ZD6474, a Novel Tyrosine Kinase Inhibitor of Vascular Endothelial Growth Factor Receptor and Epidermal Growth Factor Receptor, Inhibits Tumor Growth of Multiple Nervous System Tumors

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Abstract Purpose: Primary central nervous system (CNS) tumors represent a diverse group of tumor types with heterogeneous molecular mechanisms that underlie their formation and maintenance. CNS tumors depend on angiogenesis and often display increased activity of ErbB-associated pathways. Current nonspecific therapies frequently have poor efficacy in many of these tumor types, so there is a pressing need for the development of novel targeted therapies.

Experimental Design: ZD6474 is a novel, orally available low molecular weight inhibitor of the kinase activities associated with vascular endothelial growth factor receptor-2 and epidermal growth factor receptor. We hypothesized that ZD6474 may provide benefit in the treatment of several CNS tumor types.

Results: In mice bearing established s.c. tumor xenografts of CNS tumors (malignant glioma and ependymoma) or rhabdomyosarcoma, a limited course of ZD6474 treatment produced significant tumor growth delays and a high rate of partial tumor regression in most models examined. Mice with i.c. malignant glioma xenografts treated with ZD6474 experienced a significant prolongation of survival. Tumors from mice treated with ZD6474 displayed a lower proliferative index and disrupted tumor vascularity. Notably, some of these models are insensitive to low molecular weight kinase inhibitors targeting only vascular endothelial growth factor receptor-2 or epidermal growth factor receptor functions, suggesting that the combined disruption of both epidermal growth factor receptor and vascular endothelial growth factor receptor-2 activities may significantly increase tumor control.

Conclusions: In conclusion, ZD6474 shows significant activity against xenograft models of several primary human CNS tumor types. Consideration for clinical development in this disease setting seems warranted.
ZD6474 has displayed activity against several cancers in an orally available novel small-molecule ATP mimetic that signaling to be taken into clinical development. ZD6474 is the first inhibitor of both EGFR and VEGFR displayed additional benefit in a pancreatic cancer model (23). Both EGFR and VEGFR may have additional benefit. A combination of a single pathway may not be efficacious as other combinations. Other studies involving VEGF receptor (VEGFR) TKIs, erlotinib, another EGFR TKI, against malignant gliomas (18). Gefitinib exhibited minimal activity with a subset of EGFR TKI (gefitinib) in patients with recurrent glioblastomas (16, 17), the activity in clinical trials has been far more ineffective in many patients. As the molecular underpinnings of cancer formation are undergoing elucidation, therapies targeted at essential oncologic pathways are being developed. Of great interest are the proto-oncogenes of the tyrosine kinase growth factor receptors. Growth factor receptor pathways are frequently inappropriately activated in CNS cancers through overexpression, amplification, or mutation (reviewed in ref. 3). These pathways promote cancer development and maintenance through stimulation of cellular proliferation, resistance to apoptosis, invasion, and secretion of proangiogenic factors.

Angiogenesis is a hallmark of high-grade gliomas and has been associated with resistance of tumors to conventional therapies. The regulation of angiogenesis represents a complex process involving autocrine and paracrine growth factor loops with an interplay between tumor cells and the neovasculature (4). Several CNS tumor types exhibit striking angiogenesis with the vascular endothelial growth factors (VEGF) acting as key mediators to promote the recruitment and formation of blood vessels (5–7). VEGFs are secreted or cell-bound proteins expressed by tumor cells that function in a paracrine fashion by binding to specific high-affinity transmembrane receptors on endothelial cells (8, 9) to promote endothelial cell proliferation and survival. Antiangiogenic therapies have shown activity in brain tumor clinical trials (10, 11), but sustained tumor control remains elusive. Additional growth factor receptor pathways regulating angiogenesis include epidermal growth factor (EGF) receptor (EGFR) and other ErbB family members that are present in several CNS tumor types. EGFR is of particular interest in glioblastomas, in which the majority of tumors exhibit inappropriate activation of EGFR through overexpression, amplification, or expression of a constitutively active mutant (EGFRVIII; refs. 12, 13). EGFR activity contributes to increased tumor proliferation, resistance to therapies, angiogenesis, and invasion (14, 15).

The recognition of the contributions of VEGF and EGFR pathways in tumor malignancy have led to the development and clinical testing of small-molecule receptor tyrosine kinase ATP mimetic inhibitors (TKI) for each of these receptors. Although these agents have shown activity in preclinical trials (16, 17), the activity in clinical trials has been far more modest. We reported recently the results of a phase II trial of an EGFR TKI (gefitinib) in patients with recurrent glioblastomas (18). Gefitinib exhibited minimal activity with a subset of patients deriving significant tumor control (18). Several other institutions have also shown modest activity of gefitinib or erlotinib, another EGFR TKI, against malignant gliomas (19–21). Other studies involving VEGF receptor (VEGFR) TKIs, such as PTK787, have also been quite modest in their activities (10, 11). As antiangiogenesia may be a critical determinant of tumor response to TKIs, it has been hypothesized that targeting a single pathway may not be efficacious as other compensatory pathways may become activated (22). Thus, targeting both EGFR and VEGFR may have additional benefit. A combination of small-molecule inhibitors of EGFR and VEGFR displayed additional benefit in a pancreatic cancer model (23). ZD6474 is the first inhibitor of both EGFR and VEGFR signaling to be taken into clinical development. ZD6474 is an orally available novel small-molecule ATP mimetic that inhibits VEGFR2 and, at higher concentrations, EGFR (24–26). ZD6474 has displayed activity against several cancers in preclinical models, including cell lines that are resistant to gefitinib (25–28).

