In Vitro and In Vivo Inhibition of Neuroblastoma Tumor Cell Growth by AKT Inhibitor Perifosine

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Background

Activated AKT is a marker of decreased event-free or overall survival in neuroblastoma (NB) patients. The aim of this study was to investigate the effect of perifosine, a nontoxic AKT inhibitor, as a single agent on NB cell growth in vitro and in vivo.

Methods

Four human NB cell lines (AS, NGP, BE2, and KCNR) were treated with increasing concentrations of perifosine, and a quantitative analysis of cell death (apoptosis) was performed by using MTS and caspase-3/7 activity assays. Survival of mice carrying xenograft NB tumors that were treated with perifosine (n = 6–7 mice per group) was compared with that of untreated mice (n = 7 mice per group) using Kaplan–Meier analysis. Tumor volumes were calculated to determine the effect of perifosine on NB tumor growth. Phosphorylation of AKT and expression of cleaved caspase-3 were measured in proteins from the tumors. All statistical tests were two-sided.

Results

Perifosine, at 30 µM concentration, decreased AKT phosphorylation and increased apoptosis in all four NB cell lines in vitro. Perifosine-treated mice bearing xenograft NB tumors had longer survival than untreated mice (untreated vs treated, median survival: AS, 13 days, 95% confidence interval [CI] = 11 to 16 days vs not reached, \(P = .003\); NGP, 22 days, 95% CI = 20 to 26 days vs not reached, \(P = .013\); BE2, 24 days, 95% CI = 21 to 27 days vs not reached, \(P < .001\); and KCNR, 18 days, 95% CI = 18 to 21 days vs not reached, \(P < .001\)). Perifosine treatment induced regression in AS tumors, growth inhibition in BE2 tumors, and slower growth in NGP and KCNR tumors. Inhibition of AKT phosphorylation and induction of caspase-dependent apoptosis were noted in tumors of perifosine-treated mice in all four in vivo NB tumor models.

Conclusions

Perifosine inhibited the activation of AKT and was an effective cytotoxic agent in NB cells in vitro and in vivo. Our study supports the future clinical evaluation of perifosine for the treatment of NB tumors.

survival, as well as response to nutrient availability, intermediary metabolism, angiogenesis, and tissue invasion. All these processes represent the hallmarks of cancer, and a burgeoning literature has defined the importance of alterations of AKT activity in human cancer and experimental models of tumorigenesis (17). Constitutively activated AKT is detected in a number of adult cancers such as multiple myeloma (18), squamous cell carcinoma (19), renal carcinoma (20), endometrial cancer (21), lung cancer (22), prostate cancer (23), hepatocellular carcinoma (23), and gastric carcinomas (24) and is frequently marked as a poor prognostic factor. Treatments targeting AKT provide another therapeutic modality especially for those tumors in which activated AKT is associated with poor prognosis.

We previously demonstrated that chemoresistance induced by BDNF and its receptor TrkB was mediated by AKT, and constitutively activated AKT increased the survival of NB cells (25). More recently, multiple mutations of ALK, first identified in a chromosomal translocation associated with some anaplastic large-cell lymphomas, have been identified in 20% of sporadic NB tumors (26–29). Constitutive ALK phosphorylation and activation were observed in NB tumor tissues either with ALK mutations or with high expression of wild-type ALK (26–29). In one report, ALK protein was overexpressed in high-risk NB patients compared with low-risk NB patients (7). Activation of ALK has been shown to increase AKT activation (7,26–29). These findings make targeted therapy to AKT an important treatment modality to explore in NB tumors.

To date, several types of AKT inhibitors have been investigated, including phosphatidylinositol analog inhibitors, allosteric AKT kinase inhibitors, ATP-competitive inhibitors, and alkylphospholipids (30,31). However, the use of these inhibitors is limited either by high toxicity or by low bioavailability and stability in vivo (30,31). Perifosine, an alkylphospholipid, is perhaps the best-characterized AKT inhibitor (32–47). In clinical trials, perifosine had dose-limiting gastrointestinal toxicity, such as nausea, diarrhea, fatigue, and dehydration, but these toxic effects were ameliorated with the use of prophylactic medicine, such as 5-HT3 receptor antagonists, dexamethasone, loperamide, domperidone, and metoclopramide (35,39,41,48–50). Perifosine has been evaluated as an anticancer drug in many adult tumor types in which activated AKT is associated with poor prognosis (32–34,40,42–46). Because there are no reports that have systematically evaluated the activity of perifosine in pediatric tumors, in this study, we examined the ability of perifosine to inhibit AKT activation and assessed its functional effect on NB tumor cell growth in vitro and in vivo.

