Different Response of Human Glioma Tumor-initiating Cells to Epidermal Growth Factor Receptor Kinase Inhibitors*

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Because a subpopulation of cancer stem cells (tumor-initiating cells, TICs) is believed to be responsible for the development, progression, and recurrence of many tumors, we evaluated the in vitro sensitivity of human glioma TICs to epidermal growth factor receptor (EGFR) kinase inhibitors (erlotinib and gefitinib) and possible molecular determinants for their effects. Cells isolated from seven glioblastomas (GBM 1–7) and grown using neural stem cell permissive conditions were characterized for in vitro tumorigenicity, expression of tumor stem cell markers (CD133, nestin), and multilineage differentiation properties, confirming that these cultures are enriched in TICs. TIC cultures were challenged with increasing concentrations of erlotinib and gefitinib, and their survival was evaluated after 1–4 days. In most cases, a time- and concentration-dependent cell death was observed, although GBM 2 was completely insensitive to both drugs, and GBM 7 was responsive only to the highest concentrations tested. Using a radioligand binding assay, we show that all GBM TICs express EGFR. Erlotinib and gefitinib inhibited EGFR and ERK1/2 phosphorylation/activation in all GBMs, irrespective of the antiproliferative response observed. However, under basal conditions GBM 2 showed a high Akt phosphorylation that was completely insensitive to both drugs, whereas GBM 7 was completely insensitive to gefitinib, and Akt inactivation occurred only for the highest erlotinib concentration tested, showing a precise relationship with the antiproliferative effects of the drug. Interestingly, in GBM 2, phosphatase and tensin homolog expression was significantly down-regulated, possibly accounting for the insensitivity to the drugs. In conclusion, glioma TICs are responsive to anti-EGFR drugs, but phosphatase and tensin homolog expression and Akt inhibition seem to be necessary for such effect.

A cancer stem cell population in malignant brain tumors plays an essential role in tumor initiation, growth, and recurrence. It was demonstrated that cancer stem cells, capable of self-renewal and multilineage differentiation, are present in blood and solid tumors (1, 2). This clonogenic tumoral subpopulation is the only one able to originate a tumor mass, and for this reason, these cells were described as tumor-initiating cells (TICs) (3–6). Several groups have demonstrated the presence of TICs in various brain tumors, including gliomas (7–10).

Cerebral tumors represent 2% of all cancers, with an incidence of 14 new cases every year for 100,000 individuals, with gliomas representing the most frequent histotype (86% of total brain neoplasias) (11). These tumors, according to the World Health Organization classification, are divided into four groups, and glioblastoma (GBM) (grade IV) represents the most common and aggressive form, with a median survival time of 14 months (12). GBM is a poorly differentiated astrocytic tumor with preferential localization in the cerebral hemispheres, but it is able to infiltrate the surrounding tissues in a way reminiscent of the ability of neural stem cells to migrate to distance. This ability is the main cause of the poor prognosis and failure of conventional treatments with radio- or chemotherapy observed in this tumor (13), and it is in agreement with the hypothesis of the presence of a cancer stem cell subpopulation. In fact, glioma cells express various molecules associated with the development of neural stem cells (8, 14). In particular, CD133, one of the markers of brain TICs to date identified, is also a normal neural stem cell marker (15, 16).

The proliferation and differentiation of normal neural stem cells are under the modulation of different factors (17). It has been shown that the combination of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) is important for self-renewal and multilineage differentiation (18–20). Other growth factors, such as vascular-endothelial growth factor and platelet-derived growth factor, play a role in the maintenance and development of the central nervous system. Different studies have evaluated the importance of these growth

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‡ The abbreviations used are: TIC, tumor-initiating cell; GBM, glioblastoma; EGF, epidermal growth factor; EGFR, EGF receptor; PBS, phosphate-buffered saline; RT, reverse transcription; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; bFGF, basic fibroblast growth factor; GFAP, glial fibrillary acidic protein; PTEN, phosphatase and tensin homolog; TKI, tyrosine kinase inhibitor; PI3K, phosphatidylinositol 3-kinase.
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Factors and their receptors in glioma growth and maintenance, demonstrating that these factors are also involved in proliferation, tumorigenicity, and malignancy of the tumors (21, 22). A main issue raised by the identification of TICs in brain tumors is the efficacy of chemotherapy on this cell subpopulation. In fact, it was reported that TICs are highly resistant to radiotherapy (23, 24), likely because of increased DNA repair activity, and to many cytotoxic drugs, in virtue of the overexpression of ATP-dependent efflux pumps (25–27). Thus, this study was aimed to evaluate the sensitivity of TIC-enriched cultures to gefitinib and erlotinib, two clinically approved EGFR tyrosine kinase inhibitors (EGFR TKI).

We isolated GBM TICs from seven fresh human GBMs. Cells were expanded in vitro in a proliferation-permissive medium, and the expression of different markers, multilineage differentiation capability, and tumorigenicity in immunodeficient mice was evaluated. After this characterization, GBM TICs have been tested for their sensitivity to the antiproliferative activity of erlotinib and gefitinib and for the modulation of the intracellular signaling involved, namely the ERK1/2 and Akt pathways.

EXPERIMENTAL PROCEDURES

Isolation of Human Glioma Tumor Initiating Cells—Glioma specimens were obtained after informed consent from seven patients, 6 males and 1 female, with an average age of 62 years (range 49–74). Tumor samples were obtained during surgery from patients of the Neurosurgery Department, San Martino Hospital (Genova, Italy). All patients underwent surgery for the first time and never received chemotherapy or radiotherapy. Tumor samples, classified as gliomas grade IV (GBM) based on World Health Organization criteria, were immediately processed for isolation of single cells by mechanical dissociation and plated at the concentration of 1 × 10^5 cells/ml in Matrigel-coated culture flasks (BD Biosciences) in a proliferation permissive medium containing Dulbecco’s modified Eagle’s medium/Ham’s F-12 and Neurobasal (1:1), B27 supplement (Invitrogen), 2 mM l-glutamine (Invitrogen), recombinant human bFGF (10 ng/ml; PeproTech, London, UK), and recombinant human EGF (20 ng/ml; PeproTech). All acutely dissociated primary GBM cells gave rise to neurospheres within 1–2 weeks but also grew as a monolayer on Matrigel for more than 10 passages, without losing their spherogenic properties (28). In fact, the persistence of the stem cell features of the GBM TIC grown on Matrigel was recently demonstrated and deeply characterized by our laboratory (28). We clearly demonstrated that culturing GBM TICs on Matrigel did not affect their tumorigenic potential, their spherogenic properties when transferred to standard culture conditions, nor begin any differentiation program.

