The alkylyphospholipid perifosine induces apoptosis of human lung cancer cells requiring inhibition of Akt and activation of the extrinsic apoptotic pathway

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Abstract

The Akt inhibitor, perifosine, is an alkylyphospholipid exhibiting antitumor properties and is currently in phase II clinical trials for various types of cancer. The mechanisms by which perifosine exerts its antitumor effects, including the induction of apoptosis, are not well understood. The current study focused on the effects of perifosine on the induction of apoptosis and its underlying mechanisms in human non-small cell lung cancer (NSCLC) cells. Perifosine, at clinically achievable concentration ranges of 10 to 15 μmol/L, effectively inhibited the growth and induced apoptosis of NSCLC cells. Perifosine inhibited Akt phosphorylation and reduced the levels of total Akt. Importantly, enforced activation of Akt attenuated perifosine-induced apoptosis. These results indicate that Akt inhibition is necessary for perifosine-induced apoptosis. Despite the activation of both caspase-8 and caspase-9, perifosine strikingly induced the expression of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor, death receptor 5, and down-regulated cellular FLICE-inhibitory protein (c-FLIP), an endogenous inhibitor of the extrinsic apoptotic pathway, with limited modulatory effects on the expression of other genes including Bcl-2, Bcl-XL, PUMA, and survivin. Silencing of either caspase-8 or death receptor 5 attenuated perifosine-induced apoptosis. Consistently, further down-regulation of c-FLIP expression with c-FLIP small interfering RNA sensitized cells to perifosine-induced apoptosis, whereas enforced overexpression of ectopic c-FLIP conferred resistance to perifosine. Collectively, these data indicate that activation of the extrinsic apoptotic pathway plays a critical role in perifosine-induced apoptosis. Moreover, perifosine cooperates with TRAIL to enhance the induction of apoptosis in human NSCLC cells, thus warranting future in vivo and clinical evaluation of perifosine in combination with TRAIL in the treatment of NSCLC. [Mol Cancer Ther 2007;6(7):2029–38]

Introduction

Alkylyphospholipids are a class of antitumor agents which target the cell membrane and induce apoptosis (1, 2). Perifosine, the first orally bioavailable alkylyphospholipid, has shown antitumor activity in preclinical models and is currently in phase II clinical trials (1, 3). The mechanisms by which perifosine exerts its antitumor effect remain unclear, although it seems to inhibit Akt (2, 4) and mitogen-activated protein kinase activation (5), whereas inducing c-Jun-NH2-kinase (JNK) activation (5). Perifosine has also been shown to induce p21 expression leading to cell cycle arrest (6). In addition, perifosine, in combination with other antitumor agents such as the PDK1 inhibitor, UCN-01 (7), histone deacetylase inhibitors (8), and the chemotherapeutic agent etoposide (9), show synergistic antitumor effects. It is well known that there are two major apoptotic pathways used by mammalian cells to undergo apoptosis. One pathway involves signals transduced through death receptors known as the extrinsic apoptotic pathway; the second pathway relies on signals from the mitochondria called the intrinsic apoptotic pathway. Both pathways involve the activation of a set of caspases, which in turn, cleave cellular substrates and result in the characteristic morphologic and biochemical changes constituting the process of apoptosis (10, 11). The extrinsic pathway is characterized by the oligomerization of cell surface death receptors and activation of caspase-8, whereas the intrinsic pathway involves in the disruption of mitochondrial membranes, the release of cytochrome c, and the activation of caspase-9. Through caspase-8–mediated cleavage or truncation of Bid, the extrinsic death receptor apoptotic pathway is linked to the intrinsic mitochondrial apoptotic pathway (10, 11).