**Materials and Methods**

**Cell Culture and Reagents**

Four human NB cell lines—SK-N-AS (AS), NGP, SK-N-BE2 (BE2), SMS-KCNN (KCNR) (Table 1)—and a mouse NIH3T3 fibroblast cell line were used in this study. Cell lines NGP, BE2, and KCNR have MYCN amplification; AS, BE2, and KCNR cells have 1pLOH; AS and BE2 have TP53 mutations; and KCNR has ALK mutation (R1275Q). The cell lines were obtained from the following places: BE2 from Robert Ross (Fordham University, New York), KCNR from C. Patrick Reynolds (Texas Tech University Health Sciences Center, School of Medicine, Texas), and AS and NGP from the cell line bank of Pediatric Oncology Branch of the National Cancer Institute. All cell lines were genotyped and were genetically pure according to a single-nucleotide polymorphism–based genotype assay (S. J. Chanock, Division of Cancer Genetics and Epidemiology, National Cancer Institute). AS-luciferase cells were generated in our laboratory by transfecting pMSCV-PURO-luciferase gene (BD Biosciences, San Jose, CA) into AS cells and selecting puromycin (0.75 µg/mL)-resistant colonies to generate AS-luciferase NB cells for luciferase-based bioluminescent imaging. The cells were cultured in RPMI-1640 medium (Mediatech Inc, Manassas, VA) containing 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), 2 mmol/L glutamine (Invitrogen, Carlsbad, CA), and antibiotics penicillin (100 units/mL) and streptomycin (100 µg/mL) (Invitrogen) at 37°C in 5% CO₂ incubator.

**Table 1. Specific mutations in neuroblastoma (NB) cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MNA</th>
<th>1pLOH</th>
<th>TP53</th>
<th>ALK</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-AS (AS)</td>
<td>−</td>
<td>+</td>
<td>Δexon9</td>
<td>Wt</td>
<td>(51)</td>
</tr>
<tr>
<td>NGP</td>
<td>+</td>
<td>−</td>
<td>Wt</td>
<td>Wt</td>
<td>(52)</td>
</tr>
<tr>
<td>SK-N-BE2 (BE2)</td>
<td>+</td>
<td>+</td>
<td>C135F</td>
<td>Wt</td>
<td>(51)</td>
</tr>
<tr>
<td>SMS-KCNN (KCNR)</td>
<td>+</td>
<td>+</td>
<td>R1275Q</td>
<td>Wt</td>
<td>(51)</td>
</tr>
</tbody>
</table>

* Four NB cell lines (AS, NGP, BE2, and KCNR) used for in vitro and in vivo studies are shown. 1pLOH = loss of heterozygosity at chromosome 1p; ALK = anaplastic lymphoma kinase; MNA = MYCN gene amplification; TP53 = a tumor suppressor gene; Wt = wild type.

**CONCEPT AND CAVEATS**

**Prior knowledge**

Effective treatment of high-risk neuroblastoma (NB) patients remains a challenge. Constitutively activated AKT protein is known to increase survival of NB cells, but it is not known whether an AKT inhibitor can demonstrate a functional effect in NB tumors.

**Study design**

Four human NB cell lines were used to test the effect of perifosine, a well-characterized AKT inhibitor, on cell survival and activation status of AKT. Perifosine was also tested on the survival, tumor growth, and activation status of AKT in mice bearing human NB xenograft tumors.

**Contribution**

Perifosine showed a statistically significant reduction in NB cell survival, slowed or regressed tumor growth, and increased survival in mice bearing NB tumors. A decreased level of activated AKT was observed in perifosine-treated NB cells and xenograft tumors.

**Implications**

This study supports the evaluation of perifosine to treat NB patients.

**Limitations**

Perifosine was evaluated as a single agent; how it will perform in combination with chemotherapy was not investigated. This study was performed in an animal model and may not be predictive for humans.

*From the Editors*
Cell Survival Analysis
For cell survival assays, all four types of NB cells and mouse fibroblast cells were seeded into 96-well plates at a density of 3 × 10^4 cells per well, in three to six replicates, and cultured in RPMI-1640 containing 10% FBS. After 24 hours of culture, cells were treated with perifosine (KERYX Biopharmaceuticals, New York, NY), at concentrations ranging from 2.5 to 20 µM, for 16 hours. For AKT expression and phosphorylation assays, we used five concentrations of perifosine (2.5, 5, 10, 15, and 20 µM) for 16 hours. At the end of the treatment, cells were washed with cold phosphate-buffered saline and processed as previously described (25). Protein lysates were extracted from tumor tissues by sonicating the tumor tissues with perifosine for 15 minutes. The NB cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Sanford, ME). The membranes were incubated in 5% nonfat dry milk in Tris-buffered saline with Tween 20 (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, and 0.5% Tween 20) to block nonspecific antibody binding and then incubated at 4°C overnight with the following antibodies (1:1000)—anti-phosphorylated AKT (Serine473, shown as S473 later) (binds to all three phosphorylated AKT isoforms), anti-AKT (binds to all three AKT isoforms), anti-extracellular signal-regulated protein kinase (ERK1 or 2), anti-phosphorylated ERK1 or 2 (Threonine202 or Tyrosine204, shown as T202/Y204 later), anti-FKHL1 (also known as FOXO3), anti-phosphorylated FKHL1 (Threonine320, shown as T32 later), anti-S6, anti-phosphorylated S6 (Serine235 or 236, shown as S235/236 later), and anti-cleaved-caspase-3 (all antibodies were from Cell Signaling Technology, Beverly, MA). All the antibodies show reactivity with human proteins and were rabbit polyclonal antibodies, except anti-phosphorylated ERK1 or 2, which was a mouse monoclonal antibody. The membranes were washed with Tris-buffered saline–TWEEN 20 and incubated with the appropriate horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. Bound antibodies were detected by the enhanced chemiluminescence immunoblotting detection reagent (Amersham Biosciences Inc, Pittsburgh, PA) and exposed to x-ray film. Densitometric analysis of appropriately exposed autoradiographs was performed using NIH Image 1.63 software. The ratio of relative densitometric values of phosphorylated AKT (arbitrary units) to the relative densitometric values of the total AKT (arbitrary units) in perifosine-treated samples was normalized to the ratio of phosphorylated AKT to total AKT in the respective untreated control samples in the in vitro experiments. All experiments were performed twice.

