Celecoxib antagonizes perifosine's anticancer activity involving a cyclooxygenase-2-dependent mechanism

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Abstract

Perifosine is an orally bioavailable alkylphospholipid currently being tested in phase II clinical trials as a potential anticancer drug. In this study, we reveal a novel mechanism underlying the anticancer activity of perifosine that involves the induction of cyclooxygenase 2 (COX-2) in human cancer cells. Perifosine induced apoptosis and/or cell cycle arrest in several lung and head and neck cancer cell lines. However, the combination of perifosine with low concentrations of celecoxib rendered cells less sensitive to perifosine both in cell culture systems and in lung cancer xenograft models. Subsequently, we examined the effects of perifosine on COX-2 expression and activity in a set of lung and head and neck cancer cell lines, and found that perifosine rapidly and potently increased COX-2 levels and activity, the degrees of which correlated to the abilities of perifosine to inhibit the growth of cancer cells. We also detected increased COX-2 levels in lung cancer xenografts treated with perifosine. Moreover, blockage of COX-2 induction by both antisense and small interfering RNA approaches decreased cell sensitivity to perifosine. Collectively, these data indicate that the activation of COX-2 contributes to the anticancer activity of perifosine, including apoptosis induction and growth arrest. These data are clinically relevant as they suggest that the combination of perifosine and COX-2 inhibitors such as celecoxib, may produce a potential drug contradiction. [Mol Cancer Ther 2009;8(9):2575–85]

Introduction

Alkylphospholipids represent a class of antitumor agents that can induce apoptosis in tumors while sparing normal cells (1). These agents act at the cell membrane and disrupt membrane phospholipid metabolism (1). Miltefosine, the first alkylphospholipid to be tested in clinical trials, has been approved for topical treatment of cutaneous lymphomas and cutaneous metastases from breast cancer (2). Perifosine, in which the choline moiety of miltefosine is replaced by a cyclic aliphatic piperidyl residue, has greater oral bioavailability with limited gastrointestinal side effects and better activity in preclinical models when compared with miltefosine. Perifosine effectively inhibited the growth of a variety of human tumor cell lines, including melanoma, lung, prostate, colon, and breast cancer cells and suppressed the growth of mammary carcinomas induced by 7,12-dimethylbenz(a)anthracene in Sprague-Dawley rats (3). In addition, perifosine when combined with other cancer therapeutic agents exhibited enhanced apoptosis-inducing and anticancer activity (4–7). Perifosine is currently being tested in phase II clinical trials and has shown single-agent partial responses in certain types of cancer such as renal cell carcinoma (8).

How perifosine exerts its antitumor effect is not completely understood. Perifosine has been shown to inhibit the plasma membrane localization of Akt and Akt phosphorylation leading to antiproliferative effects in tumor cells (9). Likewise, perifosine causes cell cycle arrest in tumor cells by inducing p21WAF (10). Our recent study has shown that perifosine induces apoptosis in human lung cancer cells involving activation of the extrinsic death receptor–mediated apoptotic pathway (11).

The cyclooxygenase-2 (COX-2) inhibitor celecoxib is a marketed drug for use in humans for management of arthritis and acute pain as well as in patients with familial adenomatous polyposis for reduction of adenomatous colorectal polyps (12). In addition, celecoxib is currently being tested preclinically and clinically for its chemopreventive and therapeutic efficacy against a broad spectrum of epithelial malignancies, including lung cancers, either as a single agent or in combination with other agents (13, 14). In an effort to identify perifosine-based combination regimens, we unexpectedly found that celecoxib diminished the growth inhibitory effects of perifosine in human non–small cell lung cancer (NSCLC) and head and neck cancer cell lines.

COX-2 catalyzes the conversion of arachadonic acid to prostaglandins (PG). COX-2 expression is generally low or absent in most tissues, but its expression is inducible upon...
stimulation by such molecules as cytokines and growth factors (15). COX-2 is usually undetectable in most normal tissues, but is overexpressed in a variety of premalignant and malignant tissues (16). In general, COX-2 overexpression is associated with resistance to apoptosis, angiogenesis, tumorigenesis, and poor prognosis (15, 16), all of which are the scientific rationale for COX-2 as a preventive or therapeutic target of cancer. However, COX-2 has also been shown to suppress tumorigenesis in one model of skin cancer (17), to be associated with good prognosis in some studies (18–20), and to induce cell cycle arrest (21) and apoptosis (22–27).

