Inhibition of Akt by the alkylphospholipid perifosine does not enhance the radiosensitivity of human glioma cells

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Abstract
Akt has been implicated as a molecular determinant of cellular radiosensitivity. Because it is often constitutively activated or overexpressed in malignant gliomas, it has been suggested as a target for brain tumor radiosensitization. To evaluate the role of Akt in glioma radioresponse, we have determined the effects of perifosine, a clinically relevant alkylphospholipid that inhibits Akt activation, on the radiosensitivity of three human glioma cell lines (U87, U251, and LN229). Each of the glioma cell lines expressed clearly detectable levels of phosphorylated Akt indicative of constitutive Akt activity. Exposure to a perifosine concentration that reduced survival by ~50% significantly reduced the level of phosphorylated Akt as well as Akt activity. Cell survival analysis using a clonogenic assay, however, revealed that this Akt-inhibiting perifosine treatment did not enhance the radiosensitivity of the glioma cell lines. This evaluation was then extended to an in vivo model using U251 xenografts. Perifosine delivered to mice bearing U251 xenografts substantially reduced tumor phosphorylated Akt levels and inhibited tumor growth rate. However, the combination of perifosine and radiation resulted in a less than additive increase in tumor growth delay. Thus, in vitro and in vivo data indicate that the perifosine-mediated decrease in Akt activity does not enhance the radiosensitivity of three genetically disparate glioma cell lines. These results suggest that, although Akt may influence the radiosensitivity of other tumor types, it does not seem to be a target for glioma cell radiosensitization. [Mol Cancer Ther 2006;5(6):1504–10]

Introduction
Current strategies for enhancing tumor radiosensitivity are focused on targeting the molecules and processes that regulate cellular radioresponse. Toward this end, Akt has begun to receive considerable attention as a potential target for radiosensitization. Akt is a serine/threonine kinase activated by a variety of environmental stimuli in a phosphatidylinositol 3-kinase–dependent manner. Phosphatidylinositol 3-kinase 3-kinase generates membrane-bound phosphoinositides, which act as second messengers in the recruitment of Akt to the membrane where it becomes activated by phosphorylation (1–3). Akt can then phosphorylate and activate several proteins that have been implicated in cell survival and malignant transformation (4, 5). Most attention has been focused on the antiapoptotic properties of Akt, which are mediated through the phosphorylation and resulting inhibition of proapoptotic proteins, such as BAD (6) and caspase-9 (7). However, Akt can also promote cell survival through the phosphorylation of transcription factors, such as the forkhead family (8, 9) and CREB (10). Given the contributions of Akt to cell survival and that it is often constitutively activated in tumor cells (11), targeting Akt activity has been suggested as a strategy for cancer treatment in general (12, 13).

Akt has also been implicated as a determinant of cellular radiosensitivity. Kim et al. (14) showed that the small interfering RNA–mediated knockdown of Akt in human carcinoma cells resulted in radiosensitization, consistent with a role for Akt in radioresistance, at least in these cell lines. Pharmacologic approaches have also been used to investigate the potential of Akt as a target for radiosensitization. Many of these studies were designed to reduce Akt activity through inhibition of phosphatidylinositol 3-kinase using LY294002. Although radiosensitization was routinely detected (15–19), at the concentrations used, LY294002 inhibits not only phosphatidylinositol 3-kinase (and consequently Akt) but also members of the PIKK family, such as ATM and DNA protein kinase (20), which are critical to cell survival after irradiation (21). Thus, whether there is a causal relationship between the LY294002-mediated reduction in Akt activity and radiosensitization is unclear. Moreover, because of excessive toxicity, LY294002 is unlikely to be applicable to treatment situations (22).
Perifosine, an alkylphospholipid, is an alternative agent for inhibiting Akt activity. This compound interferes with lipid signaling and thus impedes Akt translocation to the cell membrane preventing its activation (23). Ruiter et al. reported that perifosine inhibits Akt activity and enhances radiation-induced apoptotic cell death of human leukemic cell lines (24, 25). Perifosine is orally bioavailable and in clinical trials has been shown to have favorable pharmacokinetic and toxicity profiles (26). Thus, perifosine seems to be a clinically relevant strategy for inhibiting Akt activity and consequently for enhancing tumor radiosensitivity.