A recent study has shown that ZD6474 is active in vivo against a rat glioma model (29). We have now examined the activity of ZD6474 in vitro to dissect specific aspects of its activity and tested ZD6474 in vivo against several human CNS tumor xenografts, including chemotherapy-resistant xenografts. We now show that ZD6474 has broad activity against CNS tumors even those without significant response to TKIs that block either EGFR or VEGFR alone.

### Materials and Methods

**Glioma cell line and culture.** The human malignant glioma xenograft D54MG is the Duke University subline of A-172 (30). D54MG-derived human glioma cells were maintained by culturing in 10-cm tissue culture dishes in Zinc Option medium containing 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY) and glutamine (Life Technologies) until ready for use.

**Central nervous system tumor xenografts.** D54MG malignant glioma xenografts were derived from D54MG cells implanted into athymic BALB/c nu/nu mice and maintained as xenografts. D456MG xenografts were derived from a pediatric glioblastoma biopsy specimen. D212MG was derived from a pediatric giant cell astrocytoma. D245MG was derived from an adult malignant glioma specimen, whereas D243MG-PR was derived from the parental D245MG xenograft treated with procarbazine until resistance developed. Resistance of D245MG-PR is periodically confirmed with procarbazine treatment. TE671 was purchased from the American Type Culture Collection (Manassas, VA). D612EP was derived from a pediatric ependymoma specimen. All xenografts are maintained as s.c. xenografts in athymic mice and passedaged repeatedly. Early-passage ampules are periodically thawed to remove genotypic drift.

**Drugs.** ZD6474 was generously provided by AstraZeneca (Macclesfield, Cheshire, United Kingdom). Stock solutions (10 mmol/L) were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO), stored at −80°C, and diluted in fresh medium immediately before use.

**Western blot analysis.** Cells were placed in serum-free Zinc Option medium for 24 hours before experiments, treated with either 0.1% DMSO or increasing concentrations of ZD6474 for 2 hours, stimulated with 100 pmol/L EGF for 5 minutes, lysed in lysis buffer [62.5 mmol/L Tris-HCl, 2% (w/v) SDS, 10% glycerol, 40 mmol/L DTT], vortexed for 5 seconds, and centrifuged at 14,000 rpm for 5 minutes at 4°C. An equal amount of protein was run on polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and phosphorylated extracellular signal-regulated kinase (pERK1/2) and total ERK1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Phosphorylated extracellular signal-regulated kinase (pERK1/2) and total ERK1/2 antibodies were purchased from Promega (Madison, WI). Antibodies were purchased from Cell Signaling Technology (Beverly, MA). α-Tubulin was purchased from Sigma-Aldrich. All antibodies were used according to the manufacturer’s instructions.

**Nonradioactive 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide cell proliferation assay.** CellTiter96 Cell Proliferation Assay kit was purchased from Promega. Assays were done according to the manufacturer’s instructions on a 96-well tissue culture plate. Four thousand cells per well were plated in the assay. Plates were read for absorbance at 570 nm (measure) and 650 nm (background).

**Caspase-3/7 activity assay.** Apo-ONE Caspase-3/7 Activity kits were purchased from Promega. Assays were done according to the manufacturer’s instructions on a 96-well black-walled, clear-bottomed tissue culture plate. Four thousand cells per well were plated in the assay. Fluorescent intensity was measured at emission wavelength 520 nm on SpectraMax Gemini plate scanner.
Vascular endothelial growth factor and epidermal growth factor quantification. VEGF and EGF Quantikine Assay kits were purchased from R&D Systems (Minneapolis, MN). D54MG cells were serum starved for 24 hours and then treated with DMSO (control) or increasing concentrations of ZD6474 for 24 hours in serum-free medium. Supernatants were collected and ELISA assays were done according to the manufacturer’s instructions. Plates were read for absorbance on VESSMax plate scanner at wavelength 570 nm (measure) and 650 nm (background).

Colony formation assays. Five hundred cells were plated in each well of a six-well plate in sextuplicate in medium with 10% serum. The next day, either 0.1% DMSO or increasing concentrations of ZD6474 diluted in equal final volumes of DMSO was added. After 7-day incubation, the plates were fixed and stained with Coomasie blue (0.1%) for 10 minutes and washed thoroughly in distilled water.

Migration/invasion assays. Kits were purchased from BD Biosciences (San Jose, CA) and used according to the manufacturer’s instructions. Cells were treated with 0.1% DMSO or ZD6474 in serum-free medium for 24 hours, trypsinized, and placed in migration inserts either coated with Matrigel (to assess migration and invasion through an artificial matrix) or uncoated membranes (to assess only migration) at 25,000 for each insert. Each migration insert was filled with 500 μL serum-free medium containing either DMSO or increasing concentrations of ZD6474. The wells underneath the migration inserts were filled with 500 μL. Zinc Option medium containing 10% FBS. After 48 hours, the migration inserts were fixed and stained with DiffQuik solutions provided in the kit. All invaded cells were counted manually. The invasion was calculated as a ratio of that shown by control (uncoated) inserts to that shown by Matrigel-coated inserts. Experiments were done in triplicate.

Statistical analysis. Data in all in vitro studies were compared by using the nonparametric Wilcoxon rank-sum test (31).

