Perifosine Inhibits Multiple Signaling Pathways in Glial Progenitors and Cooperates With Temozolomide to Arrest Cell Proliferation in Gliomas In vivo

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Abstract

Perifosine is an oral Akt inhibitor which exerts a marked cytotoxic effect on human tumor cell lines, and is currently being tested in several phase II trials for treatment of major human cancers. However, the efficacy of perifosine in human gliomas has not been established. As Akt is activated in ~70% of human glioblastomas, we investigated the impact of perifosine on glia in culture and on a mouse glioma model in vivo. Here we show that perifosine strongly reduces phosphorylation levels of Akt and extracellular signal-regulated kinase (Erk) 1/2, induces cell cycle arrest in G1 and G2, and causes dose-dependent growth inhibition of mouse glial progenitors in which Akt and/or Ras-Erk 1/2 pathways are activated. Furthermore, because temozolomide is a common oral alkylating agent used in the treatment of gliomas, we investigated the effect of perifosine in combination with temozolomide. We observed an enhanced effect when both were used in culture. With these results, we combined perifosine and temozolomide as treatment of platelet-derived growth factor (PDGF)-driven gliomas in mice. Animal studies showed that perifosine and temozolomide combination therapy was more effective than temozolomide treatment alone (P < 0.01). These results indicate that perifosine is an effective drug in gliomas in which Akt and Ras-Erk 1/2 pathways are frequently activated, and may be a new candidate for glioma treatment in the clinic. (Cancer Res 2005; 65(16): 7429-35)

Introduction

Gliomas are the most common primary brain tumors (1). The most aggressive subtypes, glioblastoma multiforme, makes up >50% of newly diagnosed gliomas. Despite aggressive therapy with surgery, radiation, and chemotherapy, prognosis for patients with glioblastoma multiforme is poor with a median survival of ~1 year (2). Many newer forms of treatment, including immunotherapy and gene therapy, have been tested in glioblastoma multiforme, but these have not been efficacious. Presently, the best chemotherapeutic drugs for glioblastoma multiforme are alkylating agents such as carmustine and temozolomide (2, 3).

The alkylphospholipids compose a novel class of antitumor agents with structural similarity to platelet-activating factors. Unlike most chemotherapeutic drugs which target the nuclear DNA, alkylphospholipid interacts with the cell membrane and blocks signal transduction pathways (4). Perifosine is the first oral alkylphospholipid that exerts a marked cytotoxic effect in a number of human tumor cell lines and has shown few side effects in clinical phase I trials (5, 6).

Recent studies have suggested the molecular mechanism of perifosine action and the capacity to synergize with radiation or other anticancer drugs. Perifosine interferes with recruitment of Akt to the plasma membrane and inhibits Akt phosphorylation and activation (4). This drug also causes inhibition of extracellular signal-regulated kinase (Erk) 1/2 and activation of c-Jun NH2-terminal kinase (JNK) and p21, resulting in cell cycle arrest in G1 and G2 (7–9). However, the details of the mechanisms of action remain unclear, and this agent has not been studied fully in glioma cells. The Ras-Erk 1/2 signal transduction cascade is activated in nearly all cases of glioblastoma multiforme (10, 11) and Akt is activated in ~70% (12–15). Moreover, evidence from our laboratory suggests that forced combined Ras and Akt activation in glial progenitors is sufficient to induce glioblastoma multiforme in mice (14). Therefore, we hypothesized that the blockade of Akt and Ras-Erk 1/2 pathways by perifosine would have anti-glioblastoma multiforme effects.

To develop a new paradigm for glioma therapy, we used mouse glial progenitors in vitro, in which Akt and/or Ras-Erk 1/2 pathways are activated, and mouse glioma model in vivo. In this study, we used our established mouse models of gliomas and showed the dose-dependent growth inhibitory effects of perifosine in vitro. Perifosine blocked both Akt and Ras-Erk 1/2 signaling pathways and induced cell cycle arrest in G1 and G2. We show that perifosine enhances the effect of temozolomide, and does so better than mTOR inhibitor alone. Finally, we show that perifosine and temozolomide have additive effects on cell cycle of platelet-derived growth factor (PDGF)-driven gliomas in vivo.