Interestingly, another member of the alkylphospholipid family, edelfosine, up-regulates the expression of COX-2 which contributes to apoptotic death induced by edelfosine (28). Therefore, we investigated why celecoxib antagonized the anticancer effects of perifosine by understanding the role of COX-2 in perifosine-mediated anticancer activity, including cell cycle arrest and induction of apoptosis. Our results show that perifosine induces COX-2 expression, which contributes to perifosine-induced apoptosis and cell cycle arrest. Therefore, our study reveals a novel mechanism underlying the anticancer activity of perifosine that involves COX-2 activation.

Materials and Methods

Reagents
Perifosine (Fig. 1A) was supplied by Keryx Biopharmaceuticals, Inc. with a chemical purity of >99%. This agent was dissolved in PBS and stored at −20°C. Celecoxib was purchased from LKT Laboratories. The other COX-2 inhibitors SC-58125 and DuP697 were purchased from Cayman Chemical. The celecoxib derivative 2,5-dimethyl-celecoxib (DMC), which lacks COX-2 inhibitory activity (29), was provided by Dr. Axel H. Schönthal (University of Southern California, Los Angeles, CA). These agents were dissolved in DMSO and stored at −20°C. Stock solutions were diluted to the appropriate concentrations with growth medium immediately before use.

Cell Lines and Cell Culture
The human NSCLC cell lines used in this study and their culture conditions were described previously (30). The H157 stable cell line that expresses antisense COX-2 (H157-AS) and its matched vector control line (H157-V) were described previously (31) and provided by Dr. S. M. Dubinett (University of California, Los Angeles, CA). The head and neck cancer cell line 686Ln and its derived cell lines M4c, M4d, and M4e, which have high metastatic potential, were provided by Dr. Z. G. Chen in our institute (32).

Cell Viability 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 (SRB) assay, as previously described (30). The IC50 values were calculated from a dose-response curve.

Western Blot Analysis
Preparation of whole-cell protein lysates and Western blot analysis were described previously (33, 34). Mouse anti-caspase-3 monoclonal antibody was purchased from...
Imgenex. Rabbit anti-caspase-8, anti-caspase-9, and anti-poly(ADP-ribose) polymerase (PARP) polyclonal antibodies were purchased from Cell Signaling Technology. Rabbit anti-COX-2 polyclonal antibody was purchased from Oxford Biomedical. Rabbit anti-G3PDH polyclonal antibody was purchased from Trevigen. Rabbit anti-β-actin polyclonal antibody was purchased from Sigma. Secondary antibodies goat anti-mouse and goat anti-rabbit-horseradish peroxidase conjugates were purchased from Bio-Rad.

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-PE apoptosis detection kit (BD Bioscience) following the manufacturer’s instructions and analyzed by flow cytometry using the FACScan (Becton Dickinson).

Cell Cycle Assay
Control cells or cells treated with the indicated agents for 24 or 48 h were harvested, washed with cold PBS, and fixed in 70% ethanol. DNA was stained by incubating cells with PBS containing 50 μg/mL propidium iodide and 15 μg/mL RNase A for 20 min at room temperature. Fluorescence was measured using the FACScan and analyzed using CellQuest software (BD Biosciences).

ELISA for Detection of PGE2 and 15d-PGJ2
Cells were cultured in 60-mm dishes in culture medium supplemented with 5% fetal bovine serum and treated with perifosine alone, celecoxib plus perifosine, or celecoxib alone. After a 16-h treatment, medium was collected and measured for the levels of PGE2 using the ELISA kit from Cayman Chemical and for the levels of 15d-PGJ2 using the ELISA kit from Assay Designs following the manufacturer’s instructions. Because treatment with perifosine induces significant cell death, prostaglandin levels were normalized based on protein concentration for each sample after treatment.