Although radiotherapy remains a primary treatment modality for malignant gliomas, these tumors are poorly responsive to current treatment regimens; therefore, the ability to enhance glioma cell radiosensitivity would likely be of clinical benefit. Because of mutant PTEN or abnormal phosphatidylinositol 3-kinase signaling, gliomas often express constitutively activated Akt (27, 28). Given its putative role in radioresistance, Akt has been suggested as a target for enhancing the glioma radiosensitivity (18, 29). Therefore, as a clinically relevant means of inhibiting Akt activity, we have investigated the effects of perifosine on the radiosensitivity of human glioma cells grown in vitro and in vivo as s.c. xenografts in nude mice. The data presented here indicate that although perifosine inhibits Akt activity in three human glioma cell lines, it does not enhance their radiosensitivity. Moreover, the combination of perifosine and radiation delivered to a tumor xenograft model resulted in a less than additive increase in tumor growth delay.

**Materials and Methods**

**Cell Culture and Treatments**

Three human glioma cell lines were evaluated: U87, LN229, and U251. U87 and LN229 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in DMEM supplemented with 10% fetal bovine serum. U251 was obtained from the Division of Cancer Treatment and Diagnosis Tumor Repository, National Cancer Institute (NCI)-Frederick (Frederick, MD) and grown in RPMI 1640 with 5% fetal bovine serum (Life Technologies, Inc., Rockville, MD). Each of the growth media contained glutamate (5 mmol/L) and all cells were maintained at 37°C in an atmosphere of 5% CO₂ and 95% room air. Perifosine (provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI) was dissolved in PBS at a stock concentration of 10 mmol/L and stored at −20°C. Irradiation was done using a Pantak (Solon, OH) X-ray source at a dose rate of 1.55 Gy/min.

**Immunoblot Analysis**

Cells were harvested in 4°C lysis buffer [150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), 1% NP40, 1 mmol/L EDTA] supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitors I and II (Sigma, St. Louis, MO). Following centrifugation at 4°C (14 000 rpm) for 10 minutes to remove the insoluble fraction, protein concentration in the supernatants was determined by BCA assay (Pierce, Rockford, IL). Proteins (20 µg/lane) were fractionated using NuPAGE 10% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membranes were blocked in 5% skim milk in TBS-T [500 mmol/L NaCl, 20 mmol/L Tris (pH 7.5), 0.1% Tween 20] and incubated overnight at 4°C with the appropriate antibodies followed by secondary antibody linked to horseradish peroxidase, also in 5% skim milk in TBS-T. Membranes were then developed by the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). Antibodies to phosphorylated serine Akt and total Akt were purchased from Cell Signaling Technologies (Beverly, MA). Anti-actin antibody was obtained from Chemicon (Temecula, CA) and horseradish peroxidase–conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and chemiluminescence was visualized with a Typhoon scanner (Molecular Dynamics, Sunnyvale, CA).

**Immunoprecipitation and In vitro Kinase Assay**

Cells were harvested and cell extracts were prepared in the same fashion as for immunobLOTS. Endogenous Akt was precipitated from 500 µg total protein with an anti-Akt antibody at 4°C. Coprecipitates were pulled down with A/G agarose, washed in kinase buffer, and incubated at 42°C with 1 µg recombinant glycogen synthase kinase-3β (GSK-3β) for 15 minutes. The reaction was terminated by addition of gel loading buffer and denaturation at 95°C. At this point, samples were loaded in a SDS-PAGE gel and an immunoblot against phosphorylated GSK-3β was done. The antibodies, the kinase buffer, and the recombinant GSK-3β were obtained from Cell Signaling Technologies. The A/G agarose was obtained from Santa Cruz Biotechnology.

**Clonogenic Assay**

 Cultures were trypsinized to generate a single-cell suspension and a specified number of cells were seeded into each well of six-well tissue culture plates. After allowing cells time to attach (6 hours), perifosine (9 µmol/L) or the vehicle control was added and the plates were irradiated 16 hours later. Immediately after irradiation, the growth medium was aspirated and fresh medium was added. Plates were incubated for colony formation for 10 to 14 days. Colonies were stained with crystal violet, the number of colonies containing at least 50 cells was determined, and surviving fractions were calculated. Survival curves were generated after correcting for cell killing from perifosine alone. Data are mean ± SE from at least three independent experiments.

