Radiosensitization of Squamous Cell Carcinoma by the Alkylphospholipid Perifosine in Cell Culture and Xenografts

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Abstract

Purpose: Combined modality treatment has improved outcome in various solid tumors. Besides classic anticancer drugs, a new generation of biological response modifiers has emerged that increases the efficacy of radiation. Here, we have investigated whether perifosine, an orally applicable, membrane-targeted alkylphospholipid, enhances the antitumor effect of radiation *in vitro* and *in vivo*.

Experimental Design: Several long-term and short-term *in vitro* assays (clonogenic survival, sulforhodamine B cytotoxicity, apoptosis, and cell cycle analysis) were used to assess the cytotoxic effect of perifosine in combination with radiation. *In vivo*, the response of human KB squamous cell carcinoma xenografts was measured after treatment with perifosine, irradiation, and the combination. Radiolabeled perifosine was used to determine drug disposition in tumor and normal tissues. At various intervals after treatment, tumor specimens were collected to document histopathologic changes.

Results: *In vitro*, perifosine reduced clonogenic survival, enhanced apoptosis, and increased cell cycle arrest after radiation. *In vivo*, radiation and perifosine alone induced a dose-dependent tumor growth delay. When combining multiple perifosine administrations with single or split doses of radiation, complete and sustained tumor regression was observed. Histopathologic analysis of tumor specimens revealed a prominent apoptotic response after combined treatment with radiation and perifosine. Radiation-enhanced tumor response was observed at clinically relevant plasma perifosine concentrations and accumulating drug disposition of >100 μg/g in tumor tissue.

Conclusions: Perifosine enhances radiation-induced cytotoxicity, as evidenced by reduced clonogenic survival and increased apoptosis induction *in vitro* and by complete tumor regression *in vivo*. These data provide strong support for further development of this combination in clinical studies.

Alkylphospholipids, such as alkylphosphocholines and alkyl-lysophospholipids, have been identified as synthetic antitumor agents that, in contrast to most classic chemotherapeutic drugs, primarily accumulate in the cell membrane (1). There, they interfere with signal transduction pathways and subsequently affect multiple cellular processes, including apoptosis, proliferation, and survival. Despite encouraging preclinical results, clinical use of alkylphospholipids has been limited due to severe

gastrointestinal (2, 3) and hemolytic toxicity (4). The alkylphosphocholine prototype miltefosine (hexadecylphosphocholine) is currently used as a topical formulation against cutaneous lymphomas and breast cancer metastases (5) and as an oral treatment against leishmaniasis (6). Perifosine [D-21266; octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate], a heterocyclic analogue of miltefosine, has been evaluated as an oral anticancer drug. Initial studies showed an improved therapeutic index in preclinical models (7). In several phase I and pharmacokinetic studies, gastrointestinal adverse effects were reported as dose-limiting toxicity (8, 9). Recently, phase II studies in patients with metastatic or recurrent melanoma (10) and androgen-independent prostate cancer (11) revealed no objective responses after treatment with perifosine as single agent.

Combined modality treatment has led to improved treatment results in patients with advanced solid tumors, as has been shown in several clinical studies during the last decade. In particular, the concurrent use of radiotherapy and chemotherapy resulted in reduced recurrence rates and improved survival and has become standard therapy in advanced head and neck, lung, cervical, and anal cancer (12). The combination of these classic anticancer regimens with novel biological response modifiers has emerged as an attractive strategy to further increase tumor response and limit normal tissue toxicity (13, 14). Based on their

potential to modulate signal transduction pathways involved in apoptosis, proliferation, and survival, alkylphospholipids are attractive candidates for such a combined modality approach. Indeed, perifosine shows synergistic cytotoxicity *in vitro* when combined with other cytotoxic drugs [e.g., the cyclin-dependent kinase antagonist UCN-01, 7-hydrostaurosporine (15) and histone deacetylase inhibitors (16)]. In addition, several alkyl-phospholipids have been shown to enhance radiation-induced cell death in a variety of tumor types *in vitro*. Erucylphosphocho-line enhanced radiation-induced apoptosis in glioblastoma cells (17), whereas edelfosine (Et-18-O-CH$_3$), miltefosine, and perifosine increased radiation-induced apoptosis in human leukemic cells (18). Furthermore, miltefosine and perifosine showed radiosensitizing properties in human squamous cell carcinomas (13, 19). These findings have led us to the design of a phase I trial in patients with solid tumors, where radiotherapy will be combined with daily intake of perifosine (20).