Silencing of COX-2 Expression Using small interfering RNA (siRNA)
Silencing of COX-2 was achieved by transfecting siRNA using RNAiFect transfection reagent (Qiagen) following the manufacturer's instructions. Control Stealth siRNA oligonucleotides targeting the sequence 5’-GAATTCCCTCTCGCATT-3’ and COX-2 Stealth siRNA oligonucleotides targeting the sequence 5’-AAAGACTGGTATTTCATCTGGCTGC-3’ were purchased from Invitrogen. Cells were plated in 24-well plates and the next day transfected with siRNAs. Twenty-four hours later the cells were re-plated and the next day treated with perifosine as indicated. Gene silencing effects were evaluated by Western blot analysis as described above.

Lung Cancer Xenografts and Treatments
Animal experiments were approved by the Institutional Animal Care and Use Committee of Emory University. Four- to six-week-old (about 20 g of body weight) female athymic (nu/nu) mice (Taconic) were housed under pathogen-free conditions in microisolator cages with laboratory chow and water ad libitum. 5 × 10^6 H358 cells or H460 cells in PBS were injected s.c. into the flank region of nude mice. When tumors reached certain size ranges (50–100 mm^3), the mice were randomized into four groups (n = 6/group) according to tumor volumes and body weights for the following treatments: vehicle control, perifosine in PBS (20 or 30 mg/kg/d, oral gavage), celecoxib in DMSO (50 or 100 mg/kg/d; oral gavage), and the combination of perifosine and celecoxib. Tumor volumes were measured using caliper measurements once every 2 d and calculated with the formula \( V = \pi (\text{length} \times \text{width}^2) / 6 \). After a 5-, 6-, 10-, or 11-d treatment, the mice were sacrificed with CO2. The tumors were then removed, weighed, and frozen in liquid nitrogen or fixed with 4% formaldehyde. Certain portions of tumor tissues from each tumor were homogenized in protein lysis buffer for preparation of whole-cell protein lysates as described previously (30). Western blotting results were quantitated using NIH Image J (NIH).

Immunohistochemistry for Detection of COX-2 in Tumor Tissues
Five-micrometer formaldehyde-fixed, paraffin-embedded sections from H358 tumor xenografts were dewaxed, hydrated though graded alcohols, and microwaved in 100 mmol/L sodium citrate for 5 min on high power and 10 min on low power for antigen retrieval. Immunohistochemistry analysis was done following the protocol for the DakoCytomation EnVision + Dual Link System. Sections were incubated overnight at 4°C with rabbit polyclonal anti-COX-2 (1:500 dilution; Oxford Biomedical Research). COX-2-positive cells were counted using MetaMorph Imaging Software (Universal Imaging Corporation).

Statistical Analysis
The statistical significance of differences between two groups was analyzed with two-sided unpaired Student’s t tests when the variances were equal or with Welch’s corrected t test when the variances were not equal, by use of Graphpad InStat 3 software (GraphPad Software). Data were examined as suggested by the same software to verify that the assumptions for use of the t tests held. Results were considered to be statistically significant at \( P < 0.05 \). All statistical tests were two-sided.

Results
Perifosine Inhibits the Growth of Human NSCLC Cells through Induction of Apoptosis and Cell Cycle Arrest
Human NSCLC cell lines exhibited varied sensitivities to perifosine (Fig. 1B). Among these cell lines, H460 and H358 were the most sensitive to perifosine, whereas H226 was resistant to perifosine. Both A549 and H157 exhibited intermediate sensitivities to perifosine (Fig. 1B). Detection of apoptosis and cell cycle alteration revealed that H460 cells primarily underwent apoptotic cell death (84.8 ± 1.1% in perifosine-treated cells versus 7.7 ± 1.8% in PBS-treated cells), whereas H358 cells were very sensitive to G2-M arrest by perifosine (44.8 ± 2.9 versus 21.6 ± 1.5 in PBS-treated cells) with limited sensitivity to undergo apoptotic cell death (18.4 ± 2.4% in perifosine-treated cells versus 10.8 ± 3.9% in PBS-treated cells). No apoptosis but very weak G2-M arrest (25.8 ± 1.9% in perifosine-treated cells versus 22.9 ± 0.9% in control cells) was detected in H226 cells exposed to 10 μmol/L perifosine. A549 and H157 cells underwent both...
Figure 2. Celecoxib protects NSCLC cells from perifosine-induced decrease in cell survival (A) and apoptosis (B and C) and antagonizes the anticancer activity of perifosine in mouse xenograft models (D).