Molecules that can block the extrinsic apoptotic pathway include cellular FLICE-inhibitory protein (c-FLIP). c-FLIP prevents caspase-8 activation by death receptors. There are two major isoforms of c-FLIP: FLIPα, consists of two
NH₂-terminal death effector domains and a COOH-terminal caspase homology domain devoid of enzymatic activity, whereas FLIP₅ is only composed of the NH₂-terminal death effector domains and a short COOH-terminal stretch of amino acids not found in FLIP₁. It has been shown that c-FLIP expression correlates with resistance against death receptor–induced apoptosis in a variety of cancer cells, and c-FLIP–transfected tumor cell lines develop more aggressive tumors in vivo (12, 13). In addition, many studies have shown that down-regulation of c-FLIP is sufficient to confer sensitivity against death receptor–induced apoptosis, whereas c-FLIP expression is associated with chemoresistance and down-regulation of c-FLIP using antisense oligonucleotides or small interfering RNAs (siRNA) sensitizes cells to chemotherapeutic agent–induced apoptosis (12–14).

Akt is known to be critical for tumor cell survival. One of the ways that Akt promotes cell survival is to inhibit apoptosis through its ability to phosphorylate several proapoptotic proteins such as Bad, which are involved in the regulation of the intrinsic apoptotic pathway (15). Moreover, Akt also inhibits the extrinsic death receptor–mediated apoptotic pathway through up-regulation of c-FLIP expression (16, 17). Thus, Akt negatively regulates apoptosis by suppressing both the mitochondria- and death receptor–mediated pathways.

The induction of apoptosis by perifosine has been observed in several cancer cell lines (3, 8, 9, 18). However, this effect has not been determined in non–small cell lung cancer (NSCLC) cells. Moreover, the mechanisms by which perifosine induces apoptosis is generally unknown. In this study, we examined the effects of perifosine on apoptosis in human NSCLC cells and its modulation on different apoptotic molecules in an attempt to understand its mechanisms of action. Our data show that perifosine induces apoptosis, inhibits Akt activation, up-regulates death receptor 5 (DR5) expression, and reduces c-FLIP levels in NSCLC cells. In addition, perifosine in combination with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) augments the induction of apoptosis.

Materials and Methods
Reagents
Perifosine was supplied by Keryx Biopharmaceuticals, Inc. This agent was dissolved in PBS and stored at −20°C. Stock solution was diluted to the appropriate concentrations with growth medium immediately before use. Human recombinant TRAIL was purchased from PeproTech, Inc.

Cell Lines and Cell Culture
The human NSCLC cell lines used in this study were described previously (19). H157 cell lines that stably express ectopic Lac Z (Lac Z-5) and FLIP₁ (FLIP₁-6), respectively, and A549 cell lines that stably express ectopic Lac Z (Lac Z-9) and FLIP₁ (FLIP₁-2), respectively, were described previously (20, 21). These cell lines were grown in a monolayer culture in RPMI 1640 supplemented with glutamine and 5% fetal bovine serum at 37°C in a humidified atmosphere consisting of 5% CO₂ and 95% air.

Cell Growth Assay
Cells were cultured in 96-well cell culture plates and treated the next day with the agents indicated. Viable cell number was estimated using the sulforhodamine B assay, as previously described (19).

Western Blot Analysis
Preparation of whole cell protein lysates and Western blot analysis were described previously (22, 23). Mouse anti–caspase-3 monoclonal antibody was purchased from Imgenex. Rabbit polyclonal antibodies against PTEN, Akt, phospho (p)-Akt (Ser473), phospho (p)-FKHR (Ser256), phospho (p)-GSK3β (Ser9), c-Jun, phospho (p)-c-Jun (Ser63), p44/42, phospho (p)-p44/42 (Thr202/Tyr204), survivin, caspase-8, caspase-9, poly(ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Technology. Rabbit polyclonal anti-DR5 antibody was purchased from ProSci, Inc. Mouse monoclonal anti-FLIP antibody (NF6) was purchased from Alexis Biochemicals. Rabbit anti-G3PDH polyclonal antibody and mouse anti-Bax monoclonal antibody were purchased from Trevigen. Rabbit anti-Puma polyclonal antibody and mouse anti-Bax monoclonal antibody were purchased from EMD Biosciences, Inc. Mouse anti–Bcl-2 and rabbit anti–Bcl-XL antibodies were purchased from Santa Cruz Biotechnology, Inc. Rabbit anti–β-actin polyclonal antibody was purchased from Sigma Chemicals. Secondary antibodies, goat anti-mouse and goat anti-rabbit horseradish peroxidase conjugates, were purchased from Bio-Rad.

