ZD6474, a Multitargeted Inhibitor for Receptor Tyrosine Kinases, Suppresses Growth of Gliomas Expressing an Epidermal Growth Factor Receptor Mutant, EGFRvIII, in the Brain

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Abstract

Epidermal growth factor receptor (EGFR) vIII is a mutated EGFR that is frequently overexpressed in glioblastomas and implicated in response to receptor tyrosine kinase inhibitors. In this study, we investigate the effect of ZD6474 (ZACTIMA, vandetanib), a dual inhibitor for vascular endothelial growth factor receptor 2 and EGFR on growth and angiogenesis of gliomas expressing EGFRvIII. We used two glioma xenograft models, U87MG cells overexpressing EGFRvIII and short-term cultured primary glioma GBM8 cells with EGFRvIII. ZD6474 inhibited tumor growth and angiogenesis and induced cell apoptosis in various brain gliomas. Moreover, significant inhibition of EGFRvIII-expressing U87MG and GBM8 gliomas was observed compared with their controls. Magnetic resonance imaging analysis using the apparent diffusion coefficient and three-dimensional T2*weighed measurements validated ZD6474 inhibition on tumor growth and angiogenesis in EGFRvIII-expressing GBM8 gliomas. Mechanistically, ZD6474 shows better inhibition of cell growth and survival of U87MG/EGFRvIII, GBM6, and GBM8 cells that express EGFRvIII than U87MG or GBM14 cells that have nondetectable EGFRvIII through attenuation of activated phosphorylation of signal transducer and activator of transcription 3, Akt, and Bcl-XL expression. Albeit in lesser extent, ZD6474 also displays suppressions of U87MG/EGFR and GBM12 cells that overexpress wild-type EGFR. Additionally, ZD6474 inhibits activation of extracellular signal-regulated kinase 1/2 in both types of cells, and expression of a constitutively active phosphoinositide 3-kinases partially rescued ZD6474 inhibition in U87MG/EGFRvIII cells. Taken together, these data show that ZD6474 significantly inhibited growth and angiogenesis of gliomas expressing EGFRvIII by specifically blocking EGFRvIII-activated signaling mediators, suggesting a potential application of ZD6474 in treatments for glioblastomas that overexpress EGFRvIII.

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Introduction

Malignant gliomas are the most common tumors in the central nervous system (1). Despite rapid improvements in imaging, surgery, adjuvant radiotherapy, and chemotherapy, the prognosis for patients with gliomas still remains dismal. The failure of current therapeutic approaches is rooted in the nature of high proliferation, extreme invasive behavior, and robust neoangiogenesis that confer these tumors resistant to aggressive treatments (2, 3). Acquisition of malignant growth, insidious invasion, high neovascularization, and resistance to therapies by glioma cells involve multiple genetic alterations, such as epidermal growth factor receptor (EGFR) overexpression, that activate various cellular signaling pathways (2, 3). Amplification of EGFR occurs in ~45% of high-grade glioblastomas and is often accompanied by gene mutations. The most common EGFR mutation is EGFRvIII, an in-frame deletion of exons 2 to 7 in the gene...
that encodes amino acid residues 6 to 273, resulting in a ligand-independent, constitutively active, and cell surface-retained receptor (2). Glioblastoma patients with EGFRvIII overexpression are associated with a poorer prognosis and a shorter survival time (3, 4). In U87MG glioma xenografts, overexpression of EGFRvIII significantly enhanced tumorigenicity by increasing cell proliferation and decreasing cell death (5, 6). In retrospective analysis of clinical trials using tyrosine kinase inhibitors (TKI) of EGFR, erlotinib or gefitinib, coexpression of EGFRvIII and wild-type (WT) PTEN by high-grade glioblastomas is associated with responsiveness to the TKI treatments, suggesting that EGFRvIII expression in glioma cells enhances responsiveness to TKIs (7, 8).

ZD6474 (ZACTIMA, vandetanib) is p.o. given and is a potent TKI for various receptor tyrosine kinase, in particular vascular endothelial growth factor receptor 2 (VEGFR2) and EGFR. By inhibiting VEGFR2-dependent tumor angiogenesis and EGFR-mediated cancer cell proliferation, invasiveness, and survival, ZD6474 displays potent inhibitory activities against various types of human cancer xenografts, including gliomas in animals (9, 10). ZD6474 inhibits tyrosine kinase activities of VEGFR2 in endothelial cells and EGFR in cancer cells, as well as their downstream effectors (11–16). ZD6474 suppresses tumor growth in several cancer cell lines that are resistant to gefitinib (10, 11). ZD6474 also shows better therapeutic effects versus gefitinib in clinical trials for non–small cell lung cancer and gliomas (17–21). Whereas two TKIs for EGFR, gefitinib and erlotinib, have been evaluated in completed clinical trials for malignant gliomas, the results of similar trials for ZD6474 are not yet mature (22). Moreover, in both preclinical and clinical studies, a link of the response of ZD6474-treated tumors to genetic alterations, such as EGFRvIII expression in malignant glioblastomas, has not been documented yet.