A, both H358 and H460 cells were treated with perifosine alone, celecoxib alone, or perifosine plus celecoxib at the indicated concentrations for 48 h. Cell survival was estimated using the SRB assay. Columns, means of four replicate determinations; bars, ± SD. *P values at all combination treatments are at least < 0.01 when compared with perifosine alone. B, H460 cells were treated with 10 μmol/L perifosine in the absence and presence of 10 μmol/L celecoxib for 24 h and analyzed for apoptosis by Annexin V staining. C, H460 cells were treated with 5 μmol/L perifosine in the absence or presence of increasing doses of celecoxib (5, 10, 20 μmol/L) for 12 h. The cells were harvested for preparation of whole-cell protein lysates and detection of caspase and PARP cleavage by Western blot analysis. CF, cleaved from.

D, the indicated lung cancer xenografts were treated with vehicle, 20 mg/kg perifosine, 50 mg/kg celecoxib, or the combination of celecoxib and perifosine for 9 d (H460) or 30 mg/kg perifosine, 100 mg/kg celecoxib, or the combination for 11 d (H358). Tumor sizes were measured once every 2 d. Tumor growth rates were calculated by comparing with the initial size of each individual tumor. Each measurement is a mean ± SE (n = 6). *, P < 0.05; **, P < 0.01; and ***, P < 0.001 compared with vehicle control. #, P < 0.05 compared with perifosine treatment. PRFS, perifosine; CCB, celecoxib.
Figure 3. Perifosine increases COX-2 expression (A) and prostaglandin production (B) in NSCLC cells and induces COX-2 expression in lung cancer xenografts (C and D). A, the indicated cell lines were treated with the given concentrations of perifosine for 8 h (top) or with 10 μmol/L perifosine for the indicated times (bottom). The cells were subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis. Actin served as a loading control. B, H460 cells were treated with the indicated concentrations of perifosine for 16 h and then subjected to estimation of PGE2 levels and 15d-PGJ2 levels in media using ELISA kits according to the manufacturers’ instructions. Columns, means of duplicate determinations; bars, ± SD. C, Western blot analysis of COX-2 expression in H358 tumor lysates after 6 d of treatment with vehicle control, 20 mg/kg perifosine, or 40 mg/kg perifosine by oral gavage in nude mice. These data were also quantitated using the NIH ImageJ software (bottom). D, immunohistochemistry staining of COX-2 expression in H358 tumors after 5 d of treatment with vehicle control or 30 mg/kg perifosine by oral gavage in nude mice. The COX-2–positive cells were scored using the MetaMorph Imaging Software and graphed (right). Left, the representative immunohistochemistry results of COX-2. The results in C and D are the means of triplicate tumors (n = 3). Bars, ± SDs.
G2-M arrest and apoptosis upon perifosine treatment \( (P < 0.05 \) or 0.01; Fig. 1C and D). Thus, it seems that perifosine induces apoptosis and/or G2-M arrest leading to inhibition of the growth of human NSCLC cells.

**Celecoxib Reduces the Anticancer Activity of Perifosine in Cell Culture and In vivo**

We were interested in enhancing the anticancer activity of perifosine. To this end, we tested the effects of perifosine in combination with other cancer therapeutic agents including celecoxib. Unexpectedly, the presence of celecoxib at the tested concentrations ranging from 5 to 20 \( \mu \)mol/L significantly attenuated perifosine-mediated growth-inhibitory effects in both H460 and H358 cells \( (P < 0.01 \) or 0.001; Fig. 2A). Similarly, other COX-2 inhibitors, including SC-58125 and DUP697, also significantly protected cells from perifosine-induced growth inhibition. However, the celecoxib derivative, DMC, which lacks COX-2-inhibitory activity, failed to protect cells from perifosine-induced cell death (Supplementary Fig. S1).

Furthermore, we analyzed the effects of perifosine on induction of apoptosis and G2-M arrest in the presence of celecoxib. Perifosine induced 50% of H460 cells to undergo apoptosis in the absence of celecoxib, but only 24% apoptosis in the presence of celecoxib (Fig. 2B). Perifosine alone caused cleavage of caspase-8, caspase-9, caspase-3, and PARP. However, the presence of celecoxib abrogated the ability of perifosine to cause cleavage of caspases and PARP (Fig. 2C). Thus, these results clearly show that celecoxib protects cells from perifosine-induced apoptosis.