Materials and Methods

DNA constructs and infection of primary brain cell cultures. The replication-competent ALV splice acceptor (RCAS)/tv-a system used in this work have been described previously (14). RCAS-LacZ plasmid was a gift from Yi Li (Baylor College of Medicine, Houston, TX). RCAS-K-Ras, which carries the gene encoding the G12D point mutant-activated K-Ras, was kindly provided by Galen Fisher (New York, NY). RCAS-Akt/HA, which carries the activated form of Akt designated Akt-Myr D11-60 and has a HA tag sequence added to the 3′-end of the cDNA, was a gift from Peter Vogt (Scripps Research Institute, La Jolla, CA). RCAS-PDGFB carries the entire coding sequence of PDGF-B chain. Transfection of RCAS constructs into chicken DF-1 fibroblasts and infection of primary glial progenitors from Ntv-a transgenic mice by the various RCAS virions were done as described previously (16). Ntv-a mice that express the TVA receptor from the nestin promoter have been published (17). The mice are a mixed genetic background of C57BL6, 129, Balb/C, and FVB/N.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-05-1042

"Research Article"
Cell culture. Glial progenitors transformed with RCAS-LacZ (Nh-a-LacZ), RCAS-K-Ras (Nh-a-Ras), RCAS-Akt/HA (Nh-a-Akt), RCAS-K-Ras + Akt/HA (Nh-a-Ras + Akt), and RCAS-PDG-F-B (Nh-a-PDG-F) were cultured in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 µg streptomycin. Human glioma cell lines, T98G and U87MG, were obtained from American Type Culture Collection (Manassas, VA) and were cultured in the same medium. All cells were cultured in a humidified atmosphere at 37°C and 5% CO2.

Drugs. Perifosine was provided by Keryx Biopharmaceuticals (New York, NY). Temozolomide was from Schering-Plough (Kenilworth, NJ), Rapamycin was from Wyeth (Philadelphia, PA). Perifosine stock solutions were prepared by dissolving perifosine in PBS for cell culture, and in 0.9% NaCl solution for mouse treatment both at 25 mmol/L concentration. Temozolomide stock solutions were prepared by dissolving temozolomide in DMSO at 100 mmol/L concentration for cell culture, and at 40 mg/ml concentration for mouse treatment. Temozolomide solution for mice was diluted in saline (5 mg/ml) and administered. Rapamycin stock solutions were prepared by dissolving in ethanol at 100 µmol/L concentration.

Western blot analysis. Whole-cell protein extracts were prepared by cold lysis of cell pellets. M-Per (Pierce, Rockford, IL) lysis buffer was supplemented with 30 mmol/L sodium fluoride, 1 mmol/L sodium vanadate, 0.5 mmol/L phenylmethylsulfonyl fluoride, 100 mmol/L NaCl, 1 mmol/L EDTA, and protease inhibitor cocktail tablets (Roche, Indianapolis, IN). Protein concentrations were determined by bicinchoninic acid assay (BCA) method (Bio-Rad, Hercules, CA). Samples (100 µg) were separated by 10% PAGE gel, and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk in PBS-0.1% Tween 20. Primary and secondary antibodies were diluted in the same solution. Signal was visualized using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Primary antibodies against Akt (#9272), phospho-Akt (P-Akt; Ser473; #9271), enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Primary antibodies against Akt (#9272), phospho-Akt (P-Akt; Ser473; #9271), S6 kinase (S6K; #9202), P-S6K (Thr389; #9205), 4E-BP1 (#9452), P-4E-BP1 (Ser65; #9451), Erk 1/2 (Ser98; #9212), P-Erk 1/2 (Ser217/221; #9101), P-JNK (Thr183/Tyr185; #9251), P-p38 (Thr180/Tyr182; #9211), and P-eIF4E (Ser209; #9741; Cell Biosciences) and anti-mouse antibody (Roche) were used at 1:1,000 dilution. Secondary peroxidase-conjugated anti-rabbit antibody (Amersham Biosciences) and anti-mouse antibody (Roche) were used at 1:1,000 dilution.