Adenoviral Infection
Adenovirus harboring an empty vector (Ad-CMV) or a constitutively activated form of Akt (myristoylated Akt; Ad-myr-Akt) were provided by Lily Yang (Department of Surgery, Emory University School of Medicine, Atlanta, GA). The procedure for adenoviral infection of cancer cells was described previously (24).

Gene Silencing Using siRNA
Silencing of caspase-8, DR5, c-FLIP, and PTEN were achieved by transfecting siRNA using RNAiFect transfection reagent (Qiagen) following the instructions of the manufacturer. Control, caspase-8, and DR5 siRNAs were described previously (22). These siRNAs and c-FLIP siRNA targeting the sequence 5’-AATTCTCCGAACGTCACCGT-3’ (14) were all synthesized from Qiagen. PTEN siRNA was purchased from Cell Signaling. Cells were plated in 6- or 24-well cell culture plates and transfected with the given siRNAs the next day. After 24 h, the cells were trypsinized and replated in new plates, and on the second day, treated with perifosine as indicated. Gene silencing effects were evaluated by Western blot as described above after the indicated times of treatment.

Apoptosis Assays
Apoptosis was detected either by analysis of caspase activation using Western blot analysis as described above or by Annexin V staining using Annexin V-FITC apoptosis detection kit (BD Bioscience) following the instructions of the manufacturer, and analyzed by flow cytometry using FACSScan (Becton Dickinson). In addition, we measured...
the amounts of cytoplasmic histone-associated DNA fragments (mononucleosome and oligonucleosomes) formed during apoptosis using a Cell Death Detection ELISA Plus kit (Roche Molecular Biochemicals) according to the instructions of the manufacturer.

Results

Effects of Perifosine on Cell Survival and Apoptosis in NSCLC Cells

The effects of perifosine on cell survival were examined in a panel of NSCLC cell lines (Fig. 1A). For the majority of the cell lines tested, there was a dose-dependent decrease in cell survival. The H460 cell line was the most sensitive to perifosine, showing an IC₅₀ value of ~1 μmol/L. The H226 cell line was the most resistant to perifosine, in which perifosine at 20 μmol/L decreased cell survival by <20%. Most of the tested cell lines exhibited moderate response to perifosine with IC₅₀s ranging from 8 to 15 μmol/L (Fig. 1A), which are within the clinically achievable and safe peak plasma concentrations of 12 to 15 μmol/L (25, 26). The p53 and PTEN mutation status in the NSCLC cell lines tested did not correlate with sensitivity to perifosine, suggesting that perifosine inhibits cell growth independently of p53 and PTEN mutation status (Fig. 1A). In addition, we examined the protein expression levels of PTEN, total Akt, and p-Akt in the cell lines tested. It seemed that those cell lines (e.g., H460 and H358) with low levels of p-Akt and high levels of PTEN were the most sensitive to perifosine (Fig. 1B).