In this study, we examined the therapeutic effects of ZD6474 to glioma cell lines and their brain xenografts with and without EGFRvIII expression, U87MG (no EGFRvIII), U87MG/EGFRvIII, and U87MG/EGFR (5, 6) and short-term cultured human primary glioma GBM cells (GBM6, GBM8, GBM12, GBM14; ref. 23). We evaluated the effect of ZD6474 on tumor growth, survival, and angiogenesis of various gliomas in the brains of mice using histologic and magnetic resonance imaging (MRI) analyses. Lastly, we also assessed the inhibition of ZD6474 on EGFRvIII-mediated cellular signaling in these isogenic glioma cell lines. Our data show that ZD6474 inhibits glioma growth and angiogenesis and promotes cell apoptosis of EGFRvIII-expressing glioma cells through attenuation of EGFRvIII-mediated signaling pathways in vitro and in vivo.

Materials and Methods

Cell lines, antibodies, and reagents

Human U87MG glioma cells were from American Type Culture Collection. U87MG/EGFR and U87MG/EGFRvIII cells were from Drs. F. Furnari and W. Cavenee (5). Human primary short-term cultured glioma (GBM) cells were passed in the flanks of mice and implanted to the brain of animals as previously described (23). All the cell lines were tested for their astrocytic cell lineage in vitro and gliomagenicity in vivo as previously described (24). ZD6474 was provided by AstraZeneca. The antibodies and reagents that were used in our studies are described in Supplementary Data.

Intracranial brain tumor xenograft, immunohistochemical and immunoblot analyses, and cell survival and proliferation assays

Intracranial brain tumor xenograft, immunohistochemical and immunoblot analyses, and cell survival and proliferation assays were done as described in Supplementary Data (24–26).

MRI analysis

Animal preparation. ZD6474-treated and control mice with GBM8 gliomas 2 wk postimplantation were anesthetized using isofluorane with concentrations of 5% for induction and 1% to 2% maintenance in a carrier gas mixture of 30% O2 and 60% N2O through a nose cone. Two electrodes were positioned subcutaneously bilaterally on the chest to record heart rate with ECG, and a rectal probe interfaced to a warm air circulator was used to monitor and maintain body temperature at 37.5°C throughout the imaging experiment (SA Instruments, Inc.).

Image acquisitions. Mice were placed in a cylindrical holder to stabilize the head and placed in a 36-mm Helmholtz volume transmit/receive coil for MRI. All MR data were acquired at 9.4 T on a Varian imaging spectrometer. Maps of the apparent diffusion coefficient (ADC; in units of ×10−3 mm2/s) were obtained using a spin-echo sequence (TE/TR = 25/1,000 ms) with a range of bipolar gradient strengths (up to maximum of 10 G/cm) of fixed duration (Δ = 8 ms) and separation (Δ = 14 ms) along all three orthogonal (X, Y, Z) axes simultaneously (b values of 16, 140, 389, 658, 997, and 1261 s/mm2). T2 maps were obtained using a spin-echo sequence with TE of 10, 20, 30, 40, 50, and 60 ms and TR of 1,000 ms. Both T2 and ADC map acquisitions, which also yield a range of T2 weighed and ADC images, were acquired with a 16 × 16 mm2 field of view, 64 × 64 matrix, and four to five lated on a pixel-by-pixel basis by fitting the signal intensity versus b value (ADC) or TE (T2), respectively, to single exponential functions. T2*weighted three-dimensional Turboflash images were acquired with a TE of 10 ms, a TR of 40 ms, and 16 × 16 × 16 mm3 field of view and (128)3 and (64)3 matrix size for the control and treated mouse brain, respectively, to give respective isotropic resolutions of (125 μm)3 and (250 μm)3.

Regions of interest. In Fig. 3, the maroon regions of interest (ROI) were drawn from the dark-stained tumor region on the control H&E stain. The smaller blue-tinted ROI was drawn from the darker-stained tumor region on the treated H&E stain. The ROI shown in green was
drawn from the region of reduced signal intensity on the T2*-weighed three-dimensional MR images from the control mouse, which is consistent with tumor. The darker hue of each of these colors was used in conjunction with solid lines in depicting the histograms obtained from the corresponding tumor ROIs. The lighter hue of each of these colors was used in conjunction with dashed lines to depict the corresponding histograms obtained from contralateral ROIs.

**Statistical analysis**

A one-way ANOVA with Newman-Keuls posttest or a paired two-way Student’s *t* test was done using GraphPad Prism version 4.00 for Windows (GraphPad Software). A *P* value of <0.05 was considered statistically significant.

**Results**

**ZD6474 significantly inhibits tumor growth of U87MG brain gliomas that overexpress EGFRvIII**

We examined the effects of ZD6474 on tumor growth and angiogenesis of human U87MG gliomas and their isogenic variant that overexpress EGFRvIII in the brain. As shown in Fig. 1, in control groups, 19 days post-implantation of parental U87MG or U87MG/EGFRvIII cells (Fig. 1A) into the brain of mice, mice that received U87MG/EGFRvIII cells developed tumors in the brain with an average volume of 48.7 ± 8.3 mm³. In contrast, mice that received parental U87MG cells only developed tumors in the brain with an average volume of 6.4 ± 1.5 mm³ (Fig. 1A). The growth kinetics of parental U87MG and U87MG/EGFRvIII tumors is consistent with the results as previously reported (5). In ZD6474-treated groups, mice with parental U87MG tumors only had an ∼30% decrease in tumor volume (4.4 ± 0.6 mm³ of ZD6474-treated tumors compared with 6.4 ± 1.5 mm³ of the control), whereas approximately an 85% decrease in tumor volume was seen in mice with U87MG/EGFRvIII gliomas (7.3 ± 1.2 mm³ versus 48.7 ± 8.3 mm³, *P* < 0.005; Fig. 1A, compare left with right, bar graph).