Platelet-derived growth factor-BB ELISA. Whole-cell protein was extracted using the same lysis buffer as above, and 100 µg of cell lysate was used for ELISA. PDGF-BB ELISA was done using a Quantikine kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The absorbance at 590 nm was recorded and the absorbance at 540 nm was subtracted from the readings at 590 nm using the 96-well plate reader (Thermo Electron Corporation, Vantaa, Finland).

Cell growth assay. Four thousand cells (for 10% serum) or 5,000 cells (for 1% serum) per well were plated in 96-well culture plates with 100 µL of culture medium. After applying drugs, cells were cultured for 2 days. Viable cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cell Proliferation Kit I; Roche). The absorbance at 590 nm was recorded and the absorbance at 540 nm was subtracted from the readings at 590 nm using the 96-well plate reader (Thermo Electron Corporation).

Cdk kinase assay. After treatment with perifosine, the cells were washed twice with cold PBS and lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 250 mmol/L NaCl, 5 mmol/L EDTA, 0.5% NP-40, protease inhibitor cocktail tablets (Roche), 10 µg/ml soyanb bean trypsin inhibitor, and 2 mmol/L phenylmethylsulfonyl fluoride]. The cell lysates were sonicated twice for 30 seconds, incubated on ice for 30 minutes, and supernatants were recovered by centrifuging at 14,000 rpm at 4°C for 10 minutes. Protein concentrations were determined by BCA method (Bio-Rad). Proteins (200 µg) were immunoprecipitated using specific antibodies against Cdk1 and Cdc2 (Santa Cruz Biotechnology), and collected with protein A-Sepharose slurry (Repligen, Waltham, MA) at 4°C with agitation. The immunoprecipitate was washed thrice with the lysis buffer. The Cdk kinase reactions in the immunoprecipitate were done in a kinase reaction buffer [20 mmol/L Tris-HCl (pH 7.4), 75 mmol/L MgCl2, 1 mmol/L dithiothreitol] containing 30 µmol/L ATP [γ-32P]ATP (3,000 Ci/mmol; Amersham Biosciences), and 40 µg histone H1 (Roche) at 37°C for 30 minutes. Phosphorylated histone H1 was resolved on 12% SDS-PAGE gels followed by autoradiography.

Cell cycle analysis. Cells treated with perifosine were trypsinized and washed in PBS at each time point. Aliquots of 1 × 105 cells were fixed with 70% methanol at 4°C for at least 12 hours before centrifugation. The cell pellets were treated with a solution containing 50 µg/ml propidium iodide (Sigma, St. Louis, MO) and 2 mg/ml RNase A (Roche) for 30 minutes at room temperature. Stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and evaluated with CellQuest and FlowJo software.

Giemsa staining of cultured cells. The cells were on four-well glass slides (Nagle Nunc International, Naperville, IL) and treated with perifosine. After 24 hours of incubation, medium was aspirated from the chamber. The cells were washed with PBS (pH 7.4), and fixed in methanol at room temperature for 10 minutes. Then, 75 µL of Giemsa stain (LabChem, Inc., Pittsburgh, PA) was diluted in 1 ml of water, and applied for 5 minutes. The stain was discarded and the cells were washed with water and visualized under a light microscope. The percentage of multinuclear cells was semiquantitatively analyzed by counting cells in three random fields, using a light microscope at 200× magnification.

Generation of tumor-bearing mice. DF1 cells transfected with RCAS-PDG-F and double-transgenic EF-luc Ntv-a mice have been described previously (18). Neonatal EF-luc Ntv-a mice received intracranial injections of ∼104 DF1 cells producing the RCAS-PDG-F retrovirus. Mice were then routinely screened with bioluminescence imaging and image-positive mice were followed over time, treated, and sacrificed.

Drug treatment of tumor-bearing mice. Image-positive EF-luc Ntv-a mice were treated daily with i.p. administration of buffer alone as a control, or i.p. administration of 100 mg/kg temozolomide, or oral administration of 30 mg/kg perifosine, or a combination with perifosine and temozolomide for 3 to 5 days. The mean doses of the treatments were: Control, 5 (all five); temozolomide, 3.75 (three to five); perifosine, 3.75 (three to four); and perifosine + temozolomide, 3 (all three). Control buffer solution consisted of 5% DMSO and 1% Tween 80 in distilled water.