Cell Cycle Analysis
NB cell lines were seeded into six-well plates at a density of 5 × 10^4 cells per well, in duplicates, and treated with 10–30 µM concentrations of perifosine in RPMI-1640 containing 10% FBS for 48 hours. After washing in cold phosphate-buffered saline (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, and 135 mM NaCl, pH 7.4), cells were incubated with RNase A (Roche, Indianapolis, IN) at 100 µg/mL and propidium iodide (Sigma-Aldrich Corp, St Louis, MO) at 50 µg/mL for 30 minutes in the dark at room temperature. The stained cells were analyzed for DNA content by fluorescence-activated cell sorting on a FACSScan cytometer (Becton Dickinson & Co). FlowJo software (BD Biosciences) was used to quantify the percentage of cells in different stages of the cell cycle. The experiments were performed twice. Perifosine was found to have almost no effect on mouse fibroblast cell growth; therefore, we excluded this cell line from the cell cycle analysis.

Protein Assays
NB cells were seeded into six-well plates at a density of 5 × 10^4 cells per well and cultured in RPMI-1640 containing 10% FBS. After 24 hours of culture, cells were treated with perifosine, at concentrations ranging from 2.5 to 20 µM, for 16 hours. For AKT expression and phosphorylation assays, we used five concentrations of perifosine (2.5, 5, 10, 15, and 20 µM) for 16 hours. At the end of the treatment, cells were washed with cold phosphate-buffered saline and processed as previously described (25). Protein lysates were extracted from tumor tissues by sonicating the tumor tissues in protein lysis buffer (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 µg/ml leupeptin, and 500 µM sodium orthovanadate) for 15 minutes.

Immunoblotting was performed as described previously (25). The NB cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Sanford, ME). The membranes were incubated in 5% nonfat dry milk in Tris-buffered saline with Tween 20 (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, and 0.5% Tween 20) to block nonspecific antibody binding and then incubated at 4°C overnight with the following antibodies (1:1000)—anti-phosphorylated AKT (Serine473, shown as S473 later) (binds to all three phosphorylated AKT isoforms), anti-AKT (binds to all three AKT isoforms), anti-extracellular signal-regulated protein kinase (ERK1 or 2), anti-phosphorylated ERK1 or 2 (Threonine202 or Tyrosine204, shown as T202/Y204 later), anti-FKHL1 (also known as FOXO3), anti-phosphorylated FKHL1 (Threonine320, shown as T32 later), anti-S6, anti-phosphorylated S6 (Serine235 or 236, shown as S235/236 later), and anti-cleaved-caspase-3 (all antibodies were from Cell Signaling Technology, Beverly, MA). All the antibodies show reactivity with human proteins and were rabbit polyclonal antibodies, except anti-phosphorylated ERK1 or 2, which was a mouse monoclonal antibody. The membranes were washed with Tris-buffered saline–TWEEN 20 and incubated with the appropriate horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. Bound antibodies were detected by the enhanced chemiluminescence immunoblotting detection reagent (Amersham Biosciences Inc, Pittsburgh, PA) and exposed to x-ray film. Densitometric analysis of appropriately exposed autoradiographs was performed using NIH Image 1.63 software. The ratio of relative densitometric values of phosphorylated AKT (arbitrary units) to the relative densitometric values of the total AKT (arbitrary units) in perifosine-treated samples was normalized to the ratio of phosphorylated AKT to total AKT in the respective untreated control samples in the in vitro experiments. All experiments were performed twice.

Assay of Caspase-3 and Caspase-7 Activity
NB cell lines were seeded into 96-well plates at a density of 3 × 10^4 cells per well, in duplicates or triplicates, cultured for 24 hours in RPMI-1640 containing 10% FBS, and then treated with 10 µM perifosine for 16 hours. The combined activity of caspase-3 and caspase-7 (caspase-3/7) was evaluated using the Caspase-Glo 3/7 Assay Kit (Promega Corporation) according to the manufacturer’s instruction. At the end of perifosine treatment, the Caspase-Glo 3/7 reagent was added to the cells and incubated at room temperature for 1 hour. Results were detected by a luminometer, Victor 3 (PerkinElmer Life and Analytical Sciences, Shelton, CT), at 482 nm wavelength. The experiments were performed twice. Cleaved caspase-3 in the tumor tissues was detected by immunoblotting with anti-cleaved-caspase-3 antibody.