Next, we assessed the effect of ZD6474 on vehicle treatments on tumor angiogenesis, cell growth, and apoptosis on parental U87MG and EGFRvIII tumors. As shown in Fig. 1B, neovascularization in both types of tumors was effectively suppressed by ZD6474, with an ∼94% decrease in parental U87MG tumors (15.1 ± 3.5 in treated tumors compared with 275.6 ± 5.4 pixels/mm² in the controls, *P* < 0.005) and an ∼88% decrease in U87MG/EGFRvIII gliomas (46.5 ± 3.1 in treated tumors compared with 370.3 ± 20.4 pixels/mm² in the controls, *P* < 0.005), respectively (compare left with right and bar graph). Moreover, tumor cell proliferation indices in ZD6474-treated U87MG/EGFRvIII tumors were decreased by ∼90% compared with control tumors (4.1 ± 1.2% Ki-67-positive cells in treated tumors compared with 38.3 ± 4.1% in the controls, *P* < 0.005). In contrast, only ∼45% inhibition of cell proliferation was found in ZD6474-treated parental U87MG gliomas compared with control tumors (7.7 ± 0.8% versus 16.7 ± 1.2% Ki-67-positive cells, *P* < 0.05; Fig. 1C). To a similar extent, tumor cell apoptosis in ZD6474-treated U87MG/EGFRvIII tumors was increased by 85% compared with control tumors [0.1 ± 0.03% versus 1.2 ± 0.56% terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-labeled cells, *P* < 0.005], whereas a ∼15% elevation in cell death was found in ZD6474-treated parental U87MG gliomas compared with control tumors (0.05 ± 0.001% versus 0.06 ± 0.002% TUNEL-labeled cells; Fig. 1D).

ZD6474 markedly inhibits brain tumor growth and angiogenesis of human primary short-term cultured glioma cells (GBM) that overexpress endogenous EGFRvIII

It is established that although EGFR and EGFRvIII are frequently overexpressed in clinical glioma specimens, such overexpression is lost soon after the primary tumor cells are cultured in vitro (2). To address this limitation, we chose two short-term culture primary GBM8 and GBM14 cells that retain genetic alterations found in the primary glioma specimens and form infiltrative gliomas in the brain (23) for our studies. GBM8 cells retain the overexpression of both EGFR and EGFRvIII, whereas EGFR or EGFRvIII in GBM14 cells was nondetectable (Fig. 2A; ref. 23). As shown in Fig. 2A, 23 days after GBM8 or GBM14 cells were implanted, in control groups, mice that received either of the GBM lines developed tumors in the brain with an average volume of 46.1 ± 8.5 mm³ (GBM8) or 42.2 ± 3.3 mm³ (GBM14; Fig. 2A, left; ref. 23). In ZD6474-treated groups, mice with GBM14 tumors displayed an ∼32% decrease in tumor volume (28.9 ± 4.5 mm³ compared with 42.2 ± 3.3 mm³, *P* < 0.5), whereas an ∼85% decrease in average tumor volume was seen in the brain with GBM8 gliomas (7.2 ± 0.8 mm³ versus 46.1 ± 8.5 mm³, *P* < 0.005; Fig. 2A, compare left with right, bar graph).