Because H358 cells are more sensitive to G2-M arrest when treated with perifosine, we also examined the impact of celecoxib on the ability of perifosine to induce G2-M arrest in this cell line. We found that perifosine alone induced 70% of cells in G2-M phase, but only arrested 39% of cells in G2-M phase in the presence of celecoxib (Supplementary Fig. S2). Therefore, we conclude that celecoxib also protects cells from perifosine-induced cell cycle arrest.

We also determined whether celecoxib antagonized the anticancer efficacy of perifosine in vivo using lung cancer xenografts in mice. As presented in Fig. 2D, perifosine alone significantly inhibited the growth of both H460 and H358 xenografts \( (P < 0.05) \). Celecoxib alone at the tested doses (50 or 100 mg/kg) had minimal effects on the growth of either tumors. When perifosine was combined with celecoxib, perifosine lost its activity to inhibit the growth of lung tumors in both xenograft models \( (P < 0.05) \). Thus, it is apparent that celecoxib also antagonizes the anticancer activity of perifosine in vivo.

**Perifosine Increases COX-2 Expression and Activity in NSCLC Cells and in Lung Cancer Xenografts**

Because the addition of celecoxib could abrogate perifosine-induced cell death and growth arrest, we next determined whether perifosine modulated COX-2 expression and activity in NSCLC cells. By Western blot analysis, we detected dose-dependent increases in COX-2 expression and activity in NSCLC cells. By Western blot analysis, we detected dose-dependent increases in COX-2 expression and activity in NSCLC cells.
(Fig. 3A) and are the most sensitive to perifosine (Fig. 1B), whereas the H226 cells that did not show COX-2 induction after perifosine treatment were the least sensitive to perifosine (Fig. 1B), suggesting that COX-2 induction by perifosine correlates with sensitivity to perifosine. Importantly, the induction of COX-2 in the H460 and the H358 cells occurred as early as 6 hours after perifosine treatment (Fig. 3A, bottom), suggesting that COX-2 induction is an early event in response to perifosine treatment. Perifosine had no effect on the expression of COX-1 in H460 or H358 cell lines (data not shown). In addition, significantly increased levels of the prostaglandins, PGE2 and 15d-PGJ2, were detected in cells exposed to perifosine (P < 0.05 or 0.01; Fig. 3B), indicating that perifosine increases COX-2 activity as well. Collectively, these results show that perifosine increases COX-2 expression and activity in human NSCLC cells.

To determine whether perifosine increased COX-2 expression in vivo, we treated H358 lung cancer xenografts with perifosine or vehicle control and detected COX-2 levels in these xenografts by both Western blotting (6-day treatment) and immunohistochemistry (5-day treatment). As we observed in cell cultures, we detected significant increased levels of COX-2 by Western blot analysis (Fig. 3C) and immunohistochemistry (Fig. 3D) in perifosine-treated xenografts compared with vehicle-treated tumors (P < 0.05). Thus, perifosine also increases COX-2 levels in tumor tissues in vivo.

Celecoxib Inhibits Perifosine-Induced COX-2 Expression and Activity

To determine whether celecoxib at the tested concentrations indeed inhibits perifosine-induced COX-2 activation, we analyzed the effects of perifosine on the production of PGE2 and 15d-PGJ2 in the presence and absence of celecoxib. As presented in Fig. 4A, perifosine alone increased the levels of PGE2 and 15d-PGJ2; however, addition of celecoxib significantly inhibited the perifosine-induced increase in PGE2 and 15d-PGJ2 (P < 0.05). Moreover, we found that the presence of celecoxib also abrogated the ability of perifosine to increase COX-2 expression (Fig. 4B). Collectively, these results clearly indicate that celecoxib at the tested concentration ranges inhibits perifosine-induced COX-2 activation.