Intracranial xenograft studies. Athymic male BALB/c nu/nu mice were maintained in HEPA-filtered facilities in the Duke University Cancer Center Isolation Facility according to the institutional policy approved by the Duke Institutional Animal Care and Use Committee. S.c. xenografts passedaged in athymic mice were excised, minced, and dissociated with 0.5% collagenase at room temperature in a trypsinization flask for 40 minutes. For i.c. model studies, viable cells were separated on a Ficoll density gradient, washed twice with Dulbecco’s PBS, resuspended in 2.5% methylcellulose at a concentration of 1 x 10^7 per mL, and injected into the implanted guide cannula through the 33-gauge infusion cannula in a volume of 5 μL by using a 500-μL Hamilton gas-tight syringe and injector (Hamilton, Reno, NV).

Animals were observed twice daily for signs of distress or development of neurologic symptoms at which time the mice were sacrificed.

Subcutaneous xenograft transplantation. Subcutaneous tumor transplantation was done into the right flank of the animals with an inoculation volume of 50 μL using a brei prepared from xenografts (32).

Tumor measurements. Tumors were measured twice weekly with hand-held vernier calipers (Scientific Products, McGraw, IL). Tumor volume was calculated according to the following formula: \( V = \left(\frac{\text{width}^2 \times \text{length}}{2}\right) \).

Drug tolerance. Mice were weighed twice weekly to assess weight loss and checked at least daily for general clinical condition.

Xenograft therapy. For s.c. tumor studies, groups of 10 mice randomly selected based on tumor volume were treated when the median tumor volume was in the range of 100 to 300 mm³ and were compared with control animals receiving drug vehicle. For i.c. tumor studies, groups of 10 mice were randomized 1 day after i.c. tumor implantation. ZD6474 was given at a dose of 200 mg/kg/d by oral gavage for a total of 10 days (5 days on, 2 days off, 5 days on). Previous studies have reported ZD6474 dosing in the range of 12.5 to 100 mg/kg/d to be well tolerated and active in a range of histologically diverse human tumor xenograft models (24). As brain tumors may face restricted drug delivery due to the blood-brain barrier, we maximized the potential antitumor activity in the present study by extending the dosing range to 200 mg/kg/d p.o. for a total of 10 days as described above. Using this schedule, 200 mg/kg was identified as the maximum tolerated dose in athymic male BALB/c nu/nu mice.

Tumor response assessment. The response of the s.c. xenografts was assessed by delay in tumor growth and by tumor regression. Growth delay, expressed as treated versus control (T/C), is defined as the difference in days between the median time required for tumors in treated and control animals to reach a volume five times greater than that measured at the start of treatment. Partial tumor regression is defined as a decrease in tumor volume over at least two successive measurements. The response of the i.c. xenograft studies was assessed as the difference in the median duration of survival or neurologic deterioration. Statistical analyses were done using a personalized SAS statistical analysis program, the Wilcoxon rank-order test for growth delay, and the Fisher’s exact test for tumor regression as described previously (33). Survival estimates and median survivals were determined by using the method of Kaplan and Meier.

Immunohistochemistry. Athymic nude mice with either i.c. or s.c. D54MG xenografts were treated daily with vehicle control (DMSO) or ZD6474 (200 mg/kg per gavage) for 5 days. Tumor-bearing brains or s.c. tumors were harvested simultaneously and were fixed in 10% buffered formalin for 24 hours, transferred to 70% ethanol, and then sectioned for staining with I&I or immunohistologic analysis with antibodies against Ki-67, pAKT or total-AKT, and ERK1/2. pERK1/2 (T183 and Y185), and total ERK1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; ref. 34). pAKT (S473) and total AKT antibodies and α-tubulin were purchased from Cell Signaling Technology and

| Table 1. Summary of tumor xenograft molecular characteristics |
|-------------------|----------------|----------------|----------------|
| Cell line         | p16INK4A       | p53            | PTEN           |
|                   | Wild-type      | Wild-type      | Wild-type      |
| D456MG            | Wild-type      | Wild-type      | 1.6-7.4 x 10^4 |
| D245MG            | Wild-type      | Wild-type      | 1.2-1.5 x 10^4 |
| D212MG            | Mutant         | Wild-type      | 3.3-6.4 x 10^4 |
| D54MG             | Wild-type      | Wild-type      | 5.6-46 x 10^4  |
| Rhabdomyosarcoma  | Wild-type      | Wild-type      | 0              |
| TE671             | Wild-type      | Wild-type      | 0              |
| Ependymoma        | Wild-type      | Wild-type      | 0              |
| D612MG            | Wild-type      | Wild-type      | 0              |

NOTE: Xenografts were characterized using PCR-based techniques (p16INK4A, p53, and PTEN) or flow cytometric analysis (wild-type EGFR and EGFRvIII).
Sigma-Aldrich, respectively. Formalin-fixed, paraffin-embedded sections were used for analysis with antibodies directed against EGFR, phosphor-ylated EGFR (pEGFR), ERK1/2, pERK1/2, AKT, and pAKT (as described previously in ref. 35).

Immunoreactivity was graded by a neuropathologist blinded as to treatment protocols (R.E.M.). A semiquantitative score was derived from an intensity score of the reactivity product [absent (0), mild (1), moderate (2), and strong (3)] multiplied by a distribution score [focal (1), multifocal (2), and diffuse (3)] as described previously (36). In most cases, strong immunoreactivity of the antigen in question could be identified in endothelial cells of the tumors both verifying the reactivity of the antibody and establishing an internal control. Negative controls consisted of tissues treated with the entire antibody protocol, except for a monoclonal antibody to an irrelevant antigen substituted for the positive antibody at the appropriate dilution.