Brain sectioning, H&E staining, and immunohistochemistry. These procedures were done as described previously (16). Primary antibody against Ki-67 (VP-K451; Vector, Burlingame, CA) was used at 1:500 dilution overnight. Secondary antibody was rabbit anti-mouse IgG (Cell Signaling, Beverly, MA) used at 1:1,000 dilution, and anti-Actin (I-19; Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:500 dilution. Secondary peroxidase-conjugated anti-rabbit antibody (Amersham Biosciences) and anti-mouse antibody (Roche) were used at 1:1,000 dilution.

Statistical analysis. Comparison between two groups was made using Student’s t test. Data represent the mean ± SD. P values of <0.05 were considered statistically significant.
Perifosine inhibits Akt and Ras signaling pathways in glial cells. We used Western blot analysis to determine the effect of perifosine on signaling pathways in our modified glial progenitors. We specifically investigated the PDGF-driven cells as Akt is wild-type and they have relatively low Akt activity, the RCAS-Akt infected cells as these have a myristoylated and constitutively active Akt that is highly phosphorylated, and the RCAS-LacZ infected cells as a control. Each cell type showed a unique response to perifosine over time. The Ntv-a/LacZ cells showed minimal effect of perifosine on Akt or Erk 1/2 pathways but did show some effect on 4E-BP1 phosphorylation. The effect on Erk 1/2 and 4E-BP1 was similar and found to be ~45 μmol/L (Fig. 2A). Ntv-a/LacZ control cells were more resistant to perifosine than the other cell lines at this serum concentration (P = 0.0006 at 50 μmol/L). The effect of perifosine was enhanced markedly in all cell lines when the FCS concentration in the medium was reduced to 1% (Fig. 2B). Interestingly, although the control cells were the most resistant to perifosine at high serum concentrations, the PDGF autocrine-driven cells were the most resistant to perifosine at low serum concentrations (P = 0.0045 at 5 μmol/L, P < 0.0001 at 10 μmol/L), implying that PDGF signaling could provide protection from serum deprivation in the presence of perifosine. In the following experiments in this study, we used 10% FCS to assess the molecular mechanisms of action of perifosine.

Perifosine inhibits Cdk2 and Cdc2 kinase activities and induces G1 and G2 arrest. The reduction of cell numbers seen in Fig. 2 implied that perifosine might be affecting cell proliferation. To investigate the impact of perifosine on cell cycle, we did Cdk2 and Cdc2 kinase assays. Cdk2 kinase activity was strongly inhibited in Ntv-a/PDGF cells after treatment with 45 μmol/L perifosine for 24 hours, whereas the effect on Cdc2 was milder (Fig. 4A). By contrast, the effects of perifosine on the Cdk2 in either cell type were not observed after 6 hours of treatment. As illustrated in Fig. 3B, at 6 hours, there is a strong effect on Akt, S6, 4E-BP1, and Erk 1/2 phosphorylation at later time points (Fig. 3A). The Ntv-a/PDGF cells showed a strong block in Akt and S6K phosphorylation by 3 hours with a decrease in 4E-BP1 and Erk 1/2 phosphorylation by 6 hours (Fig. 3B). The cells expressing the constitutively active Akt showed blockade of Akt phosphorylation by 3 hours but a delayed effect of S6K phosphorylation. The effect on Erk 1/2 and 4E-BP1 was similar to the Ntv-a/PDGF cells (Fig. 3C). Phospho-JNK and phospho-p38 levels did not change substantially or were below detection levels, and phospho-Elf4E levels were also did not change significantly at this drug concentration (data not shown). As Akt is activated but not mutated in human gliomas, we chose to investigate the effects of perifosine on glia using the PDGF-driven cells.
predominantly in G1 (At lower perifosine concentrations (25-50 μmol/L), Ntv-a/PDGF cells arrested predominantly in G2 (75-100 μmol/L), Ntv-a/PDGF cells arrested predominantly in G2 (P = 0.0006 at 75 and 100 μmol/L, respectively). The G2 arrest seen at high perifosine doses was also observed in the other progenitors as well (Supplementary Fig. S3). Cells with 4 N or greater DNA content might be either G2 arrested or multinucleated. Therefore, we stained the treated cells with Giemsa and calculated the percentage of multinuclear cells. Only ~6% of all treated cells were found to be multinuclear; thus, the majority of the 4 N cells were arrested in G2.