Drugs, Growth Factors, Biochemicals, Reagents and Antibodies—The low molecular weight synthetic 4-anilino-quinazoline EGFR TKIs gefitinib (ZD1839, Iressa™, C_{22}H_{24}ClFN_{4}O_{3}, M_r = 446.9) and erlotinib-HCl (OSI774, Tarceva™, C_{22}H_{23}N_{3}O_{4}·HCl, M_r = 429.9), provided by Astra-Zeneca SpA (Milano, Italy) and Hoffmann-La Roche, respectively, were diluted as stock solution at the concentration of 1 mM in DMSO and stored in small aliquots at −20 °C (29). When appropriate, the same vehicle dilution was added in the respective control samples. Lyophilized human recombinant EGF and bFGF were purchased by PeproTech EC (London, UK), reconstituted in double distilled H_2O, and stored in working aliquots of 50 ng/100 μl at −20 °C. Human recombinant 3-[¹²⁵I]iodotyrosyl-EGF, specific activity >750 Ci/mmol, (IM196) aqueous solution, was from GE Healthcare. Hanks’ balanced salt solution CaCl_2/MgCl_2-free was purchased from Invitrogen; bovine serum albumin, fraction V, was from Sigma.

Antibody directed against phospho-Akt (Ser-473), Akt, phospho-ERK1/2, and ERK1/2 were from Cell Signaling Technology (Beverly, MA); anti-phospho-EGFR (Tyr-1173) and anti-EGFR antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Differentiation Assay—To induce differentiation into different brain cell histotypes, GBM cells were seeded on Matrigel-coated coverslips at the concentration of 1 × 10^5 cells per slide in a medium without growth factors and containing 10% fetal calf serum (EuroClone, Pavia, Italy) for 2 weeks (30). To keep a better reproducibility, all the following experiments were performed using cells grown in vitro for 15–25 passages.

Immunofluorescence—For xenograft tumor analysis, mice were sacrificed, and cryopreserved brain sections were cut using a 10-μm cryostat (CM 1100; Leica, Germany). Staining with hematoxylin–eosin identified sections bearing tumors. Briefly, brain sections were mounted on slides and stained with Harris hematoxylin for 40 s and then counterstained with alcohol eosin for 30 s. Cryosections containing tumors were permeabilized in PBS containing 0.5% Triton X-100 and blocked in 10% normal goat serum/PBS. After incubation with anti-human nestin antibody (mouse monoclonal, diluted 1:1,000; Abcam (Cambridge, UK)) in NGS–PBS overnight at 4 °C, sections were washed in Tris-buffered saline. Immunopositivity was revealed using Alexa488-conjugated goat anti-mouse IgG (1:700; Invitrogen). Cells were counterstained with Hoechst 33342 (Sigma) to identify the nuclei.

To examine the expression of different brain cell lineage markers in undifferentiated and differentiated human glioma TICs, cells were plated onto Matrigel-coated glass coverslips and then fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilized and stained with different primary antibodies as follows: anti-human nestin, anti-Map2 (mouse monoclonal, 1:1,000; Chemicon/Millipore, Milano, Italy), and anti-GFAP (rabbit polyclonal, 1:10,000; Dako, Glostrup, Denmark). Following washing with PBS, cells were exposed to the appropriate secondary fluorescent antibody (Alexa488-conjugated goat anti-mouse IgG, 1:700 or rhodamine-conjugated goat anti-rabbit IgG, 1:250; Jackson ImmunoResearch Europe, Cambridge, UK) and counterstained with Hoechst 33342 dye to identify all nuclei. Data are reported as percentage of immunolabeled cells from five randomly selected microscopy fields. Images were obtained with a Nikon Eclipse 80i microscope (Nikon Europe, Ljnden, The Netherlands).

Tumorigenicity—Human glioma TICs in vivo tumorigenicity was tested by cell intracranial inoculation in 6–8-week-old NOD/SCID mice (Charles River Laboratories, Calco, Italy). Preliminary experiments showed that as few as 5,000 cells (not selected for CD133 expression) were able to induce tumors with a latency of up to 180 days. To obtain a more rapid evaluation of the results, we decided to inject 10^5 cells from each TIC.
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culture. Three mice for each GBM cell culture were used. Mice were anesthetized intramuscularly with 20 μl of ketamine (2%) and xylazine (100 mg/ml) and positioned into a stereotactic frame (David Kopf Instruments, Tujunga, CA). A hole was made in the skull using a 21-gauge needle, 2.5 mm lateral and 1 mm anterior from the intersection of the coronal and sagittal sutures (bregma), and cells were injected in the left corpus striatum using a Hamilton syringe (series 7000; Sigma) at a depth of 3.5 mm (total volume 2 μl). Mice were monitored for about 6 months for disease symptoms and were sacrificed by CO₂ asphyxiation when they showed weight loss or any severe sign of disease. All experiments, including animals, were performed in compliance with guidelines approved by the Ethical Committee for animal use in cancer research at the National Institute for Cancer Research (Genova, Italy).

**CD133⁺ Cell Isolation and Quantification**—Human GBM TICs were sorted by CD133 expression with Miltenyi microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, cells were dissociated and resuspended in PBS, 0.5% bovine serum albumin, and 2 mM EDTA. CD133/1 microbeads were used for positive magnetic cell separation using two magnetic cell separation columns in series. Aliquots of CD133⁻ and CD133⁺ cells were evaluated for purity by flow cytometry using CD133/2-PE (Miltenyi Biotec) or isotype control antibody (IgG2b-PE, Miltenyi Biotech) and analyzed on a FACS Calibur (BD Biosciences). For one-color cytofluorimetric analysis, human glioma TICs were stained with the appropriate monoclonal antibodies followed by phycoerythrin-conjugated isotypetype-specific goat anti-mouse second reagent (Southern Biotechnology Associated, Birmingham, AL).