We further examined the effects of perifosine on apoptosis in NSCLC cell lines. As shown in Fig. 2A, perifosine induced apoptosis in H460 and A459 cells as indicated by Annexin V–positive staining. At concentrations of 10 μmol/L, perifosine induced cell death in ~50% of H460 cells, whereas apoptosis was induced in 23% and 33% of the A549 cells after treatment with 10 and 15 μmol/L of perifosine, respectively, suggesting that the H460 cells were more sensitive to perifosine-induced apoptosis. The H157 cells were the least sensitive to perifosine-induced apoptosis with only 10% of H157 cells undergoing apoptosis after treatment with 15 μmol/L of perifosine. We found that perifosine at concentrations ranging from 2.5 to 10 μmol/L induced cleavage of caspase-8, caspase-9, caspase-3, and PARP in H460 cells, whereas it induced partial cleavage of the caspases and PARP only at 10 μmol/L in A549 cells (Fig. 2B). In H157 cells treated with perifosine (up to 10 μmol/L), we failed to detect cleaved bands of the caspases and PARP (Fig. 2B). Because perifosine is effective in decreasing cell number in H157 cells (Fig. 1), we further examined cell cycle alteration in H157 cells after exposure to perifosine and detected 17.9%, 35.8%, and 42.4% G2-M cells in cells treated with PBS, 10 μmol/L of perifosine, and 15 μmol/L of perifosine, respectively, after a 48 h treatment, indicating that perifosine primarily decreases cell numbers in H157 cells via induction of cell cycle arrest. In the following studies, we focused on revealing the mechanisms underlying perifosine-induced apoptosis.

Effects of Perifosine on the Phosphorylation of Akt, JNK, and ERK

Perifosine has been shown to modulate Akt as well as other signaling pathways (6, 18, 27). We therefore examined whether perifosine modulated similar signal transduction pathways in human NSCLC cells. In examining Akt phosphorylation, we observed that both H460 and A549 cells have very low basal levels of p-Akt, whereas H157 cells have much higher basal levels of p-Akt. When comparing the apoptosis results presented in Fig. 2A, it seems that low basal levels of p-Akt correlated with high sensitivity to perifosine-induced apoptosis. These cell lines exhibited a concentration-dependent decrease in p-Akt levels when exposed to perifosine (p-Akt levels were only detectable in H460 cells after a very long exposure). Interestingly, perifosine also decreased the levels of total Akt in the tested cell lines (Fig. 3A) and the degree of Akt down-regulation also seemed to correlate with cell sensitivity to perifosine-induced apoptosis. In H460 cells, decreases in both Akt and p-Akt levels occurred at 3 h after treatment with perifosine (Fig. 3B), indicating that Akt down-regulation is an early event. In H157 cells, Akt levels were only slightly decreased, whereas p-Akt levels were substantially (by 2.5 μmol/L of perifosine) and rapidly (3 h posttreatment) reduced upon perifosine treatment.
(Fig. 3A and B). These results suggest that the perifosine-mediated decrease in p-Akt levels could be due to either Akt protein down-regulation or upstream signaling suppression, depending on the cell lines used. To our knowledge, this is the first demonstration that perifosine down-regulates the levels of total Akt in human cancer cells. We also detected a decrease in the levels of p-FKHR and p-GSK3β, two well-known substrates of Akt (Fig. 3B), furthering the notion that perifosine inhibits Akt signaling in NSCLC cells.

In examining other signal transduction pathways, we observed the basal levels of p-c-Jun were very low in the tested NSCLC cells and were only slightly increased by perifosine in H157 cells, indicating that perifosine-induced JNK activation was a cell line–dependent event. Perifosine did not alter the levels of p42/44, but did decrease the levels of p-p42/44 in all three cell lines tested (Fig. 3A). These data indicate that perifosine down-regulates the ERK (or p42/44) signaling pathway in NSCLC cells.