**Immunohistochemistry**

At specified times after the initiation of perifosine treatment, tumors were fixed in 10% buffered formalin immediately after removal. Paraffin sections were mounted on glass slides and washed in PBS twice, and the anti-phosphorylated serine Akt antibody (Cell Signaling Technologies) was added at a dilution of 1:50 in 1% bovine serum albumin and incubated overnight at 4°C. Sections again were washed twice in PBS before incubating in the
In vivo Tumor Growth Delay Assay

Five-week-old female athymic nude mice (NCr nu/nu; NCI Animal Production Program, Frederick, MD) were used in these studies. Mice were caged in groups of five or less and fed a diet of sterile rodent chow and hyperchlorinated water ad libitum. Tumors were generated by s.c. injection of U251 cells (5 × 10⁶) into the right hind limb. When tumors grew to a mean volume of 172 mm³, mice were randomized into treatment groups: vehicle control (water), perifosine, radiation (4 Gy), and perifosine/radiation combination. Perifosine treatment was initiated on the day of randomization (day 0) and consisted of a loading dose of 300 mg/kg given by oral gavage in two doses of 150 mg/kg delivered 12 hours apart. A maintenance dose of 35 mg/kg was then given orally once daily for the subsequent 5 days. Radiation (4 Gy) was delivered locally on day 1 using a Pantak irradiator with animals restrained in a custom lead jig. To obtain the tumor growth curves, perpendicular diameter measurements of each tumor were measured every 2 to 3 days with a digital caliper and volumes were calculated using a formula \( V = \frac{4}{3} \pi r^3 \). Data are expressed as mean ± SE tumor volume. Tumors were measured until the mean group volume was >2,000 mm³. Each experimental group contained 6 mice and the control group contained 10 mice; absolute growth delays (in days) are expressed as the mean ± SE for all the mice in each group. All animal studies were conducted in accordance with the principles and procedures outlined in the USPHS Guide for the Care and Use of Laboratory Animals in an Association for Assessment and Accreditation of Laboratory Animal Care International–approved facility under an approved animal protocol.

Results

Effects of Perifosine and/or Radiation on Akt Phosphorylation and Activity in Human Glioma Cell Lines

Because of the role of PTEN in regulating Akt activity (30), three human glioma cell lines were evaluated in these in vitro studies: U87 and U251, which contain mutant PTEN, and LN229, which contains wild-type PTEN (31). These cell lines also expressed different forms of p53, with U251 and LN229 containing mutant p53 and U87 containing wild-type p53 (31, 32). As shown in Fig. 1A, each of the glioma cell lines constitutively expresses phosphorylated serine Akt. Exposure to 9 μmol/L perifosine, which reduces survival to ~50% (see below), resulted in a time-dependent reduction in phosphorylated Akt levels, with decreases detectable after 2 hours with the maximum decrease at 16 to 24 hours after drug addition. It should be noted that the antibody used to detect phosphorylated serine Akt recognizes phosphorylated Akt1, Akt2, and Akt3. Comparison of the cell lines suggests that the constitutive level and the perifosine-mediated decrease in phosphorylated Akt levels were independent of PTEN or p53 mutation status. These results are consistent with previous reports showing that perifosine reduces Akt phosphorylation in a variety of cell types (23, 25, 33–35). Radiation has been reported to induce Akt phosphorylation in some (16, 36–38) but not all (17, 37) cell models. Irradiation (6 Gy) had no effect on the levels of phosphorylated Akt as evaluated from 30 minutes to 6 hours after irradiation in the glioma cell lines (Fig. 1B).
To determine the effects of the combination of perifosine and radiation on phosphorylated Akt levels and Akt kinase activity, U251 cells were evaluated as a representative of the glioma cell lines. U251 cells were exposed to perifosine for 16 hours, irradiated, and then collected 6 hours later. As shown in Fig. 2A, phosphorylated Akt remained below control levels in cells receiving the combination of perifosine followed by radiation, similar to those in cells receiving perifosine only. In addition to phosphorylated Akt levels, Akt activity was determined using GSK-3β as a substrate (39). For these measurements, immunoprecipitated Akt from each of the samples was used in an \textit{in vitro} kinase assay with exogenous GSK-3β. Immunoblotting was then done for phosphorylated GSK-3β (the product of Akt kinase activity), Akt (to ensure equal efficiencies of immunoprecipitation), and GSK-3β. Similar to the results for phosphorylated Akt levels, Akt kinase activity was reduced in cells treated with perifosine alone and in cells that received the perifosine/radiation combination (Fig. 2B). Thus, measurements of phosphorylated Akt levels and Akt activity indicate that, consistent with previous studies using other cell lines, perifosine inhibits Akt activity in these three glioma cell lines.