**MTT Assay**—For MTT assay, human GBM TICs were plated in 96-well microplates previously coated with Matrigel. Because the cells employed in this study had different proliferation rates, the number of cultured cells was adjusted to a density that allowed the cells to grow exponentially for all the duration of the assay (up to 4 days) ranging from 2,500 to 10,000 cells/well. We tested the effects of erlotinib and gefitinib using three concentrations as follows: 0.5, 1, and 5 μM. After exposure to the drugs for different times (0, 24, 48, 72, and 96 h) at 37 °C, cells were incubated for 4 h in MTT solution (2 mg/ml in PBS). After removing MTT, 150 μl of DMSO were added to each well, and the absorbance was determined at 540 nm wavelength, as reported (31), using an enzyme-linked immunosorbent assay plate reader.

**Radioreceptor Assay with ¹²⁵I-Labeled EGF/Unlabeled EGF Competitive Binding to EGFR**—Human GBM TICs were plated at the concentration of 1.5 × 10⁵ in duplicate wells using the proliferation permissive medium in Matrigel pre-coated 24-well plates (28) and incubated overnight at 37 °C, before the medium was changed with a growth factor-deprived medium for an additional 48 or 96 h according to the experimental protocol. At the end of this period, cell monolayers were washed once with 1 ml/well of Binding Buffer (BB: Dulbecco’s modified Eagle’s medium, 40 mM Hepes, 0.1% bovine serum albumin) and incubated at 37 °C, 5% CO₂ for an additional 60 min. Cells were then treated with ¹²⁵I-labeled EGF (average 2 × 10⁴ cpm in 200 μl/well of BB) and serially increasing amounts of unlabeled peptide (0.195–50 ng/ml for standard curve + 400 ng/ml excess for nonspecific binding evaluation) in 200 μl/well BB (total volume/well = 400 μl). Plates were incubated for 2 h at 4 °C on a shaker with gentle agitation. After binding, medium was carefully removed from each well and monolayers were washed three times with 500 μl/well ice-cold Washing Buffer (Hanks’ balanced salt solution, 0.1% bovine serum albumin). Cells were lysed by adding 750 μl of Solubilization Solution (20 mM Hepes, 1% Triton X-100, 10% glycerol, 87% H₂O) to each well and then incubated for 20–30 min at room temperature. An aliquot (80%) of solubilized and carefully homogenized material was than measured in a γ-counter (Cobra II Autogamma, Packard Instrument Co.) to quantify the released radioactivity of the bound ligand. Specific binding was calculated after subtraction of nonspecific binding and by means of Scatchard analysis, B_max and K_d values, and sites/cell were determined.

**Western Blotting**—To avoid the activities of growth factors and other supplements present in the proliferation medium, cells were cultured for 48 h in a growth factor-free medium before being treated (32). Cells were lysed in buffer containing 1% Nonidet P-40, 20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM NaF (all from Sigma), and the “Complete” protease inhibitor mixture (Roche Applied Science) for 10 min at 4 °C. Nuclei were removed by centrifugation (5,000 rpm at 4 °C, for 10 min), and total protein content was measured using the Bradford assay (Bio-Rad). Proteins (10 μg) were resuspended in 2X reducing sample buffer (2% SDS, 62.5 mM Tris, pH 6.8, 0.01% bromophenol blue, and 1.43 mM β-mercaptoethanol, 0.1% glycerol), electrophoresed on 7.5–10% SDS-polyacrylamide gels, transferred on polyvinylidene difluoride membrane (Bio-Rad) (32), and probed with specific antibodies. The detection of immunocomplexes was performed using ECL system (GE Healthcare).

**RNA Isolation and Reverse Transcription (RT)-PCR**—Total RNA was isolated using the TRIzol reagent (Invitrogen) and the “Total RNA Purification System” (Invitrogen). cDNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad) and amplified with Taq DNA polymerase (Qiagen, Milano, Italy) by PCR (iCYCLER Bio-Rad) using the following primers: 5'-aaagcttgaaagggacga-3' and 5'-cagtaacgctgctgga-3' (GenBank™ accession number NM_000314). Amplification profile was as follows: 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by 7 min at 72 °C.

**β-Actin amplification**, used as positive control for the PCR, was performed using the following primers: sense 5'-tccg-gagacgggttc-3' and antisense 5'-tctgctgctgatc-3'. The selected sequences encompass an intronic sequence in the β-actin gene that allow the identification of genomic DNA contamination (33). Negative controls were obtained performing PCR amplification in the absence of RT reaction.

**Statistical Analysis**—Data are reported as means ± S.E. of at least two independent experiments performed in triplicate. Statistical analysis (analysis of variance) was performed using the Software GraphPad QuickCalcs. A p value ≤ 0.05 was considered statistically significant.
RESULTS

Tumorigenicity of Human Glioma-derived Cells—We analyzed post-surgical explants from seven consecutive human gliomas, histopathologically classified as grade IV according to the World Health Organization grading. Tumor samples were immediately dissociated into single cells, and plated at clonal density in a proliferation medium, in Matrigel-coated flasks (see “Experimental Procedures”). Under these conditions, these cells can be cultured in vitro retaining the ability to form neurospheres that, in limiting dilution experiments, are detected after 1 or 2 weeks, according to the tumor analyzed. To identify the presence of TICs in these cultures, we tested their in vivo tumorigenicity, to date the only test able to discriminate TICs from differentiated nontumorigenic cancer cells.

A hundred thousand cells derived from all GBM cultures analyzed (called GBM 1–7) were orthotopically injected in immunodeficient NOD/SCID mice as described under “Experimental Procedures.” After 2–6 months, according to the GBM from which the cells were isolated (see Table 1), all cultures produced tumors with highly invasive properties that resembled the primitive neoplasia, thus confirming that our cultures are enriched in TICs. In particular, intracranial tumors generated by TICs injections demonstrated various grades of infiltration into the surrounding cerebral cortex, and GBM cells showed a propensity to migrate along the corpus callosum or other brain structures. GBM generated from the injected TICs were identified by histological analysis that confirmed the exclusive presence of human tumor cells, without significant contamination from murine cells, as assessed by the positive staining detected using anti-human nestin antibody (Fig. 1) and the lack of labeling using anti-mouse antibodies (data not shown). Importantly, in agreement with our previous studies (28), no differences in tumorigenicity were observed culturing the cells as nonadherent neurosphere or in monolayer on Matrigel, in both cases injecting 10^5 cells the take rate was 100% even after more than 40 passages in vitro.