In Vivo Studies
For heterotypic subcutaneous injection, the NB cell lines (AS, NGP, BE2, and KCNR) were cultured in RPMI-1640 with 10% FBS. The cells were harvested, washed with Hanks balanced salt solution (HBSS) (Invitrogen), and resuspended in Hanks balanced salt solution and Matrigel (Trevigen, Gaithersburg, MD). One hundred microliters of cell suspension containing 2 × 10^6 cells per well, in three to six replicates, cultured for 24 hours in RPMI-1640 containing 10% FBS. After 24 hours of culture, cells were treated with perifosine, at concentrations ranging from 5 to 30 µM, or vehicle phosphate-buffered saline for 48 hours or a pan caspase inhibitor, Z-VAD-FMK (R&D Systems, Inc, Minneapolis, MN), at 10 µM concentration, for 3 hours before perifosine treatment.

The stained cells were analyzed for DNA content by fluorescence-activated cell sorting on a FACScan cytometer (Becton Dickinson & Co). When the tumor tissues of the right flank of 5- to 6-week-old female athymic nude mice (Taconic, Germantown, NY) using a 28-gauge needle were inoculated into the subcutaneous tissue of the right flank of 5- to 6-week-old female athymic nude mice (Taconic, Germantown, NY) using a 28-gauge needle (Becton Dickinson & Co). When the tumors reached 100–200 mm³, perifosine (24 mg/kg/d) or placebo was administered once
daily for up to 30 or 32 days by oral gavage (n = 8–10 mice per group). We tested four doses of perifosine (10, 15, 24, and 36 mg/kg/d) (n = 5 in each group) in a pilot experiment, and because substantial loss of body weight was noted in mice receiving 36 mg/kg/d, this dose was not used any further. There was little toxicity with all other doses of perifosine, but 10- and 15-mg/kg/d doses had little effect on tumor growth, so we selected 24-mg/kg/d dose of perifosine for our experiments. The dimensions of the resulting tumors were determined at least three times a week using a digital caliper, and the tumor volume (cubic millimeter) was calculated as \((L \times W^2)/4\), where \(L\) = length (millimeter) and \(W\) = width (millimeter). Two to three mice in each group were killed at 6 days (AS tumors) or 14 days of perifosine treatment (BE2, NGP, and KCNR tumors). The mice were killed by asphyxiation with regulated CO\(_2\), and the tumors were excised and immediately frozen at −80°C. Protein extracts from the tumors were used to assess the phosphorylation status of AKT. To determine the effect of perifosine on survival, we counted the days from the date the treatment (perifosine or control) was administered to the time the control cohort of mice were killed when the tumors reached a length of 20 mm or to the end of the perifosine treatment or until death for ethical reasons. Those mice that did not die or require to be killed by the end of the experiment were considered censored.

For orthotopic injection, 0.5 million AS cells or AS-luciferase cells (genetically modified AS cells containing luciferase gene) were inoculated into the fat pad around the left adrenal gland of BALB/c or severe combined immunodeficiency–Beige mice (Taconic) (53) (n = 6 per group). Perifosine (24 mg/kg/d) was administered by oral gavage 1 week after inoculation of the cells. The AS-luciferase tumors were imaged by bioluminescent imaging after 1 and 3 weeks of perifosine treatment. The AS tumors were excised and weighed after 35 days of treatment. All mouse experiments were performed once, except for experiments with NGP cells that were performed twice, and experiments with AS cells were performed once at subcutaneous site and twice at orthotopic site.

All xenograft studies were approved by the Animal Care and Use Committee of the National Cancer Institute, and all mouse treatments, including their housing, were in accordance with the institutional guidelines (PB–023).

**Bioluminescent Imaging**

Luciferase-based bioluminescent imaging was performed with a highly sensitive, cooled charge-coupled device camera mounted in a light-tight specimen box (Xenogen IVIS 200 Imaging System; Caliper LifeSciences, Alameda, CA). Mice (n = 6 per group) were injected intraperitoneally with luciferase substrate d-luciferin (Invitrogen) at 150 mg/kg and then anesthetized with 2% isoflurane. They were placed on the imaging chamber, and 12 minutes after injection of d-luciferin, images were acquired. Imaging signals were quantified and expressed as photon counts using the Living Image (Xenogen) software.

**Histopathology Studies**

For AS subcutaneous tumors, mice were killed after 6 days of perifosine treatment (n = 2 in control group; n = 3 in perifosine-treated group); otherwise, the mice were killed when the tumors reached 20 mm in length or at the end of the experiment day 32 (n = 7 in control group; n = 6 in perifosine-treated group). Tumor tissues were fixed in 10% formalin, embedded with paraffin, and processed to slides (n = 3) with thickness of 5 µm. The slides were stained with hematoxylin and eosin and observed under the Zeiss Axiophot microscope at ×20 and ×63 magnification.

