human Glioblastoma.** The right hind legs of the CB.17 SCID mice were inoculated s.c. with 0.5 × 106 U373 human glioblastoma cells in 0.2 ml PBS. SCID mice challenged with brain tumor cells were treated with EGF-P154 (0.5 mg/kg/dose or 1 mg/kg/dose in 0.2 ml of PBS) as daily i.p. doses for 10 treatment days, starting the day after inoculation of the glioblastoma cells. Daily treatments with PBS, unconjugated EGF (1 mg/kg/dose), and unconjugated WHI-P154 (1 mg/kg/dose) were used as controls. Mice were monitored daily for health status and tumor growth and were sacrificed if they became moribund, developed tumors that impeded their ability to attain food or water, or at the end of the 3-month observation period. Tumors were measured using Vernier calipers twice weekly, and the tumor volumes were calculated according to the following formula (10): (width2 × length)/2. For histopathological studies, tissues were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin by routine methods. Glass slides with affixed 6-µm tissue sections were prepared and stained with H&E. Primary end points of interest were tumor growth and tumor-free survival outcome. Estimation of life table outcome and comparisons of outcome between groups were done, as reported previously (11–13).

**RESULTS AND DISCUSSION**

In experiments not shown in this report, WHI-P154 was found to be a potent inhibitor of the EGF-R tyrosine kinase as well as Src family tyrosine kinases.
Activity of WHI-P154 against Human Glioblastoma Cells

Fig. 2 Cell surface expression of EGF receptor on U373 and U87 human glioblastoma cells. Cells were fixed in paraformaldehyde, immunostained with monoclonal antibody to EGF-R (green fluorescence), and counterstained with TOTO-3 (blue fluorescence). The immunostained cells were analyzed with a laser scanning confocal microscope. Blue fluorescence represents nuclei.

Enhanced Cytotoxicity of EGF-conjugated WHI-P154 against Human Glioblastoma Cells. In contrast to normal glial cells and neurons, a significant portion of glioblastomas express the EGF-R at high levels (16–20). Therefore, the EGF-R could be used as a target for delivering cytotoxic agents to glioblastoma cells with greater efficiency [reviewed by Mendelsohn and Baselga (21)]. We confirmed the surface expression of the EGF-R on the U373 and U87 human glioblastoma cell lines with immunofluorescence and confocal laser scanning microscopy using monoclonal antibodies to the extracellular domain of the EGF-R. Both cell lines showed a diffuse granular immunoreactivity with the anti-EGF-R antibody (Fig. 2).

In an attempt to enhance the antitumor activity of WHI-P154 against glioblastoma cells by improving its targeting to and cellular uptake by glioblastoma cells, we conjugated it to rhEGF. We first examined the kinetics of uptake and cytotoxicity of the EGF-P154 conjugate in U373 glioblastoma cells.
Fig. 4  Uptake of WHI-P154 and EGF-P154 by U373 human glioblastoma cells. U373 cells were incubated with unconjugated WHI-P154 (A and A') or EGF-P154 (5 μg/ml; B and B') for 24 h and processed for immunofluorescence to detect EGF-R molecules using a monoclonal antibody to the EGF-R (green fluorescence) and a polyclonal antibody to WHI-P154 (red fluorescence) as described in "Materials and Methods." Blue fluorescence represents the nuclei stained with TOTO-3.

Fig. 5  A, cytotoxic activity of EGF-P154 against brain tumor cells. U373 and U87 glioblastoma cells were incubated with EGF, EGF-P154, EGF-GEN, or unconjugated WHI-P154 for 36 h in 96-well plates, and the cytotoxicity was determined by the MTT assay. The data points represent the means from three independent experiments; bars, SE. The mean IC_{50} for EGF-P154 against U373 and U87 cells were 0.813 ± 0.139 μM and 0.620 ± 0.097 μM, respectively. The mean IC_{50} for unconjugated WHI-P154 against U373 and U87 cells were 167.4 ± 26.9 μM and 178.6 ± 18.5 μM, respectively. B, cytotoxic activity of unconjugated WHI-P154 and EGF-P154 on EGF-R-negative leukemia cells. Nalm-6 cells were incubated with unconjugated WHI-P154 or EGF-P154 for 36 h in 96-well plates, and the cell survival was determined by the MTT assay. Unconjugated WHI-P154 showed considerable cytotoxic activity on Nalm-6 cells (IC_{50} 9.1 ± 1.2 μM), whereas EGF-P154 showed no cytotoxicity. The data points represent the means from three independent experiments; bars, SE.
Activity of WHI-P154 against Human Glioblastoma Cells