Cells, isolated from dissociated intracranial xenograft tumors and re-cultured under stem cells conditions, were able to form neurospheres. When cells were injected back into the brain of new recipient mice, they not only retained the ability to generate new tumors but also showed an increased tumorigenic potential, as shown by the shorter time required for tumor development (Table 1).

Characterization and Differentiation of Human Glioma Tumor-initiating Cells—All the human glioma TICs that we isolated from GBM 1–7 displayed the potential for multilineage differentiation and clonogenicity in vitro, which are typical features of neural stem cells. In particular, immunofluorescence and cytofluorimetric analysis showed that the expression of nestin and CD133, respectively, was clearly detectable in subpopulations of these cultures (Table 1). In agreement with the stemness of these cultures, nestin was expressed in an extremely high percentage of cells (up to 97% in GBM 3, see Table 1). The identification of a CD133-positive subpopulation within these cultures was also particularly relevant, because this protein is recognized as a marker of glioma TICs (34, 35). In agreement with previously reported data (36), nestin was detected in a higher number of cells than CD133, although in two cases (GBM 4 and -5) the proportion of CD133-expressing cells was as high as 80% (see Table 1 and supplemental Fig. 1). Using microbeads for immunoselection, it was possible to obtain cultures enriched in CD133-expressing cells. However, after a few passages the level of CD133+ cells returned to baseline values because of the stem-like nature of these cells whose cell division results in the generation of a percentage of differentiated cells.

Immunofluorescence experiments showed that a high percentage of all TIC cultures also expressed percentage of Sox2 (data not shown). All these features were reported to be specific for both neural stem cells and brain tumor TICs.

| Tumor code | In vivo tumorigenicity (survival, days) | Percentage of positive cells in vitro |
|------------|----------------------------------------|-------------------------------------|
|            | Primary tumor | Secondary tumor | Nestin short term | Nestin long term | CD133 short term | CD133 long term | GFAP | Map2 |
| GBM1       | 150–180      | 90–110          | 93 (±2)            | 95 (±2.5)       | 3 (±1.5)         | 3 (±1.5)       | 27 (±3) | 75.5 (±5.5) |
| GBM2       | 80–90        | 55–65           | 78 (±2.5)          | 75 (±5)         | 1.8 (±0.8)       | 1.6 (±1)       | 4.5 (±0.5) | 8.5 (±3.5) |
| GBM3       | 40–120       | 40–60           | 95 (±3.5)          | 97 (±2.5)       | 4.5 (±1.5)       | 5.2 (±2)       | 15 (±5)  | 7.5 (±2.5) |
| GBM4       | 100–120      | 80–90           | 78 (±2.5)          | 78 (±4)         | 83 (±3)          | 82 (±4)        | 9.5 (±1)  | 23 (±2.5)  |
| GBM5       | 70–80        | 60–70           | 40 (±6)            | 35 (±5)         | 85 (±4.5)        | 80 (±3)        | 3 (±1)   | 40 (±5)    |
| GBM6       | 120–170      | 90–110          | 69 (±3)            | 65 (±5)         | 2.5 (±1)         | 5.1 (±0.5)     | 7.5 (±2.5) | 75 (±2)    |
| GBM7       | 64–110       | 55–70           | 93 (±2)            | 90 (±5)         | 1.8 (±0.5)       | 2.1 (±0.5)     | 80 (±6)  | 12.5 (±2.5) |


**EGFR Inhibitors and Glioblastoma Tumor Initiating Cells**

![Graph](image)

**FIGURE 2. In vitro proliferation of human glioma tumor-initiating cells.** Time-dependent proliferation of human glioma TICs derived from 7 patients (GBM 1–7). Cells were cultured at different cell concentrations/well to obtain a linear cell proliferation for all the experimental observations (96 h). In particular, 2,500 cells/well were plated for GBM 2 and GBM 3; 5,000 cells/well were plated for GBM 4; 10,000 cells/well were plated for GBM 1, GBM 4, GBM 5, and GBM 6. The graph reports the average of two independent experiments performed in triplicate. Data represent mean values of three independent experiments. In all samples S.E. was lower than 7% of the value.

Analysis of the basal expression of differentiation markers showed that these cells were also positive for microtubule-associated protein 2 (Map2, neuronal marker) and GFAP (astroglial marker), although at very different levels among the various GBMs (see Table 1). Interestingly, a small (but in some cases significant) percentage of the tumor cells we analyzed co-expressed both neural and glial markers (GBM 1, 13.6%; GBM 2, 0.1 ± 0.1%; GBM 3, 5.5 ± 1.5%; GBM 4, 2.7 ± 0.8%; GBM 5, 1.1 ± 0.3%; GBM 6, 3.4 ± 2.8%; and GBM 7, 5.6 ± 0.6%).

However, culturing the cells under differentiating conditions resulted in the acquisition of typical astroglial morphology and a high expression of GFAP (data not shown). These data suggest that when the differentiation program is activated, TIC cultures are mainly oriented to astroglial commitment.

Importantly, we periodically evaluated the expression levels of nestin, CD133, GFAP, and Map2, as well as their tumorigenicity, and we found that it was quite stable over time in all the selected TIC cultures (see Table 1), confirming that it represents a specific feature of each GBM TIC culture.

**Effects of the EGFR Inhibitors Erlotinib and Gefitinib on Human Glioma TIC Proliferation**—Before studying the sensitivity of TICs in response to EGFR TKI, we set up conditions of cell culture that allowed exponential growth of all the GBM TICs for the entire length of the experiments (4 days). Thus, in preliminary experiments we determined the appropriate cell number to be used for each individual TIC culture, plated in 96-multiwell plates in proliferation medium. From these experiments we defined a cell number range (from 2,500 cells/well for GBM 2 and -3 to 10,000 cells/well for GBM 1, -4, and -5) that allows the cells to keep a linear proliferation rate during the entire experimental period (up to 4 days, Fig. 2).