Next, we evaluated the effect of ZD6474 or vehicle treatments on tumor angiogenesis, cell growth, and apoptosis on GBM8 and GBM14 tumors. As shown in Fig. 2B, neoangiogenesis in both types of tumors was suppressed by ZD6474 treatments compared with their control tumors. Moreover, there was a greater decrease in vessel densities in ZD6474-treated GBM8 tumors than that in ZD6474-treated GBM14 tumors compared with their control tumors. There was an ∼70% decrease in CD31-positive vessels in GBM8 tumor group (386.7 ± 21.5 versus 118 ± 5.6 pixels/mm², *P* < 0.01), whereas there was only an ∼38% decrease in GBM14 groups (305.7 ± 8.1 versus 192.8 ± 12.7 pixels/mm², *P* < 0.03; Fig. 2B, compare left with right, bar graph). When tumor cell proliferation indices were assessed by an anti-Ki-67 antibody staining, ZD6474-treated GBM14 tumors showed an ∼65% decrease (15.1 ± 1.3% versus 6.1 ± 0.2% proliferating cells, *P* < 0.05) whereas ZD6474-treated GBM8 tumors showed an ∼90% decrease in Ki-67 staining compared with the control tumors (34.3 ± 4.1% versus 3.2 ± 0.2% proliferating cells, *P* < 0.005; Fig. 2C, compare
Figure 1. Effects of ZD6474 on tumor growth, angiogenesis, and tumor cell proliferation and apoptosis of U87MG brain gliomas with or without EGFRvIII expression. A, left, histologic analysis. H&E staining was done on brain sections with the maximal length and width of the tumors. Arrows, tumors mass. Total magnification, 12.5×. Top right, immunoblot analysis of U87MG cell lysates for WT EGFR and EGFRvIII expression. U87MG/EGFRvIII expresses exogenous EGFRvIII and U87MG/EGFR expresses WT EGFR, whereas U87MG cells have minimal levels of WT EGFR. The anti-EGFR antibody recognizes both WT EGFR and EGFRvIII. WT, WT EGFR; vIII, EGFRvIII. β-Actin was used as a loading control. Right, bar graph, estimated average tumor volume in each group with five mice per group. B, immunohistochemistry of CD31 in brain sections with gliomas. Arrows, stained tumor vessels. Total magnification, 200×. Right, bar graph, vessel densities were analyzed as described in Materials and Methods from five randomly selected fields (total magnification, 200×) from intracranial tumors from three mice per group. C, Ki-67 staining of brain sections with the indicated tumor types. Arrows, Ki-67–positive nuclei; blue staining, Hoechst-stained nuclei. Right, bar graph, cell proliferation index was assessed by the percentage of Ki-67–positive cells against total cells stained with Hoechst from five randomly selected high-power fields (200×) in intracranial tumors with three mice per group. D, TUNEL staining of the sequential brain sections with various gliomas analyzed in A. Arrows, TUNEL-positive nuclei. Blue staining, Hoechst-stained nuclei. Right, bar graph, apoptotic index was assessed by the ratio of TUNEL-positive cells versus total Hoechst-stained cells from four randomly selected high-power fields (200×) in intracranial tumors with three mice per group. The ZD6474-treated tumors show significant reduction in tumor volume, vessel densities, cell proliferation, and higher cell apoptosis compared with the control groups. *, P < 0.05, determined by ANOVA. Data in A to D are representative from three independent experiments with similar results.
left with right, bar graph). When tumor cell apoptosis was evaluated by TUNEL staining, ZD6474-treated GBM14 tumors showed an ∼65% increase in cell death (0.125 ± 0.02% versus 0.36 ± 0.03% apoptotic cells, P < 0.05), whereas ZD6474-treated GBM8 tumors showed an ∼95% increase in cell apoptosis compared with that in their control tumors (0.06 ± 0.01% versus 1.12 ± 0.22% apoptotic cells, P < 0.005; Fig. 2D compare left with right, bar graph).
MRI analysis of GBM brain gliomas correlates with efficacy of ZD6474 treatment

MRI has been widely used to characterize brain tumor growth, progression, and response to various treatments in clinical and preclinical studies (27, 28). Among the various MRI techniques, diffusion MRI allows quantification of water movement within tumors by measuring the ADC (in units of \(10^{-3}\) mm\(^2\)/s, herein), which is negatively correlated with tumor cell density. A shift in ADC to higher values has been indicated as a quantifiable indicator for antitumor treatment efficacy (27, 29). We used this technique to characterize the responses of GBM8 brain tumors to ZD6474 treatment compared with vehicle-treated gliomas. As shown in Fig. 3 and Supplementary Tables S1 and S2, in a vehicle-treated control GBM8 tumor, the distribution of tumor ADC values are decreased by \(\sim 8\)% relative to normal contralateral brain. In contrast, in a ZD6474-treated GBM8 tumor, the distribution of ADC values is \(6\)% higher relative to normal brain in the ZD6474-treated mouse (Fig. 3A, c, d, e, and f). The corresponding ADC histograms indicate larger tumor volumes of control tumors with reduced ADC values (units of \(10^{-3}\) mm\(^2\)/s; Supplementary Table S1, 0.68 when fit with one-gauss function and 0.63 when fit with two-gauss functions) compared with smaller volume tumors with higher ADC values in ZD6474-treated GBM8 gliomas (0.76 when fit with one-gauss function and 0.74 when fit with two-gauss functions). These measurements are corroborated with H&E staining of the same brains (Fig. 3A, a and b). Interestingly, in the control untreated tumor, there were twice as many pixels in the ROI (indicated as red circles in a and b) in tumor side versus the contralateral side of the same brain (34% versus 18%; Supplementary Table S1) with the lower ADC of 0.63. In contrast, in ZD6474-treated tumor, the ROI values are similar (Supplementary Table S1, 74% versus 77%) with higher ADC values in a similar range (0.74 versus 0.70).