Perifosine acts on multiple cellular targets that contribute to the mechanism of enhanced radiation-induced cell death. The increased apoptotic response of U937 leukemic cells and Jurkat T cells treated with alkylphospholipids has been shown to depend on the activation of stress-activated protein kinase/c-Jun NH$_2$-terminal kinase (18). Moreover, these drugs were found to interfere with signaling pathways crucial for cell survival, like the protein kinase B (21, 22), protein kinase C (23, 24), and mitogen-activated protein kinase (25, 26) signaling cascades. More recently, perifosine was identified as a potent cyclin-dependent kinase 2 inhibitor, causing a p53-independent but p21-dependent cell cycle arrest (27). In this context, it has been suggested that inhibition of cyclin-dependent kinase activity may promote apoptosis, depending on the cellular context (28).

Thus far, improved efficacy of radiotherapy combined with alkylphospholipids has been limited to *in vitro* studies. We recently showed a high degree of metabolic stability of perifosine after oral administration and a relatively high drug uptake in a panel of squamous cell carcinomas *in vivo* (29). Here, we have studied the effect of perifosine treatment in combination with ionizing radiation on different determinants of cytotoxicity *in vitro* and antitumor response *in vivo*, using the alkylphosphocholine-responsive KB tumor model.

Materials and Methods

Antibodies. Antibody against active caspase-3 used for flow cytometry was purchased from BD Biosciences (San Jose, CA). FITC-labeled goat anti-rabbit IgG antibody was purchased from Molecular Probes, Inc. (Eugene, OR). Mouse anti-bromodeoxyuridine was purchased from DakoCytomation (Glostrup, Denmark). Anti-mouse IgG-FITC was derived from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Cleaved caspase-3 (Asp^{175})–specific antibody and labeled polymer-horseradish peroxidase anti-rabbit used for immunohisto-chemistry were purchased from Cell Signaling Technology (Beverly, MA) and DakoCytomation (Carpinteria, CA), respectively.

Reagents. Perifosine and [2,6-^{14}C]perifosine (66.8 μCi/mg) were kindly provided by Zentaris AG (Frankfurt, Germany). Crystal violet and glutardialdehyde were obtained from Merck KgaA (Darmstadt, Germany). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture. The human head and neck squamous cell carcinoma cell line KB, routinely tested for absence of *Mycoplasma*, was cultured in DMEM supplemented with 50 units/mL penicillin, 50 μg/mL strepto-mycin, and 10% fetal bovine serum.

Clonogenic survival assay. Cells (200-3,200) in 10 mL DMEM were plated in 8-cm diameter dishes, incubated for 4 hours for the cells to attach, and irradiated using a Pantak X-ray machine, operating at 250 kV$_p$ and 12 mA with a 0.6-mm Cu filter with a dose rate ranging from 0.9 to 1.7 Gy/min. Perifosine was added at a final concentration of 0.4 μmol/L, immediately before irradiation. After 3 days, medium was removed and replaced with either control medium or with medium containing 0.4 μmol/L perifosine. Cells were allowed to form colonies over a period of 14 days after irradiation, which were subsequently fixed and stained by 0.2% crystal violet/2.5% glutardialdehyde. The number of colonies were counted with a Colcount (Oxford Optronix, Oxford, United Kingdom) and visually confirmed under a light microscope to contain at least 50 cells. Cell survival was corrected for plating efficiency.

Sulforhodamine B cytotoxicity assay. KB cells (500) in 200 μL/well were plated in 96-well plates. After perifosine was added in serial dilutions, the plates were irradiated (0-8 Gy). After 5 days of incubation, cells were washed and stained with sulforhodamine B (30). Extinction was measured at 540 nm with a microplate reader (Bio-Tek Instruments, Winooski, VT). The data were fitted to a sigmoidal concentration-response curve, and IC$_{50}$ calculation was done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). For each radiation dose, control wells (medium) were set at 100% survival.