**Fig. 1.** ZD6474 inhibits EGFR and downstream effectors and blocks glioma cell proliferation, VEGF secretion, and invasion in a human glioma cell line. A, D54MG cultures were pretreated for 1 hour with 0.1% DMSO or increasing concentrations of ZD6474 followed by a 5-minute incubation with 10 pmol/L EGF. Whole-cell lysates were collected, resolved by SDS-PAGE, and immunoblotted with phosphospecific antibodies. Membranes were stripped and reprobed with antibodies to measure total levels of each protein. Equal protein loading was confirmed by α-tubulin immunoblotting. B, D54MG cells were plated in a 96-well plate. After attachment overnight, cells were treated in sextuplicate wells with 0.1% DMSO control or increasing concentrations of ZD6474 for 24 hours. Cellular proliferation was assessed by fluorometric assay.*, P < 0.05, versus control (nonparametric Wilcoxon rank-sum test). C, ZD6474 blocks colony formation of human glioma cells. D54MG cells were plated in sextuplicate wells of a six-well plate and treated with either 0.1% DMSO control or increasing concentrations of ZD6474. The cells were treated after 4 days of incubation and then stained after 7 days of total incubation time. Representative wells are displayed. D and E, ZD6474 blocks EGF and VEGF expression. D54MG cultures were indicated either in the absence or presence of 0.1% serum with either 0.1% DMSO control or increasing concentrations of ZD6474 for 48 hours. EGF (D) and VEGF (E) levels were determined by ELISA according to the manufacturer's directions. *, P = 0.062, versus control (nonparametric Wilcoxon rank-sum test); **, P < 0.02, versus control. F, ZD6474 blocks in vitro malignant glioma invasion through an artificial extracellular matrix. D54MG cells were trypsinized and placed in Transwell inserts either uncoated (control) or coated with Matrigel containing either 0.1% DMSO or increasing concentrations of ZD6474 in the insert. Cells were allowed to migrate for 24 hours; nonmigrated cells were removed and the remaining (migrated) cells were fixed with 4% paraformaldehyde and stained with 0.01% toluidine blue. Cells treated with DMSO underwent rapid migration, whereas the ZD6474-treated cells were inhibited. The migration of the cells was reduced in a concentration-dependent fashion. *, P < 0.05, versus control (nonparametric Wilcoxon rank-sum test).
Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay was done as described earlier (37). VEGF staining was done as described earlier (38). VEGFR2 (Cell Signaling Technology) was used in 1:250 dilution in 2-nitro-5-thiobenzoate (Perkin-Elmer, Boston, MA) according to the manufacturer’s protocol. CD31 and Ki-67 stainings were done as described earlier (39). When proteinase K treatment was done, instead of 30 μg/ml, we used a concentration of 180 μg/ml at 37 °C. For tissue fixation, we used 4% paraformaldehyde in PBS overnight (12-16 hours) in 4 °C. Tissue samples are then rinsed in PBS, moved to 70% ethanol, and sent for processing.

**Microvascular area and density.** To better quantify the microvessels, CD31 experiments above were repeated using a fluorescent-tagged secondary antibody to CD31. Images were photographed at ×10 and loaded into IPLab software (Scanalytics, Inc., Fairfax, VA). The number of discrete vessel segments, the thickness of each vessel, and the total area of all vessels on the images were calculated. Results are reported as the average with SDs from five fields per slide, three slides per treatment in both treated and control groups. Representative results are shown.

**Apoptosis and Ki-67 quantification.** To calculate the percentage of cells within the tumor mass that were positive for apoptosis or proliferation (Ki-67), slides stained as detailed above were photographed in nonoverlapping fields at ×40. Grids were placed on the pictures, and the number of positive cells and total number of cells (based on the presence of nuclei) were counted. Multiple fields (a minimum of five) per slide were analyzed and results were repeated for other tumors in both control and treated groups. Results are reported as averages of the five fields with SDs and representative experiments are shown. For every tumor evaluated, 20 high-power fields with areas of tumor without significant necrosis were selected by an observer blinded as to treatment protocol. Positive nuclei were quantified using ImageJ software. Data were compared by using the nonparametric Wilcoxon rank-sum test.

**Results**

**ZD6474 inhibits epidermal growth factor receptor phosphorylation of a human glioma cell line.** ZD6474 disrupts the activities of several tyrosine kinases, with VEGFR2 (KDR/Flk-1) and EGFR as the targets most commonly expressed in gliomas. Based on expression and activity data, we expect the effects of ZD6474 in cell culture to be predominantly due to EGFR targeting. We undertook several experiments on the well-characterized D54MG human glioma cell line (30, 35) to confirm this impression on our specific model. The D54MG cell line expresses ~9 × 10^4 wild-type EGFR receptors per cell and significant levels of VEGF but does not express VEGFR2 (data not shown), consistent with the paracrine role of VEGF in which tumor cells are the source of VEGF and endothelia are the target of VEGF (through their expression of VEGFR2). Notably, D54MG glioma xenografts express both VEGF in areas of tumor and VEGFR2 in the vasculature (data not shown). D54MG glioma cells express wild-type p53 and a nonfunctional form of the tumor suppressor PTEN with deletion of exons 3 to 9 (ref. 40; Table 1). Of significance, PTEN loss has been associated with resistance to EGFR TKIs (41, 42). Indeed, D54MG xenografts are resistant to gefitinib monotherapy in animal studies (data not shown). We began these experiments by measuring the effect of ZD6474 on the level of activated phosphorylation of EGFR and the downstream targets ERK1/2 and AKT, suggesting that the activities of these pathways are not fully dependent on EGFR activity in D54MG cells. The loss of EGFR expression and dependence in glioma cell lines when cultured is nearly universal (43).