Additionally, we observed changes in T2*-weighed signal intensity that reflect alterations in the microscopic magnetic susceptibility gradients and can occur when there are changes in tissue structure at the microscopic level, including changes in cell density, necrotic regions, and/or regions containing accumulated products of hemorrhage, such as RBC, hemoglobin, and denatured proteins. This could provide an additional means of characterizing tumor progression and treatment efficacy. To this end, we found that vehicle-treated GBM8 tumor exhibits a decreased and broadened distribution of T2*-weighed signal intensities relative to tissue in the normal control contralateral side. The T2*-weighed signal from the entire untreated tumor volume is 10% less and 1.85-fold broader than the corresponding contralateral brain (Supplementary Table S2). It was possible to identify a volume of \(\sim 25\) mm\(^3\) from pixels of reduced T2*-weighed signal that seem to derive from tumor tissue (Fig. 3B, a, green circle); these regions coregistered well with tumor as indicated on the corresponding H&E-stained areas (Fig. 3A, a, green circle). In contrast, it was not possible to distinguish between tumor and normal brain in the treated mouse on the T2*-weighed images nor to coregister with the much smaller tumor ROI from the ZD6474-treated H&E-stained brain (small blue ROI on Fig. 3A, b). The T2*-weighed signal was the same at 0.13 (the same as contralateral signal in control brain), and the distribution of T2*-weighed signal intensities was only slightly more heterogeneous as indicated by the full width at half maximum (0.024 versus 0.02). Additionally, MRI analysis revealed a feature of necrosis in vehicle-treated GBM8 tumors (the small black ROI in Fig. 3A, c and B, a) and in ZD7674-treated tumor (yellow arrows in Fig. 3A, b). The ROI representing this necrotic feature was well registered on several different MR images and calculated maps including the T2*-weighed, T2 maps, T2*-weighed fast spin echo, T2*-weighed three-dimensional Turboflash, diffusion weighed, and ADC maps. This common feature, denoted within the small black circular ROI, corresponding to increased diffusion on the ADC map (Fig. 3A, c and B, a), higher T2 on the T2 map, and hyperintensity on the T2*-weighed fast spin echo image (data not shown). These signal properties are all consistent with regions of reduced cellularity, wherein water exists in a more uniform environment with minimal structure and macromolecular content. The hypointensity of this same ROI on the T2*-weighed three-dimensional images is consistent with a region of increased magnetic susceptibility gradients. It is interesting to note that the necrosis apparent in the control tumor has excellent concordance between ADC and T2*-weighed, but this is less apparent for necrosis in ZD6474-treated tumor, wherein the ADC map does not so clearly pick up the T2*-weighed hypointense area. This could possibly be due to localized, heterogeneously distributed products of hemorrhage in the tumor, such as RBC, hemoglobin, and others.

ZD6474 inhibits glioma cell proliferation and survival by suppressing phosphorylation of EGFRvIII and EGFRvIII activation of signal transducer and activator of transcription 3, Akt, extracellular signal-regulated kinase 1/2, and Bcl-X\(_L\)

EGFRvIII overexpression in gliomas promotes cell proliferation and survival by stimulating signal transducer and activator of transcription 3 (Stat3), Akt, and Bcl-X\(_L\) in vitro and in vivo (2, 6, 30). Thus, we first examined the effect of ZD6474 treatment on cell proliferation, survival, and EGFRvIII-stimulated signaling in U87MG and U87MG/EGFRvIII by WST-1 assays (25). As shown in Fig. 4A, treatment of glioma cells with 2.0 \(\mu\)mol/L ZD6474 effectively decreased cell growth and survival of U87MG/EGFRvIII compared with U87MG cells. At a concentration of 3.0 \(\mu\)mol/L, the inhibitory effect of ZD6474 on U87MG/EGFRvIII and parental U87MG cells showed a similar efficacy (Fig. 4A). When the molecular components of the EGFRvIII signaling pathway were
Figure 3. MRI analysis of the effect of ZD6474 on tumor growth of GBM8 tumors in the brain of mice. Nude mice with intracranial GBM8 (EGFRvIII) were treated daily by gavage with 0.1% Tween 80 (control) or ZD6474 (75 mg/kg/d) for 2 wk. The mice were subjected to MRI analysis and then euthanized. Brain sections were analyzed by immunohistochemistry. A, H&E and diffusion MRI analysis of the brain with GBM8 tumors. a, c, and e, control tumors; b, d, and f, ZD6474-treated tumors. a and b, H&E-stained brain sections of control (a) and ZD6474-treated tumors (b). Line circles with different colors corresponding with that in c and d. Circles in red, ROI that are in c and d and analyzed in e and f; circles in black, ROI that denotes necrotic areas in c and also seen in B, a. Yellow arrow in b denotes a necrotic region shown (also seen in B, b, c and d, ADC maps; e and f, histograms obtained using the same ROI. The red ROI was drawn from the tumor region as indicated on H&E-stained histologic image in a to d. The blue ROI was drawn from treated tumor region on H&E-stained sections. The green ROI was drawn from the largest in-plane region of hypointensity from the three-dimensional T2*w images. Histograms for both untreated and treated, in red and blue, respectively, from tumor and contralateral brain, in solid and dashed lines, respectively, are shown on the same graph for the ADC map shown in c and d. Vertical color bar next to d, a Lut bar using the “Fire Lut” with the images scaled between 0.3 and 1.3 (×10^−3 mm^2/s). B, a and b, three-dimensional T2*weighed (TE/TR = 10/40 ms) anatomic images of control (a) and ZD6474-treated (b) brains. A yellow arrow indicates the same necrotic region shown in A, a. The ROI-labeled ZD6474-treated tumor was drawn from the largest in-plane region of hypointensity from the three-dimensional T2*weighed images shown in a and b. The images shown are all registered to show the same slice from the middle region of tumor. The histograms were obtained from all of the adjacent slices where tumor was evident on MR images (primarily for the T2*weighed three-dimensional data sets). Data are representative in two independent experiments with similar results.
analyzed, we found that 2.0 μmol/L ZD6474 effectively inhibited tyrosine autophosphorylation of EGFRvIII in U87MG/EGFRvIII cells. Significantly, ZD6474 effectively suppressed EGFRvIII-stimulated protein phosphorylation of Stat3 and Akt and protein expression of Bcl-X\textsubscript{l} in U87MG/EGFRvIII glioma cells that retained EGFRvIII expression \textit{in vitro} and \textit{in vivo} (Fig. 4B), whereas minimal effect was found in parental U87MG glioma cells lacking EGFRvIII (Fig. 4B). In parallel, ZD6474 inhibited protein phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in both types of U87MG isogenic cells (Fig. 4B). We also determined whether ZD6474 has an effect on U87MG cells expressing high levels of WT EGFR (U87MG/EGFR; ref. 5). As shown in Fig. 4C, without EGF stimulation, treatment of U87MG/EGFR cells with 2.0 μmol/L ZD6474 has minimal effect on cell growth and survival, whereas moderate inhibition of EGF-stimulated U87MG/EGFR cells was seen when compared with U87MG/EGFRvIII cells. Consistently, 5 minutes after EGF stimulation, ZD6474 significantly inhibited tyrosine phosphorylation of EGFR and EGF-stimulated signaling mediators in U87MG/EGFR cells, whereas