Apoptosis measurement. KB cells (1.25 × 10^4 per well) were plated in six-well plates in 2 mL DMEM (10% FCS) and incubated overnight to allow the cells to attach. Perifosine was added, and the cells were irradiated using a ^{137}Cs radiation source at an absorbed dose rate of ~ 1 Gy/min. After 120 hours, cells and supernatant were collected, washed, and resuspended in Nicoletti buffer (ref. 31; 50 μg propidium iodine/mL, 0.1% sodium citrate, 0.1% Triton X-100). The apoptotic fraction was assessed as the percentage of cells present in the sub-G$_1$ population. To confirm the findings by nuclear staining, cells were alternatively stained for active caspase-3. In brief, cells were fixed in 4% formal-dehyde/PBS and permeabilized in 0.1% saponin/0.5% bovine serum albumin/PBS. Thereafter, cells were incubated with a rabbit-anti–active caspase-3 antibody (1:50) and stained with goat-antirabbit FITC (1:100). All measurement were done using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Cell cycle analysis. KB cells (2.5 × 10^5 per well) were plated in six-well plates and incubated overnight. Treatment consisted of either addition of perifosine at a final concentration of 2 μmol/L, irradiation to 5 Gy, or the combination. After 8, 24, and 48 hours of incubation, cells were labeled with IUdR as described previously (32). In brief, nuclei were isolated and incubated with a mouse anti-bromodeoxyuridine antibody, which also binds to IUdR (1:50), followed by 30 minutes of incubation with a FITC-conjugated anti-mouse antibody (1:50). Finally, the nuclei were incubated with propidium iodine to stain total DNA. Flow cytometry was carried out using a FACScan flow cytometer.

In vivo tumor growth delay assay. Female BALB/c *nu/nu* mice, 6 to 10 weeks old (18-28 g), were obtained from the animal department of the Netherlands Cancer Institute. Animals were kept and handled according to institutional guidelines complying with Dutch legislation under a 12/12-hour light/dark cycle at a temperature of 22°C, receiving a standard diet and acidified water *ad libitum*. Mice were injected s.c. at the lower back with 3 × 10^6 KB cells in 50 μL PBS, and tumor volume was measured regularly, using calipers. Tumor size was calculated using the formula: volume = π/6 × length × width × height, where tumor volume at the start of treatment was normalized to 100%. When the tumor reached a mean diameter of ~ 6 mm (measured in three orthogonal directions), treatment was started. Four treatment groups (*n* = 5-9 animals/group) were distinguished: Control (no perifosine, no radiation), perifosine (oral administration), radiotherapy (local tumor irradiation), and combined therapy (oral administration of perifosine and local tumor irradiation). *Drug adminstration:* Mice received, by way of gastric intubation, one to three oral doses of 40 mg/kg perifosine every 48 hours. Control animals received a PBS injection, orally. *Irradiation:* Animals treated according to a combined treatment schedule were irradiated 48 hours after the first perifosine administration. This time

interval corresponds to approximately the t_{max} in tumor tissue after a single administration of 40 mg/kg (29). For irradiation, mice were immobilized in custom designed jigs, which allowed specific irradiation of the dorsal tumor while shielding the rest of the animal. Irradiations were carried out using a Pantak X-ray machine, with a dose rate of ~ 4 Gy/min. To ensure homogeneous dose distribution, mice were rotated through 180 degrees half way during the irradiation procedure.

Histopathologic analysis. At 96, 120, and 144 hours after start of treatment, animals were sacrificed, and tumors were excised, fixed in ethanol/acetic acid/formol saline fixative (40:5:10:45, v/v), embedded in paraffin, and sectioned at 3 to 4 μm onto slides. Sections were stained using an antibody against cleaved caspase-3 (1:100) and a labeled polymer-horseradish peroxidase antirabbit, according to standard protocols. The percentage of cells expressing caspase-3 was determined by counting immunoreactive cells in three different optical fields. Because the KB tumors grow rapidly and show central areas of necrosis when untreated, these analyses were done on the peripheral rim of vital tumor tissue.

Tumor and normal tissue pharmacokinetics. Mice bearing s.c. KB tumors (with an initial mean diameter of at least 6 mm) received one, two, or three doses of 40 mg/kg perifosine, traced with [14C]perifosine (0.05 μCi/g) dissolved in PBS in a volume of 5 μL/g body weight. At various time points after administration, mice were anesthetized, and blood was collected by way of a heart puncture and sacrificed by cervical dislocation. Blood was centrifuged at 14,000 rpm for 5 minutes (4°C), and plasma was collected. Tumors and major organs were excised and dissolved in 1 to 6 mL Solvable (Packard Instrument Co., Groningen, the Netherlands) at 60°C overnight, bleached with 30% hydrogen peroxide, and diluted in Ultima Gold scintillation liquid (PerkinElmer,

Wellesley, MA). All [14C]perifosine measurements were done using a TRI-CARB liquid scintillation analyzer. The area under the curve up to the last measured concentration-time point was determined by applying the linear-logarithmic trapezoidal method.