Enforced Akt Activation Attenuates Perifosine-Induced Apoptosis

To decipher the role of Akt inhibition in perifosine-induced apoptosis, we artificially activated Akt in H460 cells by infecting the cells with adenoviruses carrying a myr-Akt gene that codes a constitutively activated form of Akt, and then examined the response of these cells to perifosine treatment. Using Western blot analysis, we detected high levels of myr-Akt, p-Akt, and p-GSK3β in cells infected with Ad-myr-Akt (Fig. 3C). In addition, infection of cells with Ad-myr-Akt also elevated the levels of c-FLIP, which has been shown to be regulated by Akt (refs. 16, 17; Fig. 3C). In Ad-CMV–infected control cells, treatment with perifosine caused 37% apoptotic cell death (9% in PBS-treated cells) plus 15.2% necrotic cell death. However, we detected only 16% apoptosis (~12% in PBS-treated cells) and ~2% necrosis in cells infected with Ad-myr-Akt after treatment with perifosine (Fig. 3D). These results clearly show that enforced Akt activation restores cell resistance to perifosine-induced apoptosis, thus indicating that Akt inhibition is necessary in mediating perifosine-induced apoptosis.

Given that there is an inverse relationship between PTEN expression and p-Akt levels (Fig. 1B), we further determined whether down-regulation of PTEN affects p-Akt levels and cell sensitivity to perifosine. Knockdown of PTEN using PTEN siRNA in H460 cells, which is the most sensitive cell line to perifosine and have the highest levels of PTEN (Fig. 1), increased basal levels of p-Akt. However,
the p-Akt increase caused by PTEN knockdown could be abrogated by perifosine treatment. Surprisingly, perifosine decreased PTEN levels, which itself did not result in an increase of p-Akt levels, probably because perifosine also inhibits Akt phosphorylation (see Supplemental Fig. S1A).1 As a result, down-regulation of PTEN by siRNA did not alter cell sensitivity to perifosine as shown by measuring cell number change (Supplemental Fig. S1B),1 caspase activation (Supplemental Fig. S1A),1 and apoptotic cells (Supplemental Fig. S1C).1

Effects of Perifosine on the Expression of Key Molecules Involved in the Regulation of Apoptosis

To further explore how perifosine induces apoptosis, we next examined the effects of perifosine on the expression of several key genes involved in either the extrinsic apoptotic pathway (e.g., DR5 and c-FLIP) or the intrinsic apoptotic pathway (e.g., Bax, Bcl-2, Bcl-XL, PUMA, and survivin). Perifosine increased the levels of DR5, particularly in H460 cells (Fig. 4). It seems that DR5 induction is associated with increased sensitivity of cell lines to perifosine. c-FLIP is another key protein that inhibits the extrinsic apoptotic pathway by blocking caspase-8 activation (12). The H460 cells, which are the most sensitive to perifosine, had very low basal levels of c-FLIP, particularly FLIP L, which were further down-regulated by perifosine, whereas the less sensitive A549 and H157 cells have high basal levels of c-FLIP, particularly FLIP L, which were only weakly decreased by perifosine. Perifosine also decreased FLIP S levels in these cell lines (Fig. 4). It seems that low levels of c-FLIP and their further down-regulation by perifosine were associated with high sensitivity to perifosine-induced apoptosis. Collectively, these results suggest that activation of the DR5-mediated extrinsic apoptotic pathway is important in perifosine-induced apoptosis.

In examining the signaling molecules involved in the intrinsic apoptotic pathway, we found that perifosine did not significantly alter the levels of Bcl-2 in NSCLC cells (Fig. 4). Surprisingly, perifosine decreased Bax levels in all the tested NSCLC cell lines. Perifosine decreased Bcl-X L levels in H460 cells that were very sensitive to perifosine, but not in A549 and H157 cells that were less sensitive to perifosine (Fig. 4). These data suggest that Bcl-X L down-regulation may affect cell sensitivity to undergo perifosine-induced apoptosis. H460 cells had low basal levels of survivin, which were further decreased by perifosine,
whereas H157 and A549 cells had high levels of survivin, which were apparently not altered by perifosine (Fig. 4). Thus, it seems that low basal levels of survivin and its further down-regulation with perifosine are also associated with increased cell sensitivity to perifosine-induced apoptosis. PUMA was slightly increased in A549 and H157 cells, but not in H460 cells, suggesting that PUMA was not important in perifosine-induced apoptosis.