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**Figure 4.** Analysis of effects of ZD6474 on cell proliferation, survival, and EGFRvIII-stimulated signaling mediators in various U87MG glioma cell lines. A and C, WST-1 assays of U87MG and U87MG/EGFRvIII (A) and U87MG, U87MG/EGFRvIII, and U87MG/EGFR (C) cells \textit{in vitro}. Serum-starved glioma cells were treated with ZD6474 at indicated concentrations (0–3.0 μmol/L) without (all types of cells) or with (U87MG/EGFR cells) EGF treatment for 48 h and analyzed using a WST-1 assay kit. A, maximum difference in inhibition of ZD6474 on U87MG/EGFRvIII cells was observed at 2.0 μmol/L compared with that in parental U87MG cells. The difference was diminished at 3.0 μmol/L. B, moderate inhibition of ZD6474 on EGF-treated U87MG/EGFR cells was observed compared with non-EGF treated U87MG/EGFRvIII cells. ZD6474 showed minimal effect on non-EGF stimulated U87MG/EGFR cells. B and D, immunoblot analysis of the effects of ZD6474 inhibition on EGFRvIII-induced (B) or EGFR-induced (D) signaling transducers in glioma cells. Serum-starved U87MG, U87MG/EGFRvIII, and U87MG/EGFR cells with or without EGF stimulation were treated with DMSO (−) or 2.0 μmol/L ZD6474 for 24 h (B) or at indicated times (D). Levels of expression of EGFRvIII, Stat3, ERK1/2, Akt, and β-actin were used as loading controls. EGFRvIII expression by U87MG cells and EGF stimulation of U87MG/EGFR cells induce phosphorylation of EGFRvIII, Stat3 (Tyr\textsuperscript{705}), Akt (Ser\textsuperscript{473}), and protein expression of Bcl-X\textsubscript{l} compared with controls. ZD6474 inhibits stimulation of these proteins in U87MG/EGFRvIII and EGF-treated U87MG/EGFR cells as well as phosphorylation of ERK1/2 (Thr\textsuperscript{202}/Tyr\textsuperscript{204}) in both types of cells. Bcl-2 expression was not altered in both types of cells (not shown). WT, WT EGFR; vIII, EGFRvIII. D, right, similar effects of ZD6474 on cell signaling were found in tumor lysates prepared from ZD6474-treated U87MG brain gliomas. Data in A to D are representative from three independent experiments with similar results.
EGF stimulation and ZD6474 inhibition of these mediators were markedly decreased in a 24-hour EGF treatment (Fig. 4D).

Next, we assessed the inhibition of ZD6474 of cell proliferation, survival, and EGFRvIII-stimulated signaling in GBM cells. As shown in Fig. 5A, treatment of GBM cells with 2.0 μmol/L ZD6474 effectively decreased cell growth and survival of GBM8 cells that express EGFRvIII when compared with GBM14 cells that lack EGFRvIII (23). Moreover, at a concentration of 3.0 μmol/L, preferential inhibition of cell growth and survival was still seen in GBM8 cells compared with that in GBM14 cells. Both in vitro and in vivo, ZD6474 effectively inhibited tyrosine autophosphorylation of EGFRvIII as well as protein phosphorylation of Stat3 and Akt and protein expression of Bcl-X<sub>l</sub> in GBM8 cells that retain EGFRvIII expression in vitro and in vivo compared with GBM14 cells lacking EGFRvIII (Fig. 5B). In parallel, ZD6474 inhibited protein phosphorylation of ERK1/2 in both GBM8 and GBM14 cells (Fig. 5B). Because GBM8 expresses both EGFR and EGFRvIII (Fig. 5B; ref. 23), we determine whether ZD6474 has an effect on GBM6 cells expressing high levels of EGFRvIII and low levels of WT EGFR and GBM12 cells expressing high levels of WT EGFR and low levels of EGFRvIII (23). As shown in Fig. 5C, treatment of GBM cells with 2.0 or 3.0 μmol/L ZD6474 effectively decreased cell growth and survival of GBM6 cells compared with GBM12 cells. Moreover, ZD6474 had similar inhibition on tyrosine autophosphorylation of EGFRvIII as well as EGFRvIII-stimulated signaling mediators except phosphorylated Akt in GBM6 and GBM12 cells (Fig. 5D).

A plausible explanation for the differences of ZD6474 inhibition on cell signaling in GBM8, GBM14 and GBM6, GBM12 cells is that GBM8 and GBM14 cells are PTEN deficient whereas GBM6 and GBM12 express WT PTEN that affects phosphoinositide 3-kinase–Akt activity (23).