Results

In vitro results

Perifosine-induced radiosensitization is dependent on prolonged drug exposure. The effect of drug exposure time on the clonogenic capacity of KB cells after radiation was determined by applying a 3-day and a 14-day exposure to 0.4 μmol/L perifosine. Continuous exposure to this drug concentration reduced the plating efficiency by 36 ± 11% compared with the untreated cells. Incubation of the cells with perifosine for 14 days reduced clonogenic survival after irradiation. At doses of ≥6 Gy, this reduction was statistically significant. This prolonged exposure time seemed to be essential, because removal of perifosine 3 days after irradiation led to loss of this radiosensitizing effect (Fig. 1A).

Radiation increases sensitivity of KB cells to perifosine. To test whether irradiation enhanced the sensitivity of KB cells to perifosine, dose-response curves and corresponding IC$_{50}$s of KB cells treated with increasing doses of irradiation were obtained using the sulforhodamine B cytotoxicity assay. Whereas nonirradiated cells showed an IC$_{50}$ of 0.38 ± 0.04 μmol/L after 5 days of incubation, radiation induced a dose-dependent
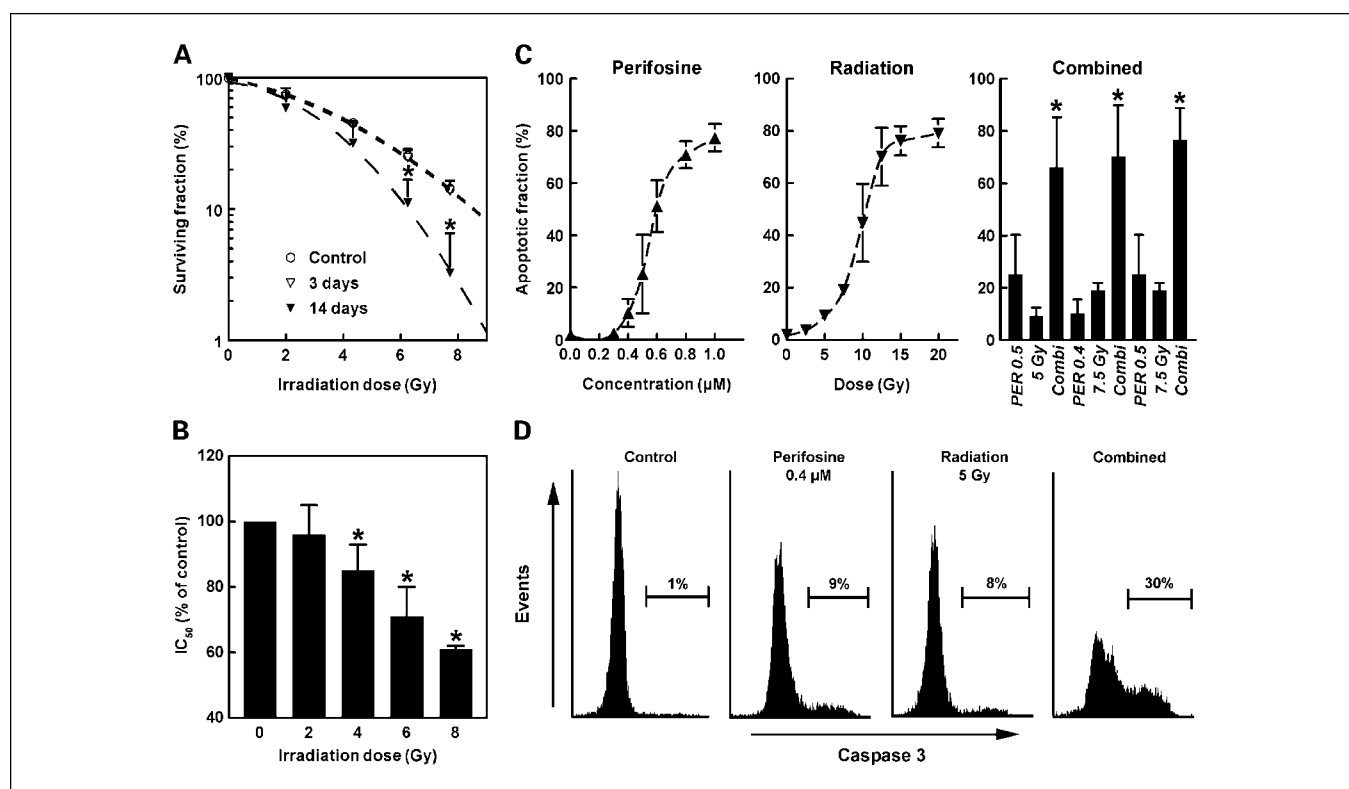