Blockade of COX-2 Induction via Genetic Manipulations Attenuates the Anticancer Activity of Perifosine

To robustly show the role of COX-2 induction on the anticancer activity of perifosine, we further evaluated the ability of perifosine to inhibit the growth of NSCLC cells when COX-2 induction was blocked using either antisense or gene silencing approaches. Perifosine exerted a dose-dependent

![Figure 5](https://example.com/fig5.png)

**Figure 5.** Inhibition of COX-2 induction by expression of antisense COX-2 (A and B) or transfection of COX-2 siRNA (C and D) protects against perifosine-induced growth inhibition. A, Western blot analysis of COX-2 expression in H157 vector control (H157-V) and H157 antisense COX-2 (H157-AS) cells after treatment with the indicated concentrations of perifosine for 8 h. B, the indicated cell lines were treated with the given concentrations of perifosine for 24 or 48 h. The cells were subjected to estimation of cell survival using the SRB assay. Points, means of four replicate determinations; bars, ± SD. ***, P < 0.01. ****, P < 0.001; and *****; P < 0.0001 compared with H157-P or H157-V. C and D, H460 cells were transfected with control siRNA (Ctrl) or COX-2 siRNA using RNAiFect transfection reagent for 48 h and then treated with 10 μmol/L perifosine. After 24 h, the cells were subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis for the given proteins (C) or to Annexin V staining and flow cytometry for detection of apoptosis (D). Columns, means of duplicate determinations; bars, ± SD. ***, P < 0.01 compared with perifosine in control siRNA–transfected cells.
effect on COX-2 induction in vector control H157-V cells; however, this effect was substantially abrogated in H157-AS cells, which express antisense COX-2 gene (Fig. 5A). Accordingly, the H157-AS cells were significantly less sensitive to perifosine compared with parental (H157-P) or H157-V cells (P < 0.01 or less; Fig. 5B). Because H157 cells are more susceptible to undergo G2-M arrest upon perifosine treatment (Fig. 1), we also compared the effects of perifosine on G2-M arrest in these cell lines. As expected, the effect of perifosine on G2-M arrest was abrogated in H157-AS cells compared with H157-P and H157-V cells (Supplementary Fig. S3). In addition, we silenced COX-2 expression in H460 cells through transfection of COX-2 siRNA and then examined its impact on the apoptosis-inducing effect of perifosine. Transfection of COX-2 siRNA successfully inhibited perifosine-mediated COX-2 induction (Fig. 5C). Correspondingly, we detected much less cleaved PARP (Fig. 5C) and Annexin V-positive cells (Fig. 5D) in COX-2 siRNA-transfected cells than in control siRNA-transfected cells. Specifically, we detected 29.6 ± 0.8% of apoptotic cells in

![Figure 6](https://example.com/fig6.png)

**Figure 6.** Effects of perifosine on the growth of human head and neck cancer cells (A) and COX-2 expression (B) and protective effects of celecoxib on perifosine-induced decrease in cell survival (C) and apoptosis (D). A, the indicated cell lines were treated with increased concentrations of perifosine (1–20 μmol/L) for 3 d and cell numbers were estimated using the SRB assay. B, the indicated cell lines were treated with the given concentrations of perifosine for 8 h (top). The cells were subjected to preparation of whole-cell protein lysates and subsequent Western blot analysis. Actin served as a loading control. C, the indicated cell lines were treated with the given concentrations of perifosine alone, celecoxib alone, and their respective combinations for 48 h and then subjected to the SRB assay for estimation of cell survival. Columns, means of four replicate determinations; bars, ± SD. **P** < 0.01 and ***P** < 0.001 compared with perifosine alone. D, the indicated cell lines were treated with 5 μmol/L perifosine alone, 10 μmol/L celecoxib alone, or their combination for 48 h (left). The cells were harvested and subjected to flow cytometric analysis for measuring apoptosis using Annexin V staining (left). In addition, the same treatments were done in M4c cells for 24 h to prepare whole-cell protein lysates for detection of the indicated proteins by Western Blot analysis (right). Tubulin served as a loading control. CF, cleaved form.
control siRNA–transfected cells, but only 15.8 ± 0.2% of apoptotic cells in COX-2-transfected cells (Fig. 5D), which is significantly less than that in control siRNA–transfected cells (P < 0.01). Collectively, these results provide a robust support for a critical role of COX-2 activation in mediating the anticancer activity of perifosine.