Lastly, we determine whether expression of a constitutively active (CA) catalytic subunit of P38k (p110.CA), one of the downstream mediators of EGFRvIII signaling, could rescue ZD6474 inhibition of glioma cell growth and survival. As shown in Fig. 6A, exogenous expression of p110.CA by U87MG/EGFRvIII cells rendered the resistance to ZD6474 inhibition of Akt phosphorylation, whereas mock transfectant was sensitive to the inhibition. Expression of p110.CA by U87MG/EGFRvIII cells partially rescued ZD6474 growth inhibition compared with the controls (Fig. 6B).

Discussion

In this study, we determined the antitumor effects of ZD6474 (ZACTIMA, vandetanib), a dual TKI for VEGFR-2 and EGFR in two separate orthotopic human brain glioma models. We show that brain gliomas expressing EGFRvIII responded better to ZD6474 treatment than gliomas lacking EGFRvIII. ZD6474 significantly inhibits tumor growth, cell proliferation, and angiogenesis and induces cell apoptosis in EGFRvIII-expressing tumors when compared with EGFRvIII nonexpressing tumors. MRI analysis of short-term cultured primary GBM8 tumors using ADC and T2* weighed as measurements corroborates the inhibitory effects of ZD6474 assessed by immunohistochemical analysis, showing changes consistent with reduced cellularity and angiogenesis in ZD6474-treated tumors when compared with vehicle-treated tumors. In vitro, at 2.0 μmol/L concentration, ZD6474 provided greater suppression of cell proliferation and survival on EGFRvIII-expressing glioma cells. Mechanistically, ZD6474 inhibits autotyrosine phosphorylation of EGFR and EGFRvIII, suppresses EGFR-dependent and EGFRvIII-dependent phosphorylation of Stat3, Akt, and ERK1/2, and reduces expression of Bcl-X<sub>l</sub> in EGFRvIII-expressing glioma cells. Additionally, expression of a constitutively active PI3K (p110.CA) by U87MG/EGFRvIII cells partially rescued ZD6474 inhibition. These data support our hypothesis that EGFRvIII expression by gliomas confers sensitivities to the dual TKI ZD6474.

The dual TKI ZD6474 for VEGFR and EGFR has been shown to inhibit growth of various types of human tumor xenografts in mice (10, 11, 31, 32). In these studies, although ZD6474 had effects on inhibition of tumor growth in animals, the response of ZD6474-treated tumors was not linked to genetic alterations, such as EGFRvIII, which frequently occur in clinical gliomas. One recent study showed that treatment of intracranial U87MG tumors without EGFRvIII with 50 and 100 mg/kg/d ZD6474 had a minimal effect of brain glioma growth in mice (33). In another report, growth kinetics of D456MG glioma that overexpress EGFRvIII in the flanks of mice that were treated with 200 mg/kg/d ZD6474 was similar to other glioma xenografts (12). Our results corroborate and also differ from these studies. We found that treatment of 75 mg/kg/d ZD6474 on brain glioma-bearing mice had a moderate effect of inhibition on tumor growth, cell proliferation, survival, and tumor angiogenesis of intracranial parental U87MG or short-term cultured primary GBM14 tumors that lack EGFRvIII. In contrast, administration of ZD6474 to mice with intracranial U87MG/EGFRvIII or GBM8 tumors that overexpress EGFRvIII significantly inhibited tumor growth, angiogenesis, and cell survival in the brain. This exciting observation prompted us to perform survival studies of ZD6474-treated brain glioma-bearing animals. However, we observed an unexpected high toxicity in mice with fast-growing U87MG and GBM tumors 2 weeks after they were treated daily with ZD6474 in 1% Tween 80 or vehicle alone. This prevented us from performing survival studies of these animals using a Kaplan-Meier curve analysis. Moreover, to a similar extent, effect of animal survival was not described in several previous studies of ZD6474 inhibition of brain glioma-bearing mice (12, 32–34), whereas treatments with similar dosages of ZD6474 had moderate effects on animal survival of subcutaneous tumor-bearing mice of various types of cancer xenografts...
This warrants a further investigation of ZD6474 inhibition of EGFRvIII-expressing brain gliomas in mice by combination of ZD6474 with other drugs or modality, such as radiation with lower dose of ZD6474 (2, 35–38). Together, our data are highly significant, as this is the first study showing that a dual inhibitor for EGFR and VEGFR2, ZD6474, significantly inhibits orthotopic human gliomas that overexpress a common EGFRvIII mutant. Distinct responses to ZD6474 by primary GBM8 gliomas in the brain is highly relevant to the clinic, because these GBM cells retain overexpression of endogenous EGFRvIII and EGFR and are highly malignant with rapid growth and insidious invasion in the brain (23).