**Statistical Analysis**

Analysis of variance (ANOVA) was performed on continuous data (except for tumor weights, see below). A two-way factorial ANOVA was performed on the caspase-3 and caspase-7 activity data; the factors tested were cell line and perifosine (positive vs negative). A three-way factorial ANOVA was performed on the cell survival data; the three factors tested were cell line, perifosine (positive, negative), and Z-VAD (positive, negative). The growth rate was estimated for each mouse within each cell line by using linear regression on the cube root of the volumes, with data restricted to times at which adequate data existed (days 1–11 for AS, days 1–19 for NGP and KCNR, and days 1–21 for BE2). ANOVA was then performed on the estimated slopes, with cell line (AS, NGP, KCNR, and BE2), treatment (control, perifosine), and their interaction (cell line × treatment) as fixed effects in the model. Mean slope estimates were compared between treatments within a cell line using a two-tailed t test. A two-way repeated measures ANOVA was performed on the photon counts data; treatment group (control, perifosine) and weeks (1 and 3) were the effects tested. Tumor weights were compared between control and perifosine-treated groups using a two-sided Wilcoxon rank sum test because the data in the groups were not normally distributed. Means and 95% confidence intervals (CIs) are reported unless indicated otherwise.

The Kaplan–Meier method was used to determine the probability of survival as a function of time. A two-sided log-rank test was used to test the statistical significance of the difference between the two treatment groups.

All P values less than .05 were considered to be statistically significant. All data were analyzed with SAS and STAT 9.1 (SAS and STAT 9.1 User’s Guide, 2004; SAS Institute Inc, Cary, NC).

**Results**

**Effect of Perifosine on the Survival of NB Cells In Vitro**

To study the effect of perifosine on NB cell survival, four NB cell lines (AS, NGP, BE2, and KCNR) that are representative of the heterogeneity typically found in NB tumors and cell lines were used (Table 1). After 48 hours, all perifosine-treated NB cell lines displayed a concentration-dependent decrease in cell survival, regardless of their genetic alterations (Figure 1, A). The increase in cell death was evident (<10% cell death in NIH3T3 cells vs approximately 70% cell death in NB cells) at 30 µM perifosine concentration (Figure 1, A).

To determine the possible events that may have resulted in increased cell death in the NB cells, changes in cell cycle were analyzed by flow cytometry in the four cell lines exposed to increasing concentrations of perifosine for 48 hours (Figure 1, B). Increased concentrations of perifosine caused an initial accumulation of cells in the G2 and M phase of the cell cycle, followed by a reduced percentage of cells in the G1 phase, and finally an increase in the percentage of cells in the sub-G1 phase of the cell cycle (Figure 1, B).

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Figure 1. Effect of perifosine on the survival of neuroblastoma (NB) cells. A) Perifosine induced cell death in NB cells. AS, NGP, BE2, and KCNR cells and the control NIH3T3 fibroblasts were treated with different concentrations of perifosine for 48 hours. MTS assay was used to detect cell survival. The percentage of surviving cells was calculated by normalizing the absorbance value of the treated cells by the
To assess whether perifosine-induced cell death was mediated via a caspase-dependent apoptotic pathway, we performed a caspase-3/7 activity assay. Caspase-3 and caspase-7 are the common effector caspases of both the intrinsic and the extrinsic apoptotic pathways. Perifosine-treated NB cells showed an increase in caspase-3/7 activity in AS cells (approximately 1.7-fold increase compared with untreated controls; mean = 167% of control; 95% CI = 165% to 170% of control; P < .001), NGP cells (approximately 3.2-fold increase compared with untreated controls; mean = 319% of control; 95% CI = 297% to 341% of control; P < .001), BE2 cells (approximately 2.4-fold increase compared with untreated controls; mean = 234% of control, 95% CI = 231% to 237% of control; P < .001), and KCNR cells (approximately four-fold increase compared with untreated controls; mean = 398% of control; 95% CI = 391% to 405% of control; P < .001), respectively (Figure 1, C).

To assess whether activation of caspases was critical for perifosine-induced apoptosis, NB cells were treated with pan caspase inhibitor Z-VAD-FMK before treatment with perifosine. All four NB cell lines, albeit to different extents, were rescued from perifosine-induced apoptosis. Compared with control treatment, the mean cell survival in the Z-VAD-FMK–treated group was 73% higher in AS cells (95% CI = 68% to 78%), 54% higher in NGP cells (95% CI = 49% to 59%), 42% higher in BE2 cells (95% CI = 37% to 47%), and 25% higher in KCNR cells (95% CI = 9% to 41%), respectively (Figure 1, D). Treatment with the inhibitor alone showed 26% and 15% increase in baseline cell survival in NGP and BE2 cells but had no effect on baseline survival in AS or KCNR cells. The results indicated that perifosine treatment induced caspase-dependent apoptosis in NB cells.

**Effect of Perifosine on AKT Phosphorylation in NB Cells In Vitro**

To test whether perifosine altered the expression and phosphorylation of AKT in NB cells, we treated each of the four NB cell lines (AS, NGP, BE2, and KCNR) with varying concentrations of perifosine (2.5, 5, 10, 15, and 20 µM). After 16 hours, there was a noticeable decrease in the level of phosphorylated AKT (S473) in all cell lines (Figure 2). Total AKT levels declined in AS, NGP, and BE2 cells at the higher concentrations of 15 and 20 µM of perifosine.