Perifosine Cooperates with TRAIL to Enhance the Induction of Apoptosis

Because perifosine induces DR5 expression and down-regulates c-FLIP levels, we hypothesized that perifosine would cooperate with TRAIL, a DR5 ligand, to enhance the induction of apoptosis. Thus, we examined the effects of perifosine in combination with TRAIL on apoptosis induction in NSCLC cells. As shown in Fig. 5A, perifosine in combination with TRAIL induced higher levels of DNA fragments than did each single agent alone. Moreover, increased amounts of cleaved caspase-8, caspase-9, caspase-3, and PARP were detected in cells treated with the perifosine and TRAIL combination, but were only minimally detected in cells treated with either perifosine or TRAIL alone (Fig. 5B). Thus, we conclude that perifosine cooperates with TRAIL to enhance the induction of apoptosis.

Perifosine Induces Apoptosis Requiring Caspase-8 Activation and DR5 Up-regulation

The data presented above strongly suggest a role for the activation of the extrinsic apoptotic pathway in perifosine-induced apoptosis. Thus, we determined whether perifosine induces apoptosis requiring activation of caspase-8 and up-regulation of DR5. To this end, we silenced the expression of caspase-8 and DR5 using caspase-8 and DR5 siRNAs, respectively, and then examined cell sensitivity to perifosine. By Western blotting, we detected substantially reduced levels of caspase-8 levels including cleaved forms in H460 cells transfected with caspase-8 siRNA compared with those in control siRNA-transfected cells (Fig. 2C), indicating successful caspase-8 knockdown or inhibition of caspase-8 activation. Accordingly, cleavage of caspase-3 and PARP and an increase in DNA fragmentation were also attenuated in caspase-8 siRNA-transfected cells in comparison with control siRNA–transfected cells (Fig. 2C and D). These results indicate that perifosine induces a caspase-8–dependent apoptosis. Similarly, silencing of DR5 expression using DR5 siRNA abrogated DR5 induction (Fig. 4B) and impaired the ability of perifosine to induce cleavage of caspase-8, caspase-3, and PARP (Fig. 4B). In addition, an increase in DNA fragmentation in DR5 siRNA-transfected cells was also reduced compared with control siRNA-transfected cells (Fig. 4C). These data show that DR5 up-regulation is also involved in perifosine-induced apoptosis.

Manipulation of c-FLIP Levels Regulates Cell Sensitivity to Perifosine-Induced Apoptosis and Enhancement of TRAIL-Induced Apoptosis

To further show that the activation of the extrinsic apoptotic pathway participates in perifosine-induced apoptosis, we examined the sensitivity of cell lines that express ectopic FLIP1 to perifosine-induced apoptosis. As presented in Fig. 6A, perifosine increased DNA fragmentation in a dose-dependent fashion in H157-Lac Z-5 cells, but only minimally in H157-FLIP1-6 cells. As a positive control treatment, TRAIL–induced increase in DNA fragmentation
was abolished in H157-FLIPL-6 cells. Similarly, perifosine-induced increase in DNA fragmentation was also abrogated in A549-FLIPL-2 cells in comparison with A549-Lac Z-9 cells (Fig. 6B). Together, these results clearly show that overexpression of ectopic c-FLIP protects cells from perifosine-induced apoptosis.

Because c-FLIP down-regulation was often associated with the enhancement of TRAIL-induced apoptosis (20, 21, 28), we further compared apoptosis induction by the combination of perifosine and TRAIL between A549-Lac Z-9 and A549-FLIPL-2 cell lines. In agreement with the results presented in Fig. 5, the combination of perifosine and TRAIL was much more potent than either perifosine or TRAIL alone in increasing DNA fragmentation in A549-Lac Z-9 cells. However, not only perifosine and TRAIL alone but also their combination exhibited minimal effects on increasing DNA fragmentation in A549-FLIPL-2 cells (Fig. 6B). These results clearly show that overexpression of ectopic c-FLIP confers cell resistance to the combination of perifosine and TRAIL, indicating that c-FLIP down-regulation contributes to perifosine-mediated enhancement of TRAIL-induced apoptosis.