MRI and spectroscopy have been used as a common modality to examine histology and pathology of tumor progression and the acute and chronic responses of various treatments in brain gliomas, both clinically (27, 39–41) and preclinically (29, 33, 42–44). In these studies, diffusion MRI using ADC values, which measures restrictions to water movement due primarily to increased cellularity.
in tumors, was used as a potential imaging marker to determine the correlation to tumor histology and response of antiglioma treatments in the brain. In a genetically engineered mouse model of gliomas, during the course of tumor progression, a significant progressive increase of ADC values for low-grade gliomas was found whereas a constant ADC measurement for high-grade gliomas was seen. When mice bearing these high-grade gliomas were treated with temozolomide, a significant increase in ADC was observed in the treated group, indicating increase in the extent of apoptosis when compared with the vehicle-treated group. The results of our ADC measurements for control and ZD6474-treated GBM8 gliomas corroborate with these studies. In control groups, tumor ROIs had twice as many pixels with lower ADC values compared with the contralateral ROI. In ZD6474-treated tumors, there were essentially no pixels with lower ADC values in tumor compared with contralateral brain ROIs. In fact, most of the pixels in the tumor ROI had markedly higher ADC values compared with the contralateral ROI. There are regions of high and normal cellularity and vascular density in the ZD6474-treated GBM8 tumors, indicating that the tumor was heterogeneous. Additionally, our T2 and T1 weighted MRI as well as ADC maps in control GBM reveal necrosis in the fast-growing GBM brain tumors. These signal properties are consistent with a region of reduced cellularity, wherein water exists in a more uniform environment with minimal structure, cell membranes, and macromolecular content. The data of MRI imaging strongly support the further evaluation of these MRI techniques as a noninvasive means to examine the vascular and cellular features in fast-growing malignant GBM brain gliomas and evaluate the responses of antiglioma treatments.

In addition to suppression of tumor angiogenesis, ZD6474 also inhibits EGF-stimulated phosphorylation of EGFR, Akt, and ERK1/2 in cancer cells (12, 13, 16). EGFRvIII is a constitutively active EGFR mutant that persistently activates the PI3K-Akt signaling compared with the WT EGFR (45, 46). Stat3 is activated in primary gliomas and is required for astrocyte transformation when EGFRvIII is expressed (47–49). We observed that ZD6474 significantly inhibits phosphorylation of EGFRvIII, Stat3, and Akt and downregulates Bcl-XL in EGFRvIII-expressing U87MG/EGFRvIII (6) and GBM8 cells in vitro and in vivo compared with parental U87MG or GBM14 cells that lack EGFRvIII expression. Interestingly, 2.0 μmol/L ZD6474 showed better inhibition of cell proliferation and survival of U87MG/EGFRvIII compared with parental cells. At the concentration of 3.0 μmol/L, a similar effect was seen in both types of cells. This effect could be explained by a similar inhibition of ERK1/2 phosphorylation in these isogenic cells. In contrast, at concentrations of 2.0 and 3.0 μmol/L, ZD6474 showed better inhibition of phosphorylation of ERK1/2 and growth and survival of GBM8 cells with EGFRvIII and EGFR compared with GBM14 cells with undetectable EGFRs. Additionally, at concentration of 2.0 μmol/L, although ZD6474 inhibits phosphorylation of EGFR, Akt, Stat3, ERK1/2, and Bcl-XL expression in EGF-stimulated U87MG/EGFR or nonstimulated GBM12 cells that only overexpress WT EGFR, lesser inhibition of cell growth and survival was seen compared with U87MG/EGFRvIII and GBM6 cells that only express EGFRvIII. In EGF-stimulated U87MG/EGFR cells, ZD6474 inhibition of these mediators was significant in 5 minutes, whereas both EGF stimulation and ZD6474 inhibition were decreased in 24 hours. Without EGF stimulation, ZD6474 had a minimal effect on cell growth and survival of these cells. The differences of ZD6474 inhibition of EGFRvIII signaling and basal WT EGFR signaling could be attributed to in vivo observations that EGFRvIII robustly promotes U87MG glioma growth whereas WT EGFR overexpression
showed lesser enhancement in tumor growth in the brain (50). Lastly, exogenous expression of a CA PI3K by U87MG/EGFRvIII cells only partially rescued ZD6474 inhibition, reinforcing the thesis that activation of Stat3 and ERK1/2 are also important in EGFRvIII-promoted cell growth and survival. Taken together, ZD6474 displays a better inhibition of EGFRvIII-activated cellular survival gene pathway signaling, resulting in suppression of glioma cell growth and survival in vitro and in the brain.

In summary, the key finding of this study is that a dual TKI ZD6474 inhibits orthotopic human gliomas expressing EGFRvIII in the brain. Treatment of brain tumor-bearing mice with ZD6474 suppressed tumor cell proliferation and angiogenesis while inducing cell apoptosis by attenuating EGFRVIII-activated cellular signaling in EGFRvIII-expressing gliomas compared with gliomas without EGFRvIII. It is important to note that this treatment effect was more pronounced in a clinically relevant GBM model. Our data also provide evidence that ADC and T2*-weighed MRI analyses are reliable methods to assess the effect of this dual TKI on inhibition of brain gliomas. This is the first study showing the clinical efficacy of ZD6474 to the inhibition of EGFRVIII-expressing glioma in the brain. Our study shows that the dependence of malignant growth of gliomas in the brain on constitutively activated EGFRVIII may render tumors susceptible to the dual TKI ZD6474 inhibition of VEGFR2 and EGFR, suggesting a potential application for this potent TKI in clinical treatments for selected patients with gliomas harboring an activating EGFRvIII mutation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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ZD6474, a Multitargeted Inhibitor for Receptor Tyrosine Kinases, Suppresses Growth of Gliomas Expressing an Epidermal Growth Factor Receptor Mutant, EGFRvIII, in the Brain

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