**Perifosine Treatment Does Not Enhance the Radio-sensitivity of Human Glioma Cell Lines \textit{In vitro}**

The effect of perifosine on glioma cell radiosensitivity was determined using the treatment protocol established to inhibit Akt activity. Each cell line was exposed to perifosine for 16 hours, the time of maximum reduction in Akt activity (Fig. 1), and irradiated, and the clonogenic assay done. Cell survival curves were generated after normalization for the level of cell killing induced by perifosine alone. Perifosine exposure only resulted in surviving fractions of 0.58 ± 0.130, 0.58 ± 0.073, and 0.42 ± 0.047 for U87, LN229, and U251 cells, respectively. As shown in Fig. 3, perifosine had no effect on the radiosensitivity of U87 or LN229 cells. For U251 cells, the combination of perifosine and radiation actually resulted in a less than additive increase in cell killing. These data indicate that the perifosine-induced reduction in Akt activity does not enhance the radiosensitivity of glioma cells \textit{in vitro}.

**Effects of Perifosine on Radiation-Induced Tumor Growth Delay**

Whereas the data presented above indicate that perifosine does not enhance the intrinsic radiosensitivity of glioma cells, previous reports suggested that Akt inhibition enhances the radioresponse of tumors via the radiosensitization of endothelial cells (16, 40). The possibility thus existed that perifosine enhances the \textit{in vivo} radioresponse of glioma xenografts through an inhibitory effect on tumor vasculature. Clinical perifosine treatment protocols typically involve an initial loading dose followed by daily maintenance doses (41). Therefore, in an attempt to simulate a clinically relevant treatment protocol, perifosine...
was delivered as a loading dose followed by five daily maintenance doses. Specifically, animals bearing U251 s.c. leg tumors were given perifosine in a loading dose of 300 mg/kg (2 × 150 mg/kg separated by 12 hours) followed by daily maintenance doses of 35 mg/kg for 5 days. This perifosine treatment protocol was shown to result in similar perifosine levels and pharmacokinetics as in humans (42). As illustrated by immunohistochemistry (Fig. 4), control U251 tumors express clearly detectable levels of phosphorylated Akt consistent with the immunoblot analysis of U251 cells grown in Fig. 1. However, 12 hours after the second dose of 150 mg/kg perifosine (the loading dose), phosphorylated Akt expression in U251 tumors was substantially reduced. Therefore, to determine the effects of a perifosine-mediated decrease in Akt activity on the radiosensitivity of U251 tumor xenografts, radiation was delivered 1 day after the initiation of perifosine treatment.

Mice bearing 172 mm³ s.c. U251 leg tumor xenografts were randomized into four groups: vehicle, perifosine, radiation, and perifosine plus radiation. Perifosine treatment as described above was initiated on the day of randomization (day 0) and radiation (4 Gy) delivered on the next day (day 1). The tumor growth rates for each treatment group are shown in Fig. 5. The absolute growth delay induced by each treatment was calculated as the time in days for tumors in treated mice to grow from 172 to 2,000 mm³ minus the time in days for tumors to reach the same size in vehicle-treated mice. The perifosine treatment protocol resulted in a growth delay of 11.5 ± 1.2 days and 4 Gy alone induced a growth delay of 14.4 ± 1.1 days. If the combined modality resulted in an independent, additive effect on tumor growth, a growth delay of ~26 days would be expected. However, the growth delay induced by the combination of the Akt inhibiting protocol of perifosine and radiation resulted in a growth delay of only 14.9 ± 1.4 days. Given that the growth delay induced by the combination treatment was not even additive in terms of the individual modalities, these data suggest not only that perifosine fails to radiosensitize in vivo tumors but also that the two treatments may be antagonistic with respect to their antitumor effects.