**Relative effects of mTOR inhibition and perifosine-mediated Akt inhibition.** One effect of Akt blockade is a reduction of mTOR activity, however, perifosine has several other effects than simply mTOR inhibition such as affecting non-mTOR pathways downstream of Akt as well as mitogen-activated protein kinase. Several mTOR inhibitors are available (rapamycin) or in clinical trials. We reasoned that given the central role of Akt activity in gliomas, blockade of the Akt pathway would ultimately be part of a combination of drugs used to treat these tumors. Therefore, we compared the effects of rapamycin and perifosine as single agents and in combination with the cytotoxic chemotherapy currently used for glioma temozolomide on our cell cultures. First, we determined doses of rapamycin and perifosine that equally inhibited phosphorylation of S6K. Western blot analysis of cells treated with various concentrations of the drug doses showed that 0.1 nmol/L of rapamycin and 45 μmol/L of perifosine achieved equal blockade of S6K phosphorylation (Fig. 5A). We noted that at these doses, rapamycin showed a slight increase in P-Akt levels consistent with feedback effects, as has been reported previously (20, 21), and did not see inhibition of Erk 1/2 or 4E-BP1 phosphorylation. This limited effect of rapamycin contrasts with the effect of perifosine on P-Akt, P-S6K, and P-4E-BP1, and P-Erk 1/2 as noted earlier in this manuscript.

We then investigated the effects of these drugs and combinations on our cells in culture. We found that as single agents, perifosine had a greater effect on Ntv-a/PDGF cells (Supplementary Fig. S4; P < 0.0001) but had minimal effects on the Ntv-a/LacZ cells (P = 0.1034). Finally, whereas rapamycin had a minimal effect on cells treated with temozolomide (P = 0.0252), perifosine substantially enhanced the inhibitory effects of temozolomide in Ntv-a/PDGF cells (P < 0.0001). Therefore, perifosine results in blockade of several components of signal transduction and seems to cooperate with temozolomide better than inhibition of mTOR alone with rapamycin.

We then did flow analysis of Ntv-a/PDGF cells with 300 μmol/L temozolomide and 45 μmol/L perifosine alone and in combination to better define the effects of the combination of these two drugs over time. The analysis indicated that at these doses, the combination of these two drugs achieved a more complete arrest of the cell cycle than temozolomide alone (Fig. 5B, P < 0.05; for further statistical analysis, see Supplementary Fig. S5). Finally, we determined the schedule of temozolomide and perifosine treatment that had the greatest effect on cells in culture. When we used temozolomide and perifosine sequentially, the growth-inhibitory effect was more pronounced when temozolomide was used first followed by perifosine than the reverse (Supplementary Fig. S6; P < 0.005). We found that simultaneous treatment with the two drugs gave the most potent effect in these cells (P < 0.005, compared with temozolomide followed by perifosine treatment).

**Combination therapy of perifosine and temozolomide reduces tumor proliferation in vivo.** The above data indicates that perifosine results in blockade of several signaling pathways and achieves a cell cycle arrest in culture that significantly enhances the effect of temozolomide in culture. Given the enhanced effect of perifosine over rapamycin in combination with temozolomide, we chose to test temozolomide and perifosine as single agents and in combination against a PDGF-driven glioma model in vivo. We identified mice with tumors by bioluminescence imaging and either treated them with 100 mg/kg temozolomide, or 30 mg/kg perifosine,
or a combination with 100 mg/kg temozolomide and 30 mg/kg perifosine (temozolomide + perifosine) for 3 to 5 days. The mice were sacrificed and tumors analyzed histologically for cell proliferation by Ki-67 immunostaining. First, we determined the baseline variability in untreated tumors of this model and found that Ki-67 staining index of tumor cells in control mice (Control) was 10.7 ± 2.0% (n = 4).