To analyze the sensitivity of these cells to target-specific anti-proliferative drugs, we studied the responsiveness of TICs to two different EGFR kinase inhibitors, erlotinib and gefitinib. The proliferation rate of TICs derived from all the seven GBM treated with the drugs at increasing concentrations (0.5, 1, and 5 μM) was compared with vehicle-treated cells at different time points (time 0, and after 24, 48, 72, and 96 h of treatment).

Erlotinib caused a time- and dose-dependent inhibition of cell proliferation in all cultures with the only exception of GBM 2 that was completely insensitive to all the concentrations used (Fig. 3A). At the concentration of 5 μM, erlotinib caused a highly significant growth arrest in GBM 3, which reached the maximal inhibition already after 24 h of treatment (−62%, p < 0.01) and lasted for the length of the experiment (−68%, after 4 days). Similar inhibitory effects were observed after 48 h in GBM 1 and after 72 h in GBM 4, GBM 5, GBM 6, and GBM 7 (ranging from −40 to −55%, p < 0.01), although a further increase in the inhibitory effects after 96 h of treatment was observed only in GBM 1 (−62%). A higher responsiveness of GBM 1 and -3 was further confirmed using a drug concentration of 1 μM that caused cell growth inhibition not statistically different (p < 0.01) from that observed using 5 μM (from −55% after 1 day of treatment to −66% after 4 days for GBM 3). Lower effects, but still statistically significant, were observed with 1 μM erlotinib in the other GBM TIC cultures, although no statistically significant effects were observed in GBM 7 cells. 0.5 μM erlotinib caused a smaller inhibitory effect in all the GBM TICs, and only GBM 3 showed a significant reduction of cell viability for all the time points analyzed, reaching a maximum after 3 days of treatment (−41%, p < 0.01). GBM 1, GBM 5, and GBM 6 cells displayed a lower inhibitory effect that reached a statistical significance (p < 0.05) after 48 h (GBM 1) or 72 h (GBM 5 and GBM 6). Using low erlotinib concentrations, no significant inhibition was observed in both GBM 4 and GBM 7.

Interestingly, GBM 2 TICs were completely insensitive to all the drug concentrations tested. All the data concerning the erlotinib effects on the seven GBM TIC cultures are summarized in Fig. 3A, and the results of the statistical analysis (p values) are summarized in supplemental Table 1.

Similar, but not identical, results were obtained after treatment with gefitinib. In fact, although GBM 3 displayed a high sensitivity to gefitinib that was comparable with what was observed with erlotinib (maximum inhibition was already reached at the concentration of 0.5 μM after 4 days of treatment, about −50%; but all time and concentration points analyzed showed a statistical significance, see Fig. 3B and supplemental Table 1), GBM 1 and -4 showed an increased responsiveness as compared with erlotinib, −73 and −62% already after 3 days of treatment with 5 μM, and a statistically significant inhibition (p < 0.05) already after 24 h of treatment with 1 and 0.5 μM gefitinib, respectively (Fig. 3B and supplemental Table 1). GBM 5 and -6 showed maximal inhibition similar to that observed after erlotinib treatment, and the antiproliferative effect was statistically significant already at the concentration of 0.5 μM after 72 h of treatment (p < 0.05, Fig. 3B). However, for the highest gefitinib concentration tested, a more significant inhibition of cell proliferation was observed in GBM 5 and -6, as compared with erlotinib treatment, being statistically significant already after 1 day of treatment (see supplemental Table 1 and Fig. 3B). Interestingly, GBM 7 showed a moderate reduction in the proliferation rate only at highest concentration (5 μM) and for prolonged times of treatment (3 and 4 days), whereas at lower concentrations, gefitinib was completely ineff-
effective. Finally, GBM 2 confirmed the absolute insensitivity to EGFR inhibition, with its proliferation rate unaffected by all the gefitinib concentrations used (Fig. 3B).

Characterization of Human Glioma TIC EGFR Site Number by EGF Competitive Binding—To better understand the differential response of the individual GBM TIC cultures to erlotinib and gefitinib, we measured EGFR expression by competitive binding of EGF to its specific transmembrane cell receptor in all the cultures. Cell monolayers were simultaneously treated (2 h at 4 °C) with unlabeled and 125I-labeled EGF. Evaluation of non-specific binding was performed by adding excess amount of EGF (400 ng/ml) that completely blocked (>95%) the binding of the iodinated isotope. Scatchard analysis of 125I-EGF binding to TIC cells resulted in a best fit according to the one-affinity model with one single class of binding sites totally in the high affinity state (see supplemental Fig. 2), ranging from 0.16 to 0.67 nM as far as the Kd and from 3.5 to 39.8 pmol regarding the Bmax after 96 h of growth factor starvation (Table 2). The number of binding sites ranged from about 2 to 12 x 10^4 per cell (Table 2). A shorter starvation period lowered the binding ability of GBM 3, whereas values remained substantially unchanged for GBM 2, -5, and -6 cells (data not shown). Thus, the increasing of sites/cell after a longer period of serum starvation (96 h versus 24–48) does not seem to be a rule, as it could have been expected considering the potential release of free available receptor sites, which is likely to happen in total absence of exogenous EGF. On the contrary, assays carried out in two TIC cultures (GBM 2 and -3) without previous growth factor starvation did not show significant values in Bmax, Kd, and sites (data not shown), possibly because of the unavailability of free receptor sites due to the exogenous EGF present in culture medium. Interestingly, we found only a partial correlation

| Tumors | Bmax (pmol) | Kd (nM) | Sites/cell |
|--------|------------|---------|------------|
| GBM1   | 30.5       | 0.17    | 124,000    |
| GBM2   | 15.4       | 0.16    | 45,000     |
| GBM3   | 39.8       | 0.36    | 112,000    |
| GBM4   | 27.5       | 0.21    | 115,000    |
| GBM5   | 17.2       | 0.67    | 68,000     |
| GBM6   | 3.5        | 0.37    | 23,000     |
| GBM7   | 12.1       | 0.20    | 37,000     |