Discussion

Given that radiotherapy is a primary treatment modality for malignant gliomas and yet results in few, if any, cures, the development of molecularly targeted radiosensitizing agents seems to offer a viable strategy for improving clinical outcome. Critical to this process is the identification of the molecular determinants of cellular radiosensitivity. However, the contribution of a molecule to the regulation of radiosensitivity can be cell type specific. There are numerous molecules, especially those involved in signaling, that have been reported to affect the radiosensitivity of some cells but not others. For example, p53 (43, 44), Chk1 (45), nuclear factor-κB (46), and epidermal growth factor receptor (47) have been shown to regulate radiation-induced cytotoxicity of some but not all tumor cells. Such results suggest that the regulatory prowess of a given molecule with respect to radiosensitivity is dependent on the genetic and/or epigenetic context of a tumor cell. Along these lines, the use of small interfering RNA provided convincing evidence of a role for Akt in the radioresistance of three human carcinoma cell lines grown in vitro (14). However, the data presented here indicate that inhibition of Akt activity does not result in the radiosensitization of a series of genetically disparate human glioma cell lines. Although a different approach was used for reducing Akt activity, the discrepancy between the carcinoma and glioma cell lines may be attributed to a cell type–specific
role for Akt in radioresponse. Understanding the specific downstream actions of Akt with respect to radioresistance may provide insight into mechanisms responsible for this cell type specificity. However, regarding potential treatment strategies, these results suggest that Akt is not a relevant target for glioma radiosensitization.

The use of perifosine to evaluate the role of Akt in radioresistance suffers from the same basic complication as using LY294002 in that the cellular effects of this alkylphospholipid are not limited to inhibiting Akt activity. However, LY294002 in addition to inhibiting phosphatidylinositol 3-kinase, also inhibits the activity of ATM and DNA protein kinase, molecules critical in the response to DNA damage (20, 21). Thus, assigning a role for Akt in LY294002-induced radiosensitization is difficult at best. Perifosine has also been reported to inhibit extracellular signal-regulated kinase 1/2 in murine glial progenitor cells (34) and human leukemic cell lines (24) yet increase extracellular signal-regulated kinase 1/2 activity in normal human keratinocytes (48). Although extracellular signal-regulated kinase activity was not measured in our studies, previous reports indicate that it does not seem to play a significant role in radioresensitivity (49, 50) and is primarily involved in the regulation of cell proliferation (51). Perifosine has also been reported to activate the stress-activated protein kinase/c-Jun NH2-terminal kinase pathway in human leukemic cells leading to an enhanced level of radiation-induced apoptosis (24). We are unaware of any reported role for stress-activated protein kinase/c-Jun NH2-terminal kinase in the radioresponse of solid tumor cells, which typically die via mitotic catastrophe after irradiation (52). Thus, as for most other pharmacologic agents, the actions of perifosine are not limited to a single molecule. However, in contrast to LY294002, the potential targets of perifosine, other than Akt, have not been established to play a major role as determinants of radioresensitivity.

The data presented here illustrate that perifosine effectively reduces active Akt levels in glioma cells grown in vitro and as s.c. xenografts. Moreover, a significant tumor growth delay was induced following a clinically relevant perifosine treatment protocol. Given that Akt has been reported to mediate the resistance to specific chemotherapeutic agents (53), many of which kill tumor cells via apoptosis, perifosine may be effective in combination with various forms of chemotherapy. Moreover, given the clinical applicability of perifosine along with results showing a role of Akt in the radioresistance of some human tumor cell lines (14), perifosine may enhance the radioresponsiveness of other tumor types. However, the data presented here indicate that, despite the inhibition of Akt, perifosine does not enhance the radioresponsivity of glioma cells to radiation. Furthermore, for the glioma cell line U251, the combination of radiation with a perifosine treatment protocol that reduces active Akt resulted in a less than additive increase in cell killing in vitro and in tumor growth delay in vivo. These data suggest that the combination of radiation and perifosine may not be effective in the treatment of patients with gliomas.

References


Inhibition of Akt by the alkylphospholipid perifosine does not enhance the radiosensitivity of human glioma cells


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