**ZD6474 inhibits proliferation of a human glioma cell line.** To determine the effect of ZD6474 on cellular proliferation, D54MG cultures were treated with ZD6474 and assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 1B). ZD6474 concentrations of 10 μmol/L were required to inhibit proliferation by 50%. As ZD6474 is likely inhibiting cellular proliferation in D54MG cells predominantly through its activity against EGFR, as VEGFRs are not expressed by these cells, the high concentration of ZD6474 required to block cellular proliferation further suggests that cellular proliferation stimulated by serum is not fully EGFR dependent in D54MG cells. ZD6474 treatment did not increase caspase-3/7 activity as measured by fluorescent substrate assay (data not shown), suggesting that ZD6474 does not directly induce tumor cell apoptosis. As further evidence of the antiproliferative effects of ZD6474, the colony formation of D54MG cells was inhibited in a concentration-dependent manner by ZD6474 (Fig. 1C). Thus, ZD6474 displays activity against D54MG cultures with predominantly antiproliferative effects.

**ZD6474 targets glioma vascular endothelial growth factor and epidermal growth factor secretion.** Although EGF is recognized as a mitogenic stimulus, the EGFR pathway activates other cellular behaviors associated with tumor progression. To address the potential effect of ZD6474 on these phenotypes, we did in vitro assays to dissect EGFR functions further. EGFR activity induces angiogenesis through the secretion of angiogenic factors, such as EGF and VEGF. ZD6474 inhibited both EGF and VEGF secretion by the tumor cells as measured by ELISA in a concentration-dependent manner (Fig. 1D and E.)

For ELISA, results were presented as averages of the five fields with SDs and representative experiments are shown.

**Table 2. Summary of ZD6474 therapy s.c. xenograft trials**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Growth delay (d)</th>
<th>T-C</th>
<th>P</th>
<th>Regressions</th>
<th>Deaths</th>
</tr>
</thead>
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<tr>
<td>Gliomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>D456MG</td>
<td>18.9</td>
<td>&lt;0.001</td>
<td>8/8</td>
<td>2/10</td>
<td></td>
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<tr>
<td>D245MG</td>
<td>25.4</td>
<td>&lt;0.001</td>
<td>10/10</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>D245MG-PR</td>
<td>19.2</td>
<td>&lt;0.001</td>
<td>6/8</td>
<td>2/10</td>
<td></td>
</tr>
<tr>
<td>D212MG</td>
<td>10.4</td>
<td>&lt;0.001</td>
<td>3/10</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>D54MG</td>
<td>14.2</td>
<td>&lt;0.001</td>
<td>1/10</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>16.6</td>
<td>&lt;0.001</td>
<td>3/10</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>TE671</td>
<td>23.2</td>
<td>&lt;0.001</td>
<td>8/10</td>
<td>0/10</td>
<td></td>
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<tr>
<td>Ependymoma</td>
<td></td>
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<td>D612EP</td>
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**NOTE:** Human tumor xenografts were implanted s.c. into nude athymic mice until tumors reached a mean volume of 100 to 300 mm³. Mice were then treated per gavage with either vehicle control or ZD6474 (200 mg/kg/d) for 5 days followed by a 2-day break and an additional 5 days of treatment. The tumor growth delay was determined by the relative time to grow to five times the original tumor volume. Ps were determined using the Wilcoxon rank-order test. Partial tumor regressions were determined by two sequential measurements with smaller total volumes. Partial regressions and deaths were only noted in the ZD6474-treated group, and all deaths are presumed to be due to drug toxicity.
respectively). Thus, ZD6474 may block angiogenesis through two mechanisms: blockade of EGF and VEGF ligand expression by tumor cells and VEGFR activity in the endothelial cells.

**ZD6474 blocks glioma tumor cell invasion.** EGFR regulates cellular motility and invasion in both normal neural stem cells and tumor cells. As measured by standard Transwell and Matrigel assays, ZD6474 inhibits both cellular motility and invasion through an artificial extracellular matrix in a concentration-dependent manner (Fig. 1F). In summary, ZD6474 exhibits significant direct inhibitory effects on tumor cell behavior independent from its effect on tumor-associated vasculature.

**ZD6474 inhibits tumor growth in animals.** Previous studies showed that ZD6474 is well tolerated and active in the 12.5 to 100 mg/kg/d dose range in several histologically diverse human tumor xenografts (24). To gain the maximum potential for therapeutic benefit in the aggressive CNS xenograft models in the present study, we extended this dose range and showed that ZD6474 at 200 mg/kg/d by oral gavage for 10 days was...
tolerated in non-tumor-bearing athymic male BALB/c nu/nu mice (data not shown). However, in occasional tumor-bearing animals, 200 mg/kg/d ZD6474 for 10 days was not tolerated (Table 2), indicating that this dose is very close to the maximum tolerated dose in these animal models and that further dose escalation is not possible. As a critical effect of ZD6474 has been measured to be against VEGFR activity, we examined the effects of ZD6474 against D54MG xenografts in vivo. Xenograft models permit the dissection of specific pathways in tumors derived from human patients (Table 1). S.c. xenograft studies allow for the direct measurement of tumor volumes and acquisition of tumor tissue directly without restriction of drug delivery by the blood-brain barrier. An i.c. xenograft model permits examination of tumor growth in a native environment with cerebral vasculature that may be differentially regulated relative to systemic vasculature. Therefore, these models used in combination offer the greatest opportunity to quantify the antitumor properties of a drug.