FIGURE 3. Inhibitory effects of EGFR tyrosine kinase inhibitors on the proliferation of human glioma TICs. TICs were plated in 96-well multiplates for 24 h and then treated with different concentrations of tyrosine kinase inhibitors. The results are reported as means from three different experiments. S.E. were constantly lower than 5% of each mean value. Individual p values obtained by analysis of variance are reported in the supplemental Table 1. A, time-dependent growth inhibition induced by erlotinib used at the concentration of 5 μM (upper panel), 1 μM (middle panel), and 0.5 μM (lower panel). B, time-dependent growth inhibition induced by gefitinib used at the concentration of 5 μM (upper panel), 1 μM (middle panel), and 0.5 μM (lower panel).
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Table 3: Inhibition of EGFR, ERK1/2, and Akt phosphorylation/activation induced after treatment with the EGFR kinase inhibitors, erlotinib and gefitinib

Data are derived by densitometric analysis of at least two independent sets of experiments and expressed as mean of the percentage values (± S.E.) of the maximal inhibition observed of the EGF-induced protein phosphorylation (set as 100%). NS indicates not statistically significant versus EGF-treated cells.

| Tumors | Erlotinib | Gefitinib |
|--------|-----------|-----------|
|        | p-EGFR    | p-ERK1/2  | p-Akt     | p-EGFR    | p-ERK1/2  | p-Akt     |
| GBM1   | 8 ± 1%    | 18 ± 5%   | 9 ± 3%    | 16 ± 2%   | 45 ± 6    | 34 ± 7%   |
| GBM2   | 10 ± 3%   | 15 ± 4%   | 101 ± 12 NS | 3 ± 1%    | 3 ± 1%    | 105 ± 9 NS |
| GBM3   | 41 ± 7%   | 32 ± 3%   | 42 ± 5%   | 21 ± 4%   | 28 ± 4%   | 28 ± 6    |
| GBM4   | 3 ± 2%    | 35 ± 8%   | 12 ± 3%   | 8 ± 2%    | 18 ± 3%   | 37 ± 11   |
| GBM5   | 46 ± 9%   | 43 ± 11%  | 39 ± 9%   | 12 ± 3%   | 42 ± 11%  | 43 ± 9    |
| GBM6   | 15 ± 4%   | 16 ± 3%   | 34 ± 12%  | 9 ± 2%    | 39 ± 9%   | 40 ± 7%   |
| GBM7   | 48 ± 6%   | 9 ± 2%    | 38 ± 8%   | 98 ± 13 NS | 90 ± 12 NS | 102 ± 10 NS |

* Values are p < 0.01.
** Values are p < 0.05.

First, to confirm the specificity of the effects of erlotinib and gefitinib on the proliferation rate of human glioma TICs, we investigated their effects on EGFR phosphorylation/activation induced by EGF. Cells were cultured for 48 h in a growth factor-free medium, pretreated for 15 min with increasing concentrations of erlotinib or gefitinib (0.5, 1, and 5 μM), and then treated for 5 min with EGF (40 ng/ml). EGF induced activation/phosphorylation of EGFR in all TIC cultures (data not shown). Importantly, Western blot analysis of the apparent EGFR molecular weight showed that in all GBM TIC cultures the EGFRvIII mutant isoform, often expressed in human GBM (37), was never expressed (data not shown). Similarly, other EGFR family members, such as HER-2, were detected in the TIC cultures (data not shown).

In Table 3 are reported the effects of erlotinib (left columns) and gefitinib (right columns) on EGF-induced phosphorylation of EGFR, and representative blots from GBM 3 and -4 (highly responsive to the antiproliferative effects of the tyrosine kinase inhibitors) and GBM 7 and -2 (slightly responsive or completely unresponsive, respectively) are reported in Fig. 4.

Erlotinib inhibited EGF-induced EGFR phosphorylation/activation in all TIC cultures (Table 3, left columns). In particular, EGFR phosphorylation induced by EGF was markedly inhibited by erlotinib pretreatment in GBM 1, -2, -4, -6, and -7 cells at all the concentrations tested (0.5, 1, and 5 μM), although in GBM 3 and -5 cells the inhibitory effects were more dose-dependent with an almost complete inhibition obtained at the highest concentration tested (5 μM).

On the other hand, gefitinib pretreatment did not reduce EGF-induced EGFR phosphorylation in all the cell lines, since...
in GBM 7 cells it was completely ineffective (see Table 3, right panel, and Fig. 4). Gefitinib completely abolished EGF-induced EGFR phosphorylation in GBM 2 and -4 cells at all the concentrations used (Fig. 4). In GBM 1, -3, -5, and -6 cells, the drug caused a highly significant reduction of EGFR phosphorylation in a dose-dependent manner, being ineffective only at the lowest concentration used (0.5 μM). Conversely, as shown in the Western blot reported in Fig. 4 and in Table 3, gefitinib did not affect EGFR phosphorylation induced by EGF in GBM 7 cells.

Effects of the EGFR Inhibitors Erlotinib and Gefitinib on Human Glioma TIC ERK1/2 Activation Induced by EGF—To assess a possible role for the activation of the mitogen-activated protein kinase cascade, via EGFR, in the proliferative effects of EGF, we performed Western blot evaluating ERK1/2 activation as a consequence of EGF treatment, in the presence or absence of the two EGFR TKIs. For this purpose, we investigated the changes in ERK1/2 phosphorylation using the same cell extracts used in the above described experiments. Interestingly, erlotinib treatment caused a significant reduction of ERK1/2 phosphorylation induced by EGF in all TICs that almost completely paralleled the effects of EGFR phosphorylation (Table 3, left panel). In particular, the drug was highly effective in all GBM TICs showing in most cases a complete abolishment of ERK1/2 phosphorylation also at low concentrations (Fig. 4).

A significant inhibition of ERK1/2 phosphorylation was also observed after gefitinib treatment in all TICs (Table 3, right panel), although the drug was less effective in GBM 7 cells with a barely detectable reduction of ERK1/2 phosphorylation only at the highest concentration tested (Table 3 and Fig. 4). Again, these effects were comparable with those observed for EGFR phosphorylation. Interestingly, in GBM 2 the significant effects of erlotinib on EGFR and ERK1/2 activation were at variance with the lack of antiproliferative effects induced by the drug.