We also tested whether perifosine altered the expression and phosphorylation of ERK1 or 2 (mitogen-activated protein kinase [MAPK])3 or MAPK1). Perifosine treatment showed a decrease in the level of phosphorylated ERK1 or 2 (T202/Y204) n AS cells, an increase in NGP cells, a transient increase followed by decrease in BE2 cells, and an unchanged level in KCNR cells. Total ERK levels were unchanged by perifosine in all four NB cell lines (Figure 2). This indicates that the effect of perifosine on ERK1 or 2 activation was cell line dependent.

The levels of phosphorylated FKHR1, a member of the Forkhead transcription factor family and a downstream target of AKT, were reduced in AS, NGP, BE2, and KCNR cell lines (Figure 2) but not in AS cells (data not shown). The levels of phosphorylated mammalian target of rapamycin (mTOR) target S6, another downstream target of AKT, were reduced in AS cells by perifosine (Figure 2). However, levels of phosphorylated S6 were not inhibited by perifosine in NGP, BE2, and KCNR cell lines (data not shown). These data indicated that levels of phosphorylated AKT were universally inhibited by perifosine in all four NB cell lines, consequently resulting in lower levels of distinct phosphorylated downstream targets in different cell lines.

**Effect of Perifosine on the Survival of NB Tumor-Bearing Mice**

We generated Kaplan–Meier curves of the survival of mice (days from the beginning of perifosine treatment until tumor growth reached a length of 20 mm or until the end of perifosine treatment) and found a statistically significant survival advantage for the perifosine-treated mice in all four groups bearing AS-, NGP-, BE2-, and KCNR-NB tumors compared with controls (AS, P = .003; NGP, P = .013; BE2, P < .001; and KCNR, P < .001) (Figure 3). All mice in the four control groups developed tumors that reached a length of 20 mm within 30 days of inception of the experiment. In the perifosine-treated groups, two of six AS tumor-bearing mice, two of seven NGP tumor-bearing mice, zero of seven BE2 tumor-bearing mice, and one of seven KCNR tumor-bearing mice reached 20 mm length before the end of the experiment. The median survival days in untreated control groups were 13 for mice bearing AS tumor (95% CI = 11 to 16), 22 for mice bearing NGP tumor (95% CI = 20 to 26), 24 for mice bearing BE2 tumor (95% CI = 21 to 27), and 18 for mice bearing KCNR tumor (95% CI = 18 to 21). The median survival days in all the perifosine-treated groups were not reached within the experiment periods. These data indicated that, as a single agent, perifosine showed a statistically significant increase in the survival of mice bearing NB tumors.

**Effect of Perifosine on Tumor Growth In Vivo**

We next determined whether perifosine affected the NB tumor growth in nude mice, for which tumors were measured three times a week. The rates of tumor growth between day 1 and day 32 are
shown in Figure 4, A. Tumors either grew slowly or regressed in perifosine-treated mice. Specifically, in AS tumors, four of six perifosine-treated mice showed tumor regression, one showed slow growth of tumors for the entire period, compared with controls, and one mouse did not response to treatment. NGP tumors grew slowly in five of seven perifosine-treated mice, compared with controls, and two of seven mice did not respond to perifosine treatment. BE2 tumors grew slowly in six of seven perifosine-treated mice compared with controls. KCNR tumors grew slowly in six of seven perifosine-treated mice compared with the control (Figure 4, A).

We also observed a statistically significant difference between untreated control and perifosine-treated groups when the slopes of the tumor volumes for each NB cell line were compared by ANOVA ($P = .042$ for AS tumor, $P = .022$ for NGP, and $P < .001$ for both BE2 and KCNR tumors). A comparison of the tumor mean volumes in untreated control and perifosine-treated groups at different time points are shown in Figure 4, B.

NB typically arises in the abdomen in association with the adrenal gland, presumably from sympathoadrenal ganglion or medullary cells of the adrenal gland. Therefore, we assessed the inhibitory effects of perifosine on tumor growth in an orthotopic periadrenal murine model (53). As shown in Figure 5, A and B, after 1 week of perifosine treatment, there was no statistically significant difference between the tumor luciferase activities in the control and perifosine-treated groups in mice carrying AS-luciferase tumors ($P = .38$). By 3 weeks of treatment, the mean luciferase activity of tumors in the control group (mean = 61 × 10$^8$ photon counts, 95% CI = 18 to 103 × 10$^8$ photon counts; $n = 6$) was 2.9-fold higher than that in the perifosine-treated group (mean = 20.9 × 10$^8$ photon counts, 95% CI = 6.4 to 35.3 × 10$^8$ photon counts; $n = 6$). The difference approached statistical significance using a repeated measures two-way ANOVA ($P = .061$). In one mouse in the control group, the photon count was not consistent with the tumor size. On inspection, we found that certain areas of the tumor were necrotic, so the low photon count was probably because of poor uptake of luciferin in the necrotic areas. In a repeat experiment, tumor weights were used to assess the inhibitory effect of perifosine on NB growth at the orthotopic site. The mice were killed after 35 days of perifosine treatment. All mice developed tumors, yet the median weight of the tumors in the perifosine-treated group ($n = 10$; median = 0.18 g; 25th percentile = 0.08 g, 75th percentile = 0.37 g) was 16.6-fold less than the median tumor weight of the control group ($n = 10$; median = 2.98 g; 25th percentile = 2.09 g, 75th percentile = 3.88 g) (Figure 5, C). There was a statistically significant difference between the control and
perifosine-treated groups using a two-sided Wilcoxon rank sum test \( (P < .001) \). These data indicated that perifosine significantly reduced the tumor burden in the heterotypic NB xenograft as well as the orthotopic NB xenograft models.