We then measured Ki-67 staining index in the mice treated with perifosine, temozolomide, and temozolomide + perifosine, and found that Ki-67 staining index was significantly reduced in mice treated with either temozolomide (Ki-67 staining index = 5.5 ± 1.2%, n = 4, P = 0.0019) or perifosine (Ki-67 staining index = 3.2 ± 1.1%, n = 3, P = 0.0010) compared with Control, demonstrating the inhibitory effect on proliferation (Fig. 6A and B). Most importantly, the tumors treated with temozolomide + perifosine had the lowest Ki-67 staining index (1.7 ± 1.2%, n = 3, P = 0.0005). The additional treatment with perifosine resulted in a significantly lower proliferation rate than temozolomide alone (P = 0.0087). In spite of the reduced proliferation rate in these tumors, the bioluminescence

Figure 3. Perifosine (PRF) inhibits Ras and Akt pathways. Cells were treated with 45 μmol/L perifosine or buffer as a control for indicated periods of time, and analyzed by Western blot. Control cells were also treated for 1 to 12 hours, and confirmed that the expression levels were not changed in all cells. In this figure, only the results of 3 hours in control cells are shown as a control. Total levels of each protein were determined after stripping the same membrane. Actin was used as loading control. Although perifosine effect was minimal in Ntva/LacZ (LacZ) cells (A), perifosine strongly inhibited the Akt pathway and Erk 1/2 phosphorylation in Ntva/PDGF (PDGF) cells and Ntva/Akt (Akt) cells (B and C).

Figure 4. Perifosine (PRF) inhibits cell cycle progression. A, H1 kinase assays in Ntva/LacZ (LacZ) and Ntva/PDGF (PDGF) cells. Cells were treated with 45 μmol/L perifosine or buffer alone for 24 hours, and immunoprecipitated with anti-Cdk2 or Cdc2 antibody. Histone H1 was used as a substrate to measure Cdk2 and Cdc2 kinase activity. Western blots for actin were done in parallel on the same immunoprecipitates to confirm equal protein loading. Cdk2 and Cdc2 kinase activities were inhibited by perifosine in PDGF cells at 24 hours. B, cell cycle analysis in LacZ and PDGF cells in one representative experiment. Cells were treated with perifosine at indicated concentrations for 24 hours and stained with propidium iodide. DNA content was determined by flow cytometry. Perifosine induced cell cycle arrest in G1 and G2. PDGF cells were treated with the same doses of perifosine, stained with Giemsa, and calculated the percentage of multinuclear cells (MNC).
imaging of the mice after perifosine treatment was quite variable (data not shown). The reason for the variability is not clear but may reflect the activity of the E2F1 promoter in the G2 phase of the cell cycle and variable G1 and G2 blockade in the tumors.

Discussion

We show growth inhibitory effects of perifosine and its molecular mechanism through signal transduction and cell cycle progression in glial progenitors in vitro. Our work shows that perifosine blocks Akt and Ras-Erk 1/2 signaling in mouse glial progenitors. These cell lines are engineered to have activated Ras and Akt signaling pathways, which are the pathways most commonly activated in human malignant gliomas. Therefore, the growth-inhibitory effects of perifosine are more significant in these cells than in control cells which are infected with LacZ gene. We have also confirmed that perifosine inhibits Cdk2 and Cdc2 activities in our cells, and blocks cell cycle progression in G1 and G2 as reported in squamous carcinoma cells (9). These inhibitory effects on Cdk2 and Cdc2 were not observed after 6 hours of treatment, and we suspect that it is a secondary effect on the signaling pathways. Interestingly, perifosine effect was dependent on serum concentration. Therefore, we think that the inhibiting effect of perifosine may be compensated for by growth factors contained in the serum.