Because the cell lines, A549 and H157, with high basal levels of c-FLIP were relatively less sensitive than H460 cells which have low basal levels of c-FLIP to perifosine-induced apoptosis, we wanted to determine whether down-regulation of c-FLIP sensitized cells to perifosine-induced apoptosis. To this end, we silenced the expression of c-FLIP (both FLIPL and FLIPS) using siRNA in A549 cells, and then examined their response to perifosine-induced apoptosis. As presented in Fig. 6C, transfection of c-FLIP siRNA reduced the levels of both FLIPL and FLIPS, which were further reduced after treatment with perifosine. Those cells whose expression of c-FLIP had been reduced with siRNA were more sensitive to caspase-8, caspase-3, and PARP cleavage after perifosine treatment compared with control cells, indicating that c-FLIP levels indeed affect cell sensitivity to perifosine-induced apoptosis.

Discussion

In this study, we have shown that perifosine exerts its growth-inhibitory effects in a panel of NSCLC cell lines, primarily through the induction of apoptosis and/or cell cycle arrest. Importantly, perifosine inhibited the growth of most of the tested NSCLC cell lines with IC50s ranging between 8 and 15 μmol/L (Fig. 1A), which are within the

Figure 5. Perifosine cooperates with TRAIL to enhance DNA fragmentation (A) and caspase activation (B) in human NSCLC cells. A, cell lines plated in 96-well plates and treated with the given doses of perifosine (PRFS) alone, 20 ng/mL TRAIL alone, or TRAIL plus perifosine on the second day. After 24 h, the cells were subjected to DNA fragmentation using the Cell Death Detection ELISA kit. Columns, means of triplicate determinations; bars, SD. B, H157 cells were treated with perifosine alone, TRAIL alone, and their combinations. After 16 h, the cells were subjected to a preparation of whole cell protein lysates and subsequent detection of the indicated proteins by Western blot analysis. c, cleaved.
Perifosine Induces Apoptosis in Human Lung Cancer Cells

Although perifosine inhibits Akt activation in different types of cancer cells including the NSCLC cells shown in the current study, enforced activation of Akt through overexpression of the constitutively activated form of Akt, myr-Akt, protects cells from perifosine-induced cell death in one type of cancer cell line (e.g., PC-3 prostate cancer cells; ref. 4) but not in another type of cancer cell (e.g., MM.1S multiple myeloma cells; ref. 18). In our study, we found that perifosine increased p-c-Jun only in one (i.e., H157 cells) of three cell lines tested (Fig. 3A). Given that H157 cells were relatively insensitive to perifosine-induced apoptosis (Fig. 2), we suggest that JNK activation is unlikely to account for the perifosine-induced apoptosis in human NSCLC cells. Perifosine was reported to either decrease or increase ERK phosphorylation depending on cancer cell type (5, 18, 27). In our study, perifosine decreased ERK phosphorylation in all of the three tested cell lines tested, regardless of cell sensitivity to TRAIL-induced apoptosis (Fig. 2), we suggest that JNK activation is also unlikely to be critical for perifosine-induced apoptosis in human NSCLC cells.

Figure 6. Overexpression of ectopically expressed c-FLIP (A and B) and silencing of c-FLIP expression (C) modulate cell sensitivity to perifosine-induced apoptosis. A, H157 cell lines that stably express Lac Z and FLIP L, respectively, were treated with the indicated concentrations of perifosine (PRFS) or TRAIL. B, A549 cell lines that stably express Lac Z and FLIP L, respectively, were exposed to 10 μmol/L of perifosine, 20 ng/mL of TRAIL, and the combination of perifosine and TRAIL (P + T). Twenty-four hours later, the cells were subjected to a preparation of whole cell protein lysates and subsequent detection of the indicated proteins using Western blot analysis. CFs, cleaved fragments.