**Apoptosis and AKT Phosphorylation in Perifosine-Treated NB Tumors In Vivo**

We observed that at 6 days of perifosine treatment, there was no histological difference between the control and perifosine-treated tumors. Histological examination revealed that tumors consisted of small, round, and blue cells, characteristic of childhood NB. However, at the end of the experiment (day 15 for control group and day 32 for perifosine group), the majority of the residual mass in the perifosine-treated AS tumors was fibrous tissue compared with the tumor cells in control mice receiving placebo (Figure 6, A).

To study whether perifosine inhibited the activation of AKT in NB xenograft tumors in vivo, we assessed the levels of phosphorylated AKT in tumors from control and perifosine-treated mice. There was a decrease in the level of phosphorylated AKT in perifosine-treated AS tumors at 6 days of treatment compared with tumors from mice receiving placebo (Figure 6, B). Similar results of perifosine-induced decrease in the level of phosphorylated AKT were also found in NGP, BE2, and KCNR tumors (Figure 6, C). We noted that the decrease in the level of phosphorylated AKT in AS tumors of perifosine-treated mice occurred within 6 days of treatment, at a time when there were no gross histopathologic differences between control and perifosine-treated tumors (Figure 6, A). There was no difference in total AKT level in the NB tumors in either control or perifosine-treated mice (Figure 6, B).

The pattern of ERK1 or 2 phosphorylation in tumors in vivo was consistent with the pattern seen in individual cell lines in vitro. The levels of phosphorylated downstream targets of AKT—FKHRL1 and S6—were also assessed. Similar to the results detected in vitro, the levels of phosphorylated S6 were decreased by perifosine in AS tumors (Figure 6, B) but not in NGP, BE2, and KCNR tumors (data not shown). In contrast, levels of phosphorylated FKHRL1 were decreased by perifosine in the NGP, BE2, and KCNR tumors (Figure 6, C) but not in AS tumors (data not shown). These results indicate that treatment with perifosine uniformly inhibited levels of phosphorylated AKT in NB tumors in vivo, whereas there was a differential modulation of downstream targets of AKT.

To assess whether perifosine-induced decrease in tumor size and inhibition of AKT activation were associated with induction of caspase-dependent apoptosis, we evaluated the levels of cleaved caspase-3, a marker of apoptosis. The level of cleaved caspase-3 increased in most of the tumors from mice treated with perifosine (Figure 6, B and C). This finding was consistent with the in vitro results of perifosine-induced increase in caspase-3/7 activities.

**Discussion**

In this study, we show that perifosine, as a single agent, inhibits the growth of NB tumors and increases the survival of mice with NB...
tumors. The perifosine-induced inhibition of cell growth was accompanied by a decrease in AKT phosphorylation in all four NB cell lines tested in both in vitro and in vivo models.

The antitumor activity of perifosine has been evaluated in xenograft models of multiple myeloma (54) and squamous cell carcinomas (47), and in these models, the perifosine-induced inhibition of tumor growth was also accompanied by inhibition of AKT phosphorylation (54). Similarly, our in vivo studies showed that perifosine induced a reduction in overall tumor growth and in some cases tumor regression in heterotypic xenograft models of NB tumors. We observed that perifosine showed a substantial decrease in tumor growth also in our orthotopic NB model.

![Figure 4](image_url)

**Figure 4.** Effect of perifosine on subcutaneous tumor growth. Subcutaneous xenografts were established as described in Figure 3. Perifosine (24 mg/kg/d) and placebo were administered once daily 7 d/wk for up to 30–32 days. The tumor sizes were measured at least three times a week. **A** The tumor size changes of individual mice were plotted according to the time of treatment. Each cell line had a plotted graph indicating the control and perifosine-treated mice. **B** The comparison of mean tumor volumes between control and perifosine-treated groups in each cell line. Data represent means and 95% confidence intervals (where n = 4–7, where n < 4, no summary statistics are shown.)
Mechanistically, it has been reported that perifosine induces apoptosis in lung tumor models via a caspase-dependent mechanism (55), whereas in tumor cells of endometrial (56) or prostate tissue (50), apoptosis is induced via a caspase-independent mechanism. This study showed that perifosine induced increased caspase-3/7 activity and Z-VAD-FMK inhibited perifosine-induced apoptosis in NB cells, thus demonstrating a caspase-dependent mechanism of apoptosis.