In a heterotopic xenograft model of established s.c. tumors, ZD6474 treatment (given as 200 mg/kg/d for a total of 10 days on a 5 days on, 2 days off, 5 days on schedule) slowed D54MG xenograft growth in two trials in a statistically significant manner (representative results; Fig. 2A). As measured by time to reach five times initial tumor volume, ZD6474 delayed D54MG tumor growth by >14.2 days (T-C; \( P < 0.001 \) relative to control). Rare partial tumor regressions were seen with ZD6474 therapy (1 of 10 treated animals), and no tumors were cured by the limited therapy. Survival of mice with implanted i.c. tumors is used as a clinical surrogate for tumor growth. In an i.c. D54MG xenograft study, ZD6474 led to moderate increases in median animal life span in replicate studies (representative trial shown in Fig. 2B) with median life span of 12 days for control mice and 44 days for ZD6474-treated animals (\( P < 0.001 \) versus control). Four mice were long-term survivors in the displayed trial. The smaller initial size of i.c. tumors at treatment initiation (with a probable lower vascular density) and differential tumor microenvironment likely account for the differential effects of ZD6474 treatment measured in s.c. and i.c. tumor assays.

**ZD6474 inhibits tumor growth of pediatric glioma xenografts.** EGFR amplification and mutation is uncommon in pediatric gliomas. To fully determine the potential utility of ZD6474 against pediatric gliomas, we examined several other well-characterized xenograft models. ZD6474 displayed activity against two s.c. pediatric gliomas: D456MG (T-C, 18.9 days; \( P < 0.001 \); 8 of 8 nondurable partial regressions; Fig. 2C) and D212MG (T-C, 10.4 days; \( P < 0.001 \); 3 of 10 nondurable partial regressions; Fig. 2D). Of note, both of these xenografts express wild-type PTEN (Table 1), reflecting the rarity of PTEN

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**Fig. 3.** Analysis of ZD6474 effects on tumor xenograft proliferation and apoptosis. Nude athymic mice with established D456MG (A) and D54MG (B) xenografts were treated with vehicle control or ZD6474 (200 mg/kg/d) per gavage for 5 days. One hour after the final dose, tumors were harvested and formalin fixed, and sections were stained for Ki-67 as a marker of proliferation. Areas of viable tumor were examined for positive cells (brown). Magnification: ×400 (A) and ×100 (B). Quantification of Ki-67-positive nuclei per high-power fields (hpf) was measured in 20 fields per tumor sample. *, \( P < 0.02 \); **, \( P = 0.011 \), versus control. Additionally, D456MG was assessed for induction of apoptosis using terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining. A representative high-power field for each condition is shown.
mutations in pediatric gliomas. Thus, ZD6474 may offer benefit for pediatric patients.

**ZD6474 inhibits tumor growth of chemotherapy-resistant xenografts.** As the activities of most novel therapies directed against gliomas are evaluated in patients with recurrent glioblastomas who have failed other therapies, we examined the activity of ZD6474 against a pair of glioma xenografts: a parental xenograft (D245MG, T-C, 25.4 days; \( P < 0.001 \); 10 of 10 nondurable partial regressions; Fig. 2E) and a matched xenograft selected for resistance to procarbazine (D245MG-PR, T-C, 19.2 days; \( P < 0.001 \); 6 of 8 nondurable partial regressions; Fig. 2F), a chemotherapeutic agent in current use for gliomas. D245MG-PR is deficient in normal DNA mismatch repair mechanisms contributing to chemotherapeutic resistance. ZD6474 delayed the growth of both parental and resistant xenografts, suggesting that ZD6474 has use in gliomas resistant to conventional chemotherapies.

**ZD6474 blocks tumor growth of other histologies.** Other primary CNS tumor types have been less clearly linked to dependence on either EGFR or VEGFR than gliomas, but all solid cancers are dependent on angiogenesis. Ependymomas are highly vascular tumors that originate in the posterior fossa or spinal cord. ZD6474 delayed tumor growth of a s.c. xenograft derived from a patient with an ependymoma in a statistically significant manner (D612EP, T-C, 23.2 days; \( P < 0.001 \); 8 of 10 nondurable partial regressions; Fig. 2G; Table 2). TE671 is a xenograft that has a small, blue round cellular appearance on histologic analysis that has been characterized as a rhabdomyosarcoma but was used previously as a model of medulloblastoma (44, 45). S.c. TE671 xenografts treated with ZD6474 were significantly delayed in tumor growth (T-C, 16.6 days; \( P < 0.001 \); 3 of 10 nondurable partial regressions; Fig. 2H; Table 2). These results suggest that ZD6474 may have a role in the treatment of ependymoma and rhabdomyosarcoma.

**Immunohistochemical analysis of tumor proliferation and apoptosis after ZD6474 treatment.** We examined the consequences of ZD6474 treatment on the histologic appearance of both i.c. and s.c. xenografts treated with a course of orally given ZD6474. In all xenografted animal models studied, the animals treated with DMSO only (control) displayed hypercellular areas with a high mitotic rate (measured by percentage of cells expressing Ki-67; Ki-67 is a proliferative marker that detects all cells not in G0), although we detected differences in degree between xenografts. In s.c. xenografts treated with ZD6474, we detected a decreased cellular proliferation as measured by Ki-67 staining (Fig. 3). The nuclei of tumors cells in the parenchyma...
of the control tumors grown s.c. stained widely for Ki-67, whereas the viable areas of ZD6474-treated tumors exhibited much more limited Ki-67 staining, and Ki-67 staining was virtually absent in those areas of the ZD6474-treated tumors that have necrosed. In a count of five fields in treated and untreated s.c. tumors, there is a 3-fold decrease in Ki-67 rate in T/C D456MG tumors (Fig. 3A) and a similar benefit in D54MG tumors (Fig. 3B). In contrast to the effects of ZD6474 on s.c. tumors, ZD6474 only modestly lowered the number of tumor cells positive for Ki-67 in i.c. tumors (Fig. 4).