**Perifosine Also Induces COX-2-Dependent Apoptosis in Head and Neck Cancer Cells**

To determine whether perifosine exerts COX-2-dependent apoptosis and shows antagonism with celecoxib in other types of cancer cells, we further examined the effects of perifosine on COX-2 expression in 666Ln and its derivative cell lines M4c, M4d, and M4e, which exhibited differential responses to perifosine (Fig. 6A). As we found in the NSCLC cell lines, the M4c, M4d, and M4e cell lines which expressed much higher levels of COX-2 after perifosine treatment compared with the 666Ln cell line (Fig. 6B) were much more sensitive to perifosine than were the 666Ln cells (Fig. 6A). Perifosine was also able to induce COX-2 expression in the M4e cells as early as 3 hours, showing that COX-2 induction is an early event. The induction of COX-2 reached peak levels at 6 hours and was maintained up to 24 hours, indicating that COX-2 induction is also a sustained event (Supplementary Fig. S4). We next examined the effects of perifosine combined with celecoxib on the growth of these head and neck cancer cells including examination of apoptosis. As presented in Fig. 6C, in the presence of celecoxib at the tested concentrations ranging from 5 to 20 μmol/L, perifosine-mediated growth-inhibitory effects were significantly attenuated in both M4c and M4e cells (P < 0.01 or 0.001). The addition of celecoxib also substantially reduced apoptosis induced by perifosine as was examined by Annexin V analysis and cleavage of caspase-3 and PARP (Fig. 6D). Thus, the protection from perifosine-induced cell death by the addition of celecoxib is also clearly shown in human head and neck cancer cell lines. Moreover, the presence celecoxib abrogated perifosine-induced COX-2 expression (Fig. 6D, right), further supporting an important role of COX-2 in perifosine-induced apoptosis.

**Discussion**

Induction of apoptosis and cell cycle arrest by perifosine in other types of cancer cells was documented previously (10, 35, 36). Perifosine was reported to induce both G1 and G2-M arrest in glioma cells and head and neck squamous carcinoma cells (10, 35). However, we found that all of the tested human NSCLC cell lines that responded to perifosine underwent G2-M arrest albeit with various degrees, indicating that perifosine primarily induces G2-M arrest in human NSCLC cells. Given that perifosine also induces apoptosis in these cell lines, we conclude that perifosine induces both apoptosis and G2-M arrest, leading to inhibition of the growth of human NSCLC cells.

Importantly, the current study reveals a novel and unique mechanism underlying perifosine-mediated anticancer activity in human NSCLC and head and neck cancer cells which is COX-2–dependent. Specifically, we show that perifosine induces apoptosis and/or cell cycle arrest in human cancer cells through induction and activation of COX-2 based on the following compelling evidence: First, perifosine increases COX-2 expression in both cell cultures and in mouse lung cancer xenografts. The degree of COX-2 induction is associated with the potency of perifosine in inhibiting the growth of cancer cells. Second, the COX-2-specific inhibitor celecoxib as well as other COX-2 inhibitors suppress perifosine-induced COX-2 up-regulation and activation and protect cells from perifosine-induced apoptosis or G2-M arrest. In addition, celecoxib’s derivative, DMC, which lacks a COX-2-inhibitory effect, failed to abrogate the antitumor effect of perifosine, suggesting that induction of COX-2 activity is important in mediating apoptosis and cell cycle arrest induced by perifosine. Finally, blockade of COX-2 induction using either antisense or siRNA approaches attenuates the ability of perifosine to induce growth arrest or apoptosis. To our knowledge, this is the first study to show a critical role of COX-2 activation in mediating the anticancer activity of perifosine.