Many genetic alterations exist in NB tumor cells, including MYCN amplification (4,5,57), or 1pLOH (loss of heterozygosity on chromosome 1p) (4,5,58), which mark poor prognosis of NB tumors. In our study, three (BE2, NGP, and KCNR) of the four NB cell lines have MYCN amplification and three (AS, BE2, and KCNR) of the four NB cell lines have 1pLOH, and notwithstanding the disruption these chromosomal alterations have on the biology of the tumor cells, all four cell lines were sensitive to perifosine.

In this study, the two NB cell lines (AS and BE2) with mutant tumor protein TP53 were also sensitive to perifosine, supporting the findings in squamous carcinoma cells in which perifosine inhibited the growth of cells containing either wild-type or mutant TP53 (32). Recently, ALK mutations have been detected in poor prognosis NB and are associated with increase in activated AKT (26–29). Inhibition of ALK leads to decrease in activated AKT (28), indicating that AKT is a downstream target of ALK. NB cells with the F1174L ALK mutation are sensitive to the ALK inhibitor TAE684 (26), whereas NB cells with wild-type ALK, or the R1275Q ALK mutation, are resistant to TAE684 (26). The KCNR cell line used in our study has R1275Q ALK mutation and is resistant to TAE684, yet the growth of KCNR cells in vitro and xenograft tumors in vivo was inhibited by perifosine. In addition to KCNR, our preliminary assessment of three additional NB cell lines with ALK mutations (SY5Y cells [F1174L], LAN5 cells [R1275Q], and SKN-FI cells [S1136S]) indicated that they were also sensitive to perifosine treatment (data not shown). Our results indicated that regardless of whether the NB cell lines have mutant or wild-type ALK, NB tumors can be sensitive to perifosine. Therefore, perifosine may be useful in the treatment of patients whose tumors have ALK mutations and are resistant to ALK inhibitors. Overall, these findings indicated that perifosine exhibited...

Figure 5. Effect of perifosine on orthotopic tumor growth. We injected $5 \times 10^5$ AS-luciferase or AS cells into the fat pad around the left adrenal gland in severe combined immunodeficiency-Beige mice. One week after inoculation, perifosine or placebo treatment (24 mg/kg) was initiated and was given once daily 7 d/wk by oral gavage. A) In mice bearing AS-luciferase tumors, luciferase-based bioluminescent imaging was performed at 1 and 3 weeks of perifosine treatment. B) Photon counts of control mice (n = 6) and perifosine-treated mice (n = 6). Values for individual mice are shown (symbols), as are the mean values for each group (bars). C) After 5 weeks of treatment, mice bearing AS tumors were killed and the tumor weight in each mouse was measured. Tumor weights of control (median = 2.98 g; n = 10) and perifosine-treated (median = 0.18 g; n = 10) groups are shown in the box-and-whiskers plot. The boxes represent the interquartile range (25th and 75th percentiles), and the horizontal line in the box represent the median value. The whiskers show the range of largest and smallest values. $P < .001$ (two-sided Wilcoxon rank sum test).
both in vitro and in vivo inhibitory activity against NB tumor cells with different genetic alterations.

One limitation of our study is that we have only evaluated perifosine as a single agent. Our future studies will be aimed at evaluating perifosine in combination with chemotherapy.

Perifosine has been tested in adult clinical trials in a number of tumor types. In phase II trials using perifosine as a single agent, consistent responses in patients with advanced, relapsed, or refractory multiple myeloma were obtained, whereas variable responses were noted in primary or recurrent prostate cancer, breast cancer, advanced soft tissue sarcoma, head and neck cancer, and melanoma. Furthermore, perifosine has synergistic antitumor growth effect when combined with bortezomib (proteasome inhibitor), rapamycin (mTOR inhibitor), lenalidomide/dexamethasone (immunomodulatory drugs), or tumor necrosis factor–related apoptosis-inducing ligand (45) in the treatment of patients with multiple myeloma. Preclinical models have also shown that perifosine sensitizes endometrial cancer cells to cisplatin treatment, epithelial adenocarcinoma cells to UCN-01 treatment, and squamous carcinoma cells to radiotherapy.

To our knowledge, perifosine has not been tested systematically in any pediatric tumors. However, a recent report in a murine medulloblastoma model showed that medulloblastoma was more sensitive to radiation therapy on treatment with perifosine. This led to a phase I clinical trial (registered at ClinicalTrials.gov, the pictures is x20 (scale bar = 50 µm). The magnification of the insets is x100 (scale bar = 10 µm). B and C) Mice in each group were killed at 6 days (AS cells) or 14 days (BE2, NGP, and KCNR cells) of perifosine treatment. Tumors were excised and proteins extracted. Total proteins (40 µg) were analyzed for phosphorylated (P)-AKT (S473), total (T)-AKT, P-ERK1/2 (T202/Y204), T-ERK, P-FKHRL1 (T32), T-FKHRL1, P-S6 (S235/236), T-S6, and cleaved caspase-3 by immunoblotting.
References


42. Richardson P, Wolf J, Jakubowiak A, et al. Phase I/II results of a multicenter trial of perifosine (KRX-0401) + bortezomib in patients with relapsed or relapsed/refractory multiple myeloma who were previously relapsed from or refractory to bortezomib [ASH annual meeting abstracts]. Blood. 2008;112:870.


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