Although we did not see evidence of direct induction of tumor cell apoptosis with ZD6474 treatment in cell culture assays, the mechanism by which ZD6474 functions in tumors grown in animals is different due to the antiangiogenic effects of ZD6474 in vivo. We did a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling stain to assess the effect of ZD6474 on s.c. tumors; ZD6474 only modestly lowered the number of tumor cells positive for Ki-67 in i.c. tumors (Fig. 4).

To evaluate the selective inhibition of the targets of ZD6474, we did immunohistochemical analysis of i.c. and s.c. xenografts. Immunohistochemical reactivity for activated (phosphorylated) EGFR, ERK, and AKT revealed moderate to strong reactivity in the control tumors in either i.c. or s.c. tumors, indicating that these kinases are activated at baseline and that immunohistochemical detection of these epitopes is a successful technique. In the ZD6474-treated i.c. tumors, treatment effectively abolished reactivity for pEGFR, more modestly affected pERK, and had little effect on the detected levels of pAKT versus nontreated controls (Fig. 5; data not shown). In the ZD6474-treated s.c. tumors, ZD6474 treatment modestly lowered pEGFR intensity, effectively abolished reactivity for pERK, and had little or no effect on the detected levels of pAKT versus nontreated controls (Fig. 6). The modest basal EGFR expression in i.c. xenografts and the limited effect on EGFR phosphorylation in s.c. tumors by ZD6474 in these studies suggest that the effect of ZD6474 in vivo may also be due to disruption of VEGFR2 function.

Fig. 5. Immunohistochemical analysis of i.c. tumor xenografts treated with ZD6474. Nude athymic mice bearing i.c. D456MG or D54MG xenografts were treated with vehicle control or ZD6474 (200 mg/kg/d) per gavage for 5 days. One hour after the final dose, brains bearing D456MG tumors were harvested and formalin fixed. Brain sections were stained with antibodies to both unphosphorylated and phosphorylated targets as described in Materials and Methods. A, representative microscopic fields of each immunohistochemical reaction (×400). B, overall semiquantitative grading scale (intensity × distribution) for pEGFR, pAKT, and pERK as described in Materials and Methods provided for each xenograft. *, P = 0.0032, versus control. Similar results were noted with D54MG xenografts (data not shown).
ZD6474 treatment disrupts vascular endothelial growth factor receptor-2 staining in subcutaneous tumors. To assess the effect of ZD6474 on the VEGF axis, we did immunohistochemical analyses on VEGF ligand and its receptor (VEGFR2). VEGF was present in both tumor-associated vasculature and tumor cells of untreated tumors, providing a continuous signal to VEGFR2 on surrounding endothelial cells. Whereas the parenchyma of control s.c. tumors displayed diffuse immunoreactivity for VEGF, the cells around blood vessels were significantly stronger in their reactivity for the antibody in the control samples within the solid component of the tumor (Fig. 7A). This suggests that the tumor or tumor-dependent stroma produces VEGF. In addition, there were areas of tumor cell death that complicate an analysis of the parenchymal distribution of the VEGF signal. This may well represent the up-regulation of VEGF as the tumor becomes hypoxic due to the effects of ZD6474. As expected, the most evident increase in VEGF signal in the treated s.c. tumors was within the viable tumor areas (Fig. 7A). VEGFR2-stained vessels in the control s.c. tumors were well organized with thin, branched linear structures as expected, but ZD6474 treatment in s.c. tumors was associated with areas of complete disorganization with disruption in the VEGFR2 staining (Fig. 7A).

In contrast, normal brain produces VEGF, so the effects of ZD6474 on VEGF expression of i.c. tumors were far less significant (data not shown). In addition, the VEGFR2 staining of the i.c. tumors also did not display the disorganization in the tumor vasculature detected in the s.c. tumors. These results suggest that targeting VEGFR2 may have significantly different effects on tumor vasculature based on the microenvironment.

ZD6474 inhibits formation of tumor vasculature in vivo. Fluorescent CD31 staining was used to evaluate for microvesel density. The CD31 immunostain, a marker of endothelial cells, was restricted to the vessels as expected. Cerebral tumor vasculature fundamentally differs from systemic vasculature (46), so we hypothesized that tumor vasculature in s.c. and i.c. locations may differ in response to ZD6474. Indeed, tumors grown in orthotopically and ectopically both responded to ZD6474 treatment but in strikingly different ways. In i.c. tumors, ZD6474 decreased the microvascular density and total vascular area but not average vessel size (Fig. 4). These vessels remained well organized despite ZD6474 treatment.

In contrast to the response of i.c. tumors treated with ZD6474, s.c. xenografts treated with ZD6474 displayed a much more limited change in microvascular density and no quantitative difference in the overall mean vascular area per vessel and total vascular area between control and treated tumors despite the altered vascular structure (Fig. 7). Control
s.c. tumors displayed well-organized, long-branching vessels with thin walls (Fig. 7A). ZD6474 treatment of s.c. tumors disrupted the appearance of the vasculature, so that the nice long-branching vessels are much less evident. Additionally, CD31 immunostaining in the ZD6474-treated s.c. tumors is present, but the positive cells were more diffuse and disorganized. The vessels of ZD6474-treated s.c. tumors were thick and multilayered (Fig. 7A) and were most disrupted in the areas where tumor cell death is evident.