Given that previous studies have shown proapoptotic or tumor suppressive roles of COX-2 in various model systems (17, 21–25, 28), our current finding on the critical role of COX-2 activation in mediating the anticancer activity of perifosine should not be surprising. The fundamental questions of how COX-2 mediates perifosine-induced apoptosis or cell cycle arrest and how perifosine increases COX-2 expression are of interest for further exploration in the future. 15d-PGJ2, one of the prostaglandins generated by COX-2, can act as a PPARγ ligand and is proapoptotic (37). A recent study suggests that edelfosine, another alkylphospholipid, induces COX-2–dependent apoptosis through production of 15d-PGJ2 (28). They showed that edelfosine could induce the expression and transcriptional activity of PPARγ in H-ras–transformed human breast epithelial cells, suggesting that 15d-PGJ2 may be acting through PPARγ to induce apoptosis. In our study, we detected increased levels of 15d-PGJ2 in cells exposed to perifosine, but perifosine had no effect on the transcriptional activity of PPARγ (data not shown). Another recent study showed that the chemotherapeutic agents paclitaxel, cisplatin, and 5-fluorouracil could induce COX-2 expression in cervical carcinoma cells, and the inhibition of COX-2 by siRNA or the addition of the selective COX-2 inhibitor, NS-398, rendered the cells less sensitive to apoptosis induced by these chemotherapeutic agents (26). They showed that the addition of 15d-PGJ2–induced apoptosis and inhibition of lipocalin-type PGD synthase, which contributes to prostaglandin synthesis, could prevent the chemotherapeutically induced apoptosis. Therefore, whether the production of prostaglandins, specifically, 15d-PGJ2, directly contributes to perifosine-induced COX-2–dependent apoptosis in our system needs further investigation. Our unpublished data show that perifosine increased the activity of the COX-2 promoter, suggesting that perifosine likely induces COX-2 expression at the transcriptional level. Therefore, a detailed analysis of the COX-2 promoter may help us reveal the mechanism by which perifosine increases COX-2 expression.
In this study, perifosine-induced caspase-8 activation is clearly inhibited by celecoxib (Fig. 2C). We previously showed that perifosine induces DR5 expression which contributes to perifosine-induced apoptosis (11). Moreover, it has been shown that 15d-PGJ2 increases DR5 expression (38). Thus, it is plausible to speculate a link between perifosine-induced COX-2 and DR5 up-regulation. Our preliminary data indeed show that celecoxib is able to abrogate perifosine-induced DR5 expression. Our ongoing work in this direction may shed light on the mechanisms underlying COX-2-induced apoptosis.

We did not see complete protection from perifosine-induced cell killing or arrest with celecoxib, or blockade of COX-2 induction using either an antisense or gene knockdown strategy, suggesting that COX-2 is not the only mechanism mediating the anticancer activity of perifosine in human NSCLC cells. It has been documented that perifosine inhibits Akt activity (9, 36). Our own study shows that perifosine inhibits Akt activity leading to perifosine-induced apoptosis in human NSCLC cells (11). Thus, further exploration of the relationship between COX-2 activation and Akt inhibition is also of interest in the future.

Celecoxib is a prescribed drug for treatment of inflammation and pain. In the field of oncology, celecoxib is an approved drug in the clinic for the adjuvant treatment of familial adenomatous polyposis, an inherited syndrome that predisposes individuals to colon cancer. In addition, celecoxib has been tested in various clinical trials for either cancer chemoprevention or therapy (13, 16). The clinically achievable peak plasma concentrations of celecoxib in humans after oral administration of a single dose of 800 mg are approximately 3.2 to 5.6 μmol/L (39). At these concentration ranges, celecoxib significantly attenuated the growth-inhibitory effects of perifosine. Consistently, celecoxib significantly diminished the activity of perifosine against the growth of lung cancer xenografts in mouse models (Fig. 2). Collectively, these results clearly indicate that celecoxib antagonizes the anticancer activity of perifosine both in vitro and in vivo. As mentioned above, the COX-2 inhibitor NS 398 can render cervical carcinoma cells less sensitive to apoptosis induced by chemotherapeutic agents (26). These data and our current study add to the clinical relevance of COX-2 inhibition by non-steroidal anti-inflammatory drugs in patients also being administered anticancer agents. The immediate clinical impact of our study is that caution should be taken when combining COX-2 inhibitors such as celecoxib with perifosine, so as not to inhibit the efficacy of perifosine. Given that COX-2 induction seems to be associated with cell sensitivity to perifosine-induced apoptosis or cell cycle arrest as shown in our study, it may also be important in the clinical practice to consider COX-2 induction or activity as a predictive biomarker for perifosine-based cancer therapy.

**References**


1 Elrod and Sun, unpublished data.


Molecular Cancer Therapeutics

Celecoxib antagonizes perifosine's anticancer activity involving a cyclooxygenase-2-dependent mechanism

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