Fig. 6  EGF-P154 induced apoptosis in brain tumor cells. A–C, U373 cells were incubated with 25 μg/ml of EGF or EGF-P154 for 24 h and processed for immunofluorescence using a monoclonal antibody to α-tubulin (green fluorescence). EGF-P154 (but not EGF)-treated cells showed marked shrinkage with disruption of microtubules and lost their ability to adhere to the substratum. Blue fluorescence represents nuclei stained with TOTO-3. D–E, U373 cells were incubated with 25 μg/ml of EGF-P154 for 36 h, processed for the in situ apoptosis assay, and analyzed with a laser scanning confocal microscope. When compared with controls treated with EGF (D), several of the cells incubated with EGF-P154 (E) showed apoptotic nuclei (yellow fluorescence). Red fluorescence represents nuclei stained with propidium iodide.

using immunofluorescence and confocal laser microscopy for the internalized EGF-R and EGF-P154 molecules as well as for evaluating the morphological changes in treated cells. EGF-P154, similar to unconjugated EGF (not shown), was able to bind to and enter target glioblastoma cells via receptor-mediated endocytosis by inducing internalization of the EGF-R molecules. Within 10 min after exposure to EGF-P154, the EGF-R/EGF-P154 complexes began being internalized, as determined by colocalization of the EGF-R (detected by anti-EGF-R antibody, green fluorescence) and EGF-P154 (detected by anti-P154 antibody, red fluorescence) in the cytoplasm of treated cells (Fig. 3). By 30 min, the internalized EGF-R/EGF-P154 complexes were detected in the perinuclear region of the treated glioblastoma cells. In contrast, cells treated with unconjugated WHI-P154 alone did not reveal any detectable redistribution of the surface EGF-R or cytoplasmic staining with the anti-P154 antibody (i.e., red fluorescence). By 24 h, WHI-P154 molecules could also be detected in cells treated with unconjugated WHI-P154 (Fig. 4). Thus, in accordance with our expectation, conjugation of WHI-P154 to EGF resulted in increased and more rapid uptake of this cytotoxic quinazoline derivative by EGF-R-positive glioblastoma cells.

We next sought to determine whether the improved delivery of WHI-P154 to glioblastoma cells by conjugation to EGF results in potentiation of its antitumor activity. To this end, we compared the cytotoxic activities of EGF-P154 and unconjugated WHI-P154 against U373 and U87 human glioblastoma cell lines in dose-response studies using in vitro MTT assays. As shown in Fig. 5A, EGF-P154 killed these glioblastoma cells in each of three independent experiments at nanomolar concentrations with mean IC_{50}s of 813 ± 139 nM (range, 588–950 nM) for U373 cells and 620 ± 97 nM (range, 487–761 nM) for U87 cells.
The IC_{50} derived from the composite cell survival curves were 881 nm for U373 cells and 601 nm for U87 cells. By comparison, unconjugated WHI-P154 killed U373 or U87 cells only at micromolar concentrations. EGF-P154 was 206-fold more potent than unconjugated WHI-P154 against U373 cells (IC_{50} = 167.4 ± 26.9 μM versus 811 ± 139 nm, P < 0.003) and 288-fold more potent than unconjugated WHI-P154 against U87 cells (IC_{50} = 178.62 ± 18.46 μM versus 620 ± 97 nm, P < 0.001; Fig. 5A). Unlike WHI-P154, which showed marked cytotoxicity against the EGF-R-negative NALM-6 leukemia cells, EGF-P154 elicited selective cytotoxicity to EGF-R-positive glioblastoma cell lines only (Fig. 5). Thus, conjugation to EGF increased the potency of WHI-P154 against human glioblastoma and at the same time restricted its cytotoxicity to EGF-R-positive targets. Unlike the EGF-P154 conjugate, EGF-GEN, a potent inhibitor of the EGF-R tyrosine kinase and EGF-R-associated Src family PTK (22, 23), failed to kill glioblastoma cells (Fig. 5A). Thus, the potent cytotoxicity of EGF-P154 cannot be explained by the tyrosine kinase inhibitory properties of its WHI-P154 moiety.