Effects of the EGFR Inhibitors Erlotinib and Gefitinib on Human Glioma TIC Akt Phosphorylation Induced by EGF—It was reported that the effects of EGFR, as a key mediator of oncogenesis in human tumors, are mediated not only through ERK1/2 signaling but also by the serine/threonine kinase Akt, activated by phosphatidylinositol 3'-kinase (PI3K) signaling. Constitutively activated Akt pathway is often associated with lack of tumor response to EGFR inhibitors (38, 39). To study the role of this downstream pathway in EGFR activation, we also performed Western blot experiments evaluating the phosphorylation/activation of Akt in basal or EGF-treated conditions in the presence or absence of erlotinib and gefitinib. In all TICs, a significant increase in Akt activation was observed after EGF treatment, except in GBM 2 cells. As reported in Fig. 4, in this TIC culture an elevated level of Akt phosphorylation was already detected in untreated cells, and EGF treatment did not modify Akt basal activation.

Table 3 details the effects of gefitinib and erlotinib on EGF-induced Akt phosphorylation. Erlotinib pretreatment caused a significant reduction of the kinase activation in all TICs but had no effect either on basal or EGF-induced Akt phosphorylation of GBM 2 cells. Gefitinib also powerfully inhibited the activation of Akt by EGF while lacking efficacy in GBM 2 and GBM 7 (Fig. 4 and Table 3).

Phosphatase and Tensin Homolog (PTEN) mRNA Expression in Cultured Human Glioma TICs—From the previous experiments it appears that the main determinant of the responsiveness to the antiproliferative activity of erlotinib and gefitinib may be represented by the ability to revert Akt activation. Thus, we examined, by RT-PCR, the levels of mRNA expression of PTEN, a phospholipid phosphatase representing the main intracellular inhibitor of the PI3K/Akt pathway.

TICs derived from all the GBM analyzed expressed significant amounts of PTEN mRNA, with only GBM 2 cells that showed a significantly lower mRNA amount (Fig. 5A). To better correlate the role of Akt activation to the EGFR-TKI response, we treated two TIC cultures with gefitinib or erlotinib in the presence or absence of the PI3K inhibitor wortmannin. In these experiments we evaluated a highly responsive TIC culture (GBM 3) and GBM 2 that displayed low PTEN expression associated with a drug nonresponsive phenotype. The inhibition of PI3K/Akt pathway resulted, per se, in slight antiproliferative effect in both TIC cultures (Fig. 5B). In the TKI-responsive GBM 3 cells the significant inhibition induced by gefitinib and erlotinib was further increased in the presence of wortmannin (Fig. 5B). More importantly, the inhibition of PI3K/Akt activity evidenced a moderate, although statistically significant, inhibitory effect of gefitinib and erlotinib also in the nonresponsive TIC culture from GBM 2 (Fig. 5B). These results strongly support the hypothesis that in the presence of constitutively active PI3K/Akt signaling, the EGFR kinase inhibitors lose their efficacy.
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DISCUSSION

The discovery in many solid tumors (including GBM) of a cancer stem cell subpopulation endowed with tumorigenic properties opened a completely new scenario in the therapeutic approach in oncology. In fact, although not yet completely characterized, these cells showed a very different pattern of resistance to the traditional pharmacological or radiation treatments, as compared with the bulk of neoplastic cells into the tumor mass (23, 25, 40). However, because of the difficulties in obtaining and maintaining in vitro cultures enriched in this TIC subpopulation, a characterization of their specific sensitivity to target-directed compounds has been performed only in limited samples.

Here we report the characterization of GBM-derived TIC cultures as far as in vivo tumorigenicity, cancer stem cell marker expression, multilineage differentiation, and sensitivity to the EGFR kinase inhibitors erlotinib and gefitinib. Interestingly, we showed that whereas most of the GBM TICs analyzed retain the in vitro responsiveness to both drugs, this effect seems to be related to PTEN activity and the ability to reduce Akt phosphorylation.

Seven human GBM TIC cultures from post-surgical specimens were isolated from patients that never received chemoradiotherapy. In agreement with previous studies, we cultured these cells in a proliferation medium containing the growth factors EGF and bFGF, according to the conditions described for the propagation of brain cancer stem cells (9). Moreover, we confirmed the crucial role of EGF in the mitogenic regulation of GBM TICs, as recently reported (41). Although all the GBM-derived cultures gave rise to tumorigenic neurospheres when cultured in nonadherent conditions according to standard protocols (9), we developed a novel in vitro growth protocol using Matrigel-coated dishes. In these experimental conditions TICs grow in monolayers, retain a good proliferation rate, and do not enter the differentiation program, as shown by the continuous and stable expression of stem cells markers (CD133, nestin, see Table 1) in a rather constant percentage of cells. More importantly, the persistence of TICs under these culture conditions was further confirmed by the retained ability to generate tumors when orthotopically injected in NOD-SCID mice. Interestingly, these tumors displayed a highly invasive pattern of growth, very much resembling the growth features of human GBM in vivo. This observation strongly supports the relevance of this cell model to evaluate the biological characteristics and pharmacological responsiveness of human GBM cells, as already demonstrated at the genetic level (9).

We show that isolated GBM TICs, cultured as detailed before, were positive for typical stem cells markers such as nestin, CD133, and Sox2. All these markers were reported to be overexpressed in gliomas as compared with normal brain (42) as well as in cancer stem cells (28). In particular, nestin was detected in most of the cultured cells from each GBM (up to 97%, in GBM 3), whereas CD133 was identified in a subset of the nestin-positive tumor cells. In agreement with previous studies on human glioma specimens (42), a certain variability in the expression of these markers was observed, with two TIC cultures that expressed an extremely high level of CD133 (GBM 4 and -5) as compared with all the others in which its content was much lower (1.6–5.2%), although not dissimilar to recent published studies (41). Previous reports suggested that increased expression of CD133 was directly related to a highly aggressive behavior of the tumor (36). Interestingly, in xenotransplant experiments we did not observe a faster tumor development injecting GBM cells in which a significant higher percentage CD133 expression was detected in vitro (i.e. GBM 4 and -5). It is likely that the number of cells injected in the CD133 less expressing cultures were already over a threshold of maximal capability to develop tumors in immunodeficient mice.