Different cell and cancer types have characteristic signal transduction pathways and might be expected to show differing responses to small molecule inhibitors of specific signaling components. In this light, we have found some characteristics of glial progenitor cell responses to perifosine that are either unique or at least different from that found in other cell types. For example, the IC50 for perifosine in our mouse glial progenitor cell lines (45 μmol/L) is substantially higher than those reported before such as glioma (IC50 = 3.1 μmol/L), or other cancer cell lines (0.2-19.9 μmol/L) including melanoma, lung, colon, squamous cell, breast, and prostate cancer (4, 9, 22). Second, in glial progenitors expressing constitutively active Akt, perifosine treatment leads to reduction in P-Akt and a slower but nonetheless substantial effect on P-S6K. This finding differs from results reported with PC3 prostate carcinoma cells which have myristoylated form of Akt and are resistant to perifosine action (4). This differential response to perifosine might be caused by several factors including different perifosine concentrations used (45 versus 5 μmol/L), different length of treatment (3 hours versus 30 minutes), and different cell types. Third, the JNK pathway is not significantly up-regulated in glial progenitor cells unlike what is seen in human leukemia cells (7). Finally, p21 expression does not increase significantly in glial cells treated with perifosine and phosphorylation of Erk 1/2 is suppressed in our cells at the IC50 in contrast to the human leukemia cell lines (23).

To evaluate the potential of perifosine for combination chemotherapy, we chose to examine temozolomide, which is a common drug for glioma therapy. We found that the addition of perifosine to temozolomide enhanced cell cycle arrest and growth inhibition. Because perifosine is known to enhance radiation-induced apoptosis in human leukemia cell lines (7), and both radiation and temozolomide cause DNA damage, perifosine may enhance the effects of temozolomide. We showed the combination effect of temozolomide and perifosine in vivo by immunohistochemistry where the results of Ki-67 staining in tumor cells support the cell culture results. Although the combination of perifosine and temozolomide only trended toward being more effective than perifosine monotherapy, it is possible that this finding would become statistically significant if more animals were used.

We previously showed that mTOR blockade results in inhibition of proliferation in PDGF-induced mouse gliomas (18). As we show here in the same tumors, blockade of the Akt signaling pathway can enhance temozolomide effect, and Akt blockade is more effective than mTOR blockade when combined with temozolomide. Furthermore, in sequential treatment with temozolomide and perifosine, we found the growth-inhibitory effect is more pronounced when temozolomide is administered before perifosine rather than after. These results may help guide schedules of drug combination when temozolomide is used with other drugs that inhibit Ras and Akt signaling pathways. Temozolomide has become the first choice of chemotherapy for gliomas because of its efficacy, convenience, and tolerability (3, 24, 25). Moreover, recent studies suggest that temozolomide may enhance the effectiveness of other chemotherapeutic agents (26, 27). However, impact of temozolomide on long-term disease control is limited and new drugs are needed.
In conclusion, perifosine blocks Akt and Ras-Erk 1/2 pathways in glial progenitors in vitro, and inhibits glioma growth in vivo. The perifosine effect is enhanced when it is used in combination with temozolomide. Although the effects of perifosine on human gliomas are not known, this class of drugs or other drugs that affect these same targets may be good choices to combine with temozolomide. This study indicates that perifosine may be useful as a novel therapy for human gliomas.

Acknowledgments
Received 3/29/2005; revised 5/19/2005; accepted 5/26/2005.
Grant support: NIH grants R01 CA58949-1 and R01 CA100688-2.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
We thank Dr. Andrew Koff and Jeff Miller for H1 kinase assay; members of the Holland laboratory for technical assistance and reading of the manuscript; and Keryx Biopharmaceuticals for generously providing us with perifosine.

References


The percentage of Ki-67-positive cells in treated tumors. Combination treatment of temozolomide and perifosine shows significant effect in vivo. *P < 0.005 compared with Control; **P < 0.01 compared with temozolomide. Columns, bars, ± SD.

Figure 6. Temozolomide (TMZ) and perifosine (PRF) suppress tumor growth in a mouse glioma model. A. H&E staining (>400) and immunohistochemical staining for Ki-67 (>400). H&E staining shows oligodendroglia-like morphology of PDGF-induced tumors. Brown nuclear staining for Ki-67 indicates proliferating cells. B. Percentage of Ki-67-positive cells in treated tumors. Combination treatment of temozolomide and perifosine shows significant effect in vivo. *P < 0.005 compared with Control; **P < 0.01 compared with temozolomide. Columns, bars, ± SD.

www.aacrjournals.org 7435 Cancer Res 2005; 65: (16) August 15, 2005

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