Clinically achievable and safe peak plasma concentration ranges (i.e., 10–15 μmol/L; refs. 25, 26), suggesting the potential of perifosine in the treatment of NSCLCs.

Modulation of Akt, JNK, and ERK signaling pathways and their involvement in perifosine-induced apoptosis has been studied in other types of cancer cells (4, 18, 27). Despite the proapoptotic role of JNK activation in perifosine-induced apoptosis in multiple myeloma (18), we found that perifosine increased p-c-Jun only in one (i.e., H157 cells) of three cell lines tested (Fig. 3A). Given that H157 cells were relatively insensitive to perifosine-induced apoptosis (Fig. 2), we suggest that JNK activation is unlikely to account for the perifosine-induced apoptosis in human NSCLC cells. Perifosine was reported to either decrease or increase ERK phosphorylation depending on cancer cell type (5, 18, 27). In our study, perifosine decreased ERK phosphorylation in all of the three tested cell lines tested, regardless of cell sensitivity to TRAIL-induced apoptosis. Thus, we suggest that ERK inhibition is also unlikely to be critical for perifosine-induced apoptosis in human NSCLC cells.
levels were lower and Bcl-2 levels were higher in the sensitive H460 cells than in less sensitive A549 and H157 cells (Fig. 4A). Moreover, we found that Bax levels were actually decreased in cells treated with perifosine, although the underlying mechanisms and its effects on perifosine-induced apoptosis are unclear. In fact, our preliminary data show that Bax or PUMA deficiency does not alter cell sensitivity to perifosine-induced apoptosis. Together, we suggest that the activation of the extrinsic apoptotic pathway is important in mediating perifosine-induced apoptosis. This observation is supported by our findings that silencing of caspase-8 or DR5, or overexpression of ectopic c-FLIP protects cells from perifosine-induced apoptosis (Figs. 2, 4, and 6), whereas down-regulation of endogenous c-FLIP using c-FLIP siRNA sensitizes cells to perifosine-induced apoptosis (Fig. 6). In agreement with our findings, a recent study has shown that perifosine induces apoptosis through activation of the Fas-mediated extrinsic apoptotic pathway in human leukemia cells (29).

To the best of our knowledge, this is the first study showing that perifosine modulates the expression of DR5 and c-FLIP in human cancer cells.

Some studies have shown that Akt also inhibits the extrinsic apoptotic pathway through the up-regulation of c-FLIP expression (16, 17). In this study, we have shown that perifosine inhibits Akt and reduces c-FLIP levels, both of which are involved in perifosine-induced apoptosis. Indeed, we detected increased levels of c-FLIP in cells infected with Ad-myr-Akt (Fig. 3C), suggesting that Akt activation indeed increases c-FLIP levels in the tested cells. Thus, it is possible that Akt exerts its inhibitory effect on perifosine-induced apoptosis through the up-regulation of c-FLIP. On other hand, perifosine may down-regulate c-FLIP levels through inhibition of Akt; this needs to be investigated in detail in the future.

It is known that TRAIL functions as the DR5 ligand and rapidly induces apoptosis in a wide variety of transformed cells but is not cytotoxic in normal cells in vitro and in vivo (10, 16, 17). Therefore, TRAIL is considered to be a tumor-selective, apoptosis-inducing cytokine with promising potential for cancer treatment and is currently being tested in phase I clinical trials. In our study, we showed that the combination of perifosine and TRAIL exhibited augmented induction of apoptosis in human NSCLC cells (Fig. 5), which is likely due to the ability of perifosine to induce DR5 expression and down-regulate c-FLIP levels. This finding warrants future in vivo animal studies and clinical evaluation of the efficacy of perifosine in combination with TRAIL for the treatment of NSCLC.

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2 Unpublished data.

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Molecular Cancer Therapeutics

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