We next used immunofluorescence staining with anti-α-tubulin antibody and the nuclear dye TOTO-3 in combination with confocal laser scanning microscopy to examine the morphological features of U373 glioma cells treated with either unconjugated EGF or EGF-P154. After 24 h of exposure to 25 μg/ml EGF-P154 (but not 25 μg/ml unconjugated EGF), most of the glioma cells showed an abnormal architecture with complete disruption of microtubules, marked shrinkage, nuclear fragmentation, and inability to adhere to the substratum (Fig. 6, A–C). These morphological changes in EGF-P154-treated glioma cells were consistent with apoptosis. To confirm apoptotic DNA fragmentation in the nuclei of EGF-P154-treated glioblastoma cells, we used an in situ apoptosis assay that allows the detection of exposed 3'-hydroxyl groups in fragmented DNA by TdT-mediated dUTP nick-end labeling. As evidenced by the confocal laser scanning microscopy images depicted in Fig. 6, D and E, EGF-P154-treated (but not EGF-treated) glioma cells examined for digoxigenin-dUTP incorporation using FITC-conjugated anti-digoxigenin (green fluorescence) and propidium iodide counterstaining (red fluorescence) showed many apoptotic yellow nuclei with superimposed green and red fluorescence at 36 h after treatment.

**In Vivo Antitumor Activity of EGF-P154 in a SCID Mouse Xenograft Model of Human Glioblastoma.** CB.17 SCID mice develop rapidly growing tumors after s.c. inoculation of 0.5 × 10^6 U373 cells. We examined the *in vivo* antitumor activity of EGF-P154 in this SCID mouse xenograft model of human glioblastoma multiforme. EGF-P154 significantly improved tumor-free survival in a dose-dependent fashion, when it was administered 24 h after inoculation of tumor cells. Fig. 7 shows the tumor growth and tumor-free survival outcome of SCID mice treated with EGF-P154 (500 μg/kg/day × 10 days or 1 mg/kg/day × 10 days), unconjugated EGF (1 mg/kg/day × 10 days), unconjugated WHI-P154 (1 mg/kg/day × 10 days), or PBS after inoculation with U373 glioblastoma cells. None of the 15 control mice treated with PBS (n = 5; median tumor-free survival, 19 days), EGF (n = 5; median tumor-free survival, 23 days), or unconjugated WHI-P154 (n = 5; median tumor-free survival, 19 days) remained alive tumor-free beyond 33 days (median tumor-free survival, 19 days; Fig. 7A). All of the five mice treated with EGF-P154 at the 500 μg/kg/day dose level developed tumors within 40 days with an improved median tumor-free survival of 33 days (Fig. 7B), and the tumors were much smaller than in control mice (Fig. 7A). Tumors reached a size of 50 mm^3 by 37.5 ± 3.3 days in PBS-treated mice, 34.0 ± 3.0 days in EGF-treated mice, 36.0 ± 5.1 days in WHI-P154-treated mice. Tumors developing in EGF-P154 (500 μg/kg/day × 10 days)-treated mice reached the 50-mm^3 tumor size ~11 days later than the tumors in control mice treated with PBS, EGF, or WHI-P154 (47.4 ± 7.1 days versus 35.8 ± 1.8 days). The average sizes (mean ± SE) of tumors at 20 and 40 days were 10.2 ± 1.4 mm^3 and 92.3 ± 6.0 mm^3, respectively, for mice in the control group (i.e., PBS + EGF groups combined). By comparison, the average sizes (mean ± SE) of tumors at 20 and 40 days were significantly different.
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smaller at 1.0 ± 1.1 mm³ (P = 0.002) and 37.6 ± 10.7 mm³ (P = 0.0003) for mice treated with EGF-P154 at the 500-µg/kg/day dose level. Notably, 40% of mice treated for 10 consecutive days with 1 mg/kg/day EGF-P154 remained alive and free of detectable tumors for > 58 days (PBS + EGF + WHI-P154 versus EGF-P154, P < 0.00001 by log-rank test). The tumors developing in the remaining 60% of the mice did not reach a size > 50 mm³ during the 58-day observation period. Thus, EGF-P154 elicited significant in vivo antitumor activity at the applied nontoxic dose levels. The inability of childhood.


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Taken together, our findings provide unprecedented evidence that WHI-P154 exhibits significant cytotoxicity against human glioblastoma cells, and its antitumor activity can be substantially enhanced by conjugation to EGF as a targeting molecule. Although WHI-P154 is a potent inhibitor of the EGFR-R kinase as well as Src family tyrosine kinases, its cytotoxicity in glioblastoma cells cannot be attributed to its tyrosine kinase inhibitory properties alone, because WHI-P79 with virtually no cytotoxicity of an anti-osteosarcoma immunotoxin containing pokeweed antiviral protein immunotoxin. Cancer Res., 2: 881–895, 1992.


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