The characteristics of these cultures, showing tumor-specific phenotype differences, well match the accepted theory that tumors are composed of mixed populations of cells in which only a fraction retaining stem cells features can maintain and perpetuate the tumor (34, 43). Importantly, the in vivo tumorigenicity and the in vitro immunolabeling experiments, reported here, provide evidence that the culture conditions used allow the persistence of a proportion of nestin and CD133-expressing cells, sustaining the in vitro “self-renewal” of a subpopulation of cells possessing stem cell characteristics. It has been shown that EGF plays an important role in the maintenance of normal and malignant cells of many tumor histotypes (including GBMs) promoting not only cell proliferation but also cell survival (44). Downstream to EGF activation, three major intracellular signaling pathways are activated to elicit these effects as follows: the Ras-Raf-MEK-ERK cascade, the PI3K/Akt pathway, and the STAT3-dependent signaling events (44–50). It was recently reported that the spherogenic and self-renewal properties of cells isolated from three human GBMs were mainly dependent on EGF receptor activation and were sensitive to gefitinib treatment (41). In this study we used two selective inhibitors of EGFR tyrosine kinase, erlotinib and gefitinib, to evaluate the role of EGF in the maintenance of human brain TICs derived from a larger sample (seven tumors), with the aim to identify possible tumor-related specific responses and the molecular determinant of such effects.

We obtained a highly significant inhibition of TIC proliferation after treatment with both erlotinib and gefitinib in five of seven cultures that showed a concentration- and time-dependent growth arrest, with a slightly higher responsiveness observed after gefitinib treatment and the expected tumor-related individual different sensitivity. In addition to a direct EGFR signaling inhibition, it was proposed that in primary cultures of human GBM (51) or other tumor cell lines (52), it was possible to induce growth arrest through a G protein-coupled receptor-mediated activation of phosphotyrosine phosphatase (53). Thus, it will be important to demonstrate that a synergism between inhibition of tyrosine kinase and an activation of phosphotyrosine phosphatases may induce a more complete growth arrest also in TIC-enriched cell cultures.

As discussed before, TICs represent only a small percentage of tumor cells, and this feature is maintained in vitro even after long term cultures using stem cell permissive conditions (see above and Ref. 41). Thus, it is difficult to assess whether drug treatments really affect this tumorigenic subpopulation or exert their effects only on the bulk of differentiated tumor cells. However, it is important to point out the following: 1) it was shown that EGF signaling is the main mediator of the self-renewal
activity of these cells (41), thus the observed cell growth arrest very likely also involves the duplication of TICs; and 2) a significant inhibition of proliferation was also observed in two TIC cultures (derived from GBM 4 and −5) in which a persistent high level of CD133+ cells (>80%) was observed, clearly indicating the sensitivity also of this subpopulation to the EGFR inhibitors.

Another important achievement of this study was the assessing of possible different responsiveness to drugs of individual GBM TICs cultures. In fact, previous studies analyzed only a very limited number of isolated tumor cells and were unable to identify GBM-specific responses.

In our study, using a larger sample, we report that only TICs derived from two patients (GBM 2 and −7) display a much less responsive profile to EGFR TKI. In particular, GBM 2 was totally unresponsive, and GBM 7 showed a slight inhibition of proliferation only at the highest concentrations of erlotinib and gefitinib and after prolonged treatment (at least 3 days). Thus, it was very relevant to identify possible molecular determinants for such unsatisfactory effects in this subset of tumors. In all responsive GBM TIC cultures, erlotinib and gefitinib caused the reversal of EGFR phosphorylation with the consequent inhibition of both ERK1/2 and Akt activation. However, in GBM 2, whose proliferation was completely unaffected by the tyrosine kinase inhibitors, unexpectedly EGFR and ERK1/2 phosphorylations induced by EGF were abolished in a way not dissimilar from that observed in the responsive cells. Conversely, Akt activation not only was not inhibited in the presence of either erlotinib or gefitinib, but the kinase was already maximally phosphorylated under basal conditions (after growth factor deprivation) without any further increase caused by the treatment with EGF. This constitutive activation pattern of Akt was likely related to the down-regulation of PTEN mRNA observed, in RT-PCR experiments, only in GBM 2 cells. In fact, the pharmacological inhibition of the PI3K/Akt pathway induced by wortmannin allowed the identification of an inhibitory effect of both gefitinib and erlotinib also in GBM 2 TIC cultures. Thus, the inhibition of Akt activation seems to be necessary for the antiproliferative effects of EGFR kinase inhibitors on GBM TIC-enriched cultures, as already shown for human GBM in vivo (54).

A more complex picture was observed in GBM 7 in which a different response to erlotinib and gefitinib was observed, and Akt was not constitutively phosphorylated. In fact, considering the response to erlotinib, the proliferation rate of these cells was affected only at the highest concentration used (5 μM), which was the only one able to abolish Akt phosphorylation, despite a significant inhibition of ERK1/2 at all the concentrations tested. No apparent alterations in PTEN mRNA expression levels were detected in these cells, although we cannot exclude the occurrence of inactivation mutations in the PTEN gene that will require further analysis. Thus, as far as the response to erlotinib is concerned, again the inhibition of Akt activity seems to be one of the main requirements for the antiproliferative effects of the drug.

On the other hand, in GBM 7 TIC cultures, the minimal response to gefitinib in terms of growth arrest, with a statistically significant inhibition only at the highest concentration of drug for at least 3 days, was not paralleled by a clear biochemical effect. Differently from what was observed with erlotinib, no reduction in the EGF-dependent phosphorylation/activation of EGFR, ERK1/2, and Akt was detected, even for high gefitinib concentrations and prolonged treatment. Thus, we cannot exclude that in this case a “nonspecific” toxic activity of the drug may occur after 3 days of exposure to high concentrations of gefitinib, as already reported in previous in vitro studies (29).

Although the different responsiveness to these drugs may be due to different causes yet undefined, our results underline the possible unpredictable differential responses occurring in human tumors treated with drugs apparently acting with the same molecular mechanism (i.e. EGFR TKI). In conclusion, we report that human TIC-enriched cultures isolated from seven GBMs undergo growth arrest in the presence of inhibitors of EGFR kinase activity and that this effect seems to be related to their ability to inhibit the PI3K/Akt pathway.

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