Discussion

We have shown that ZD6474, an orally available inhibitor of VEGFR2 and EGFR tyrosine kinase activity, displays significant activity against a selection of xenografts derived from CNS tumors. In fact, every xenograft tested displayed growth delay of at least the duration of treatment (10 days). Further, partial regressions were detected in every group of treated xenograft, but these regressions were temporary, suggesting that ZD6474 may require chronic dosing. The in vivo efficacy of ZD6474, which targets both EGFR and VEGFR activities, stands in striking contrast to the efficacy of other TKIs directed toward EGFR or VEGFR2 alone using these same glioma xenografts (data not shown). Thus, the in vivo efficacy of ZD6474 suggests that targeting both EGFR and VEGFR pathways in gliomas and their associated vasculature may be necessary to achieve significant clinical efficacy possibly by inhibiting the two pathways from compensating for each other. Multiple EGFR TKIs, including gefitinib and erlotinib, have been evaluated in clinical trials for the treatment of malignant gliomas with evidence of only modest activity (16–19). Molecular markers of glioma sensitivity to EGFR TKIs have not been definitively validated, but preclinical studies have suggested that mutation of the PTEN tumor suppressor gene and increased activation of AKT may be associated with EGFR TKI resistance (41, 42, 47–49). Our current studies show that ZD6474 is active against xenografts with both wild-type and mutant PTEN. The activity of ZD6474 in cell culture largely reflects the inhibition of EGFR as VEGFR2 is not expressed by most glioma cell lines, including D54MG. High concentrations of ZD6474 are required to block the activating phosphorylation of intracellular oncogenic pathways of AKT and ERK in the D54MG cell line (PTEN mutant) in culture beyond that required to block EGFR. Thus, ZD6474 activity against xenografts resistant to EGFR blockade suggests that the VEGF axis may represent not only a resistance mechanism of gliomas to EGFR TKIs but also a viable therapeutic target to disrupt in combination with EGFR. An additional possibility for the efficacy of ZD6474 may be that tumor cells rapidly become resistant to disruption of the EGFR pathway.

Fig. 7. ZD647(241,891),(766,944)(43,781),(733,894)

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pathway. Although endothelial cells cannot become resistant per se, they have enough VEGF signal to maintain survival and proliferation. By contrast, by targeting both VEGFR and EGFR, the tumor cell rapidly may switch to another growth factor pathway to survive, whereas the tumor-associated endothelial cell, which expresses both EGFR and VEGF mediated signaling, cannot maintain growth. Thus, tumor cell death now becomes dependent on vascular cell death.

Our results further suggest that CNS tumors not usually felt to depend on EGFR or VEGF may still have significant tumor control in response to small-molecule inhibitors targeting these pathways. To date, the expression levels of specific receptor tyrosine kinases have not been predicted responses of solid tumors to TKIs targeted to these kinases (18). Thus, the lack of increased expression of EGFR and VEGF in solid tumors may not preclude sensitivity of a broad spectrum of tumors to ZD6474. The potency of ZD6474 binding to the ATP-binding pockets of its target kinases may suppress signaling from these receptors even when they signal at low levels on tumor cells or associated endothelia. Early preclinical studies with TKIs led to the hypothesis that these agents would induce cytostasis in solid tumors, but several TKIs have shown tumor responses in clinical trials. Clinical glioma trials with TKIs targeted to EGFR or VEGFR have only rarely reported tumor responses, but the significant frequency of tumor regressions in our studies with ZD6474 suggests that the joint blockade of EGFR and VEGFR may have greater likelihood of response. The use of an agent, such as ZD6474, may offer significant advantages over the combination of two separate agents that may have pharmacologic interactions and increased toxicities.

The development of targeted therapies requires greater sophistication in evaluation with confirmation of disruption of the involved cellular pathways. Whereas cell culture assays showed that the phosphorylation of AKT was more easily blocked than that of ERK, xenograft studies show quite distinctive efficacy of ZD6474 against ERK rather than AKT. These results indicate that caution should be applied when extrapolating from lessons derived from cell culture studies alone especially with therapies that have antiangiogenic activity. Additionally, the relative resistance of AKT phosphorylation with ZD6474 treatment suggests that ZD6474 may offer combinatorial benefit with agents targeting phosphatidylinositol 3-kinase or downstream pathways. Indeed, we have shown recently that another TKI can offer additional benefit when combined with a rapamycin derivative (35). Finally, the responses of systemic and i.c. vasculature to ZD6474 differ significantly.

The composite of our results suggest that ZD6474 holds great promise in the treatment of nervous system tumors, including those that are resistant to gefitinib or cytotoxic chemotherapy. Many mechanisms have been linked to the resistance of gliomas to conventional chemotherapies and radiotherapy, and EGFR and VEGF may be prominent among them. Thus, the therapeutic benefits of ZD6474 may be seen not only as monotherapy but also in combination with traditional cytotoxic therapies (50). The design of clinical trials of ZD6474 may incorporate tissue acquisition and pharmacodynamic measures of tumor response. Ultimately, it will only be with the coordinated assessment of patient response and tumor characteristics that predictive components of tumor cell signaling will become elucidated. Unlike studies of more common solid tumors, such as lung carcinomas, in which many patients were treated with TKIs to derive rare marked responses, clinical trials with relatively uncommon nervous system tumors may yield far more modest clinical benefit due to small trials with unselected populations. Long-term development in this patient population that remains desperate for improved therapies will require an iterative process in which lessons from both other solid tumor trials and preliminary brain tumor trials will inform trials with selected patient populations.

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