Fig. 1. *In vitro* cytotoxicity induced by perifosine combined with radiation. *A,* radiosensitization is dependent on prolonged exposure time after radiation. KB cells were irradiated in the absence (control) or in the presence of 0.4 μmol/L perifosine and exposed to the drug for either 3 or 14 days. At day 14, cultures were fixed and stained for assessment of colony formation. Note: Markers representing survival of untreated cells (control) and cells exposed to perifosine for 3 days almost completely overlap. *B,* radiation increases the sensitivity of KB cells to perifosine. Dose-response curves were generated after a 5-day incubation period with a serial dilution of perifosine combined with 0 to 8 Gy radiation. Expressed is the relative perifosine sensitivity after irradiation (IC$_{50}$ irradiated cells/IC$_{50}$ nonirradiated cells). *C,* perifosine enhances radiation-induced apoptosis. KB cells were treated with perifosine, radiation, or a combination at doses indicated. After 5 days, cells were stained for DNA content by propidium iodine, and nuclear fragmentation was quantified using flow cytometry. *D,* apoptosis analyzed by the detection of active caspase-3–positive cells, 5 days after treatment. *Points/columns,* means; *bars,* SD. *, *P* < 0.05, one-tailed Student's *t* test.

decrease of the IC$_{50}$ down to 0.23 ± 0.02 μmol/L for KB cells treated with 8-Gy irradiation. This corresponds to a maximum decrease of 39 ± 1% (Fig. 1B).

Perifosine enhances radiation-induced apoptosis. The effect of perifosine, radiation, and the combination on apoptosis induction was assessed using flow cytometry. Both nuclear fragmentation with propidium iodine staining and caspase-3 staining using an active caspase-3–specific antibody were measured. Both perifosine and radiation induced a significant dose-dependent apoptotic response. When radiation and perifosine were combined, the number of apoptotic cells was strongly increased and resulted in a more than additive effect in the dose range between 0.3 and 0.6 μmol/L perifosine (Fig. 1C). Similar results were obtained when cells were treated with perifosine, radiation, or the combination and stained with an active caspase-3–specific antibody (Fig. 1D). It should be noted that the steep dose-response relationship of KB cells after treatment with perifosine or radiation hampers the calculation of a supra-additive interaction between both stimuli over the full dose ranges according to the concept of Steel and Peckham (35).

Perifosine prolongs radiation-induced cell cycle arrest, mainly in G$_2$. Cell cycle perturbations induced by treatment with either perifosine, radiation, or a combination were analyzed using IUdR labeling and flow cytometry. Representative dot plots at 24 hours after treatments are shown in Fig. 2A. At this time point, the most pronounced cell cycle arrest was observed after combined treatment. Both perifosine and radiation caused a block in G$_2$-M and a decrease in S phase. The S-phase population was reduced by ~80%, whereas the G$_2$-M population increased with >300% compared with control cells. At 48

hours after treatment, the cell cycle distribution after irradiation was restored, whereas perifosine and combined treated cells still displayed an impaired cell cycle progression (Fig. 2B).

In vivo results

Perifosine enhances the antitumor effect of radiation. To investigate whether perifosine improves the tumor response after radiation *in vivo*, BALB/c nude mice bearing KB tumor xenografts were treated with either perifosine, radiation, or with both modalities, and tumor size was measured regularly. Table 1 shows the normalized tumor growth delay. Three treatment schedules were used to determine the *in vivo* enhancement of radiation by orally administered perifosine. In the first schedule, 10-Gy irradiation induced a growth delay of ~21 days; a single dose of 40 mg/kg was ineffective. This drug dose did not lead to enhancement of the radiation effect (Fig. 3A), although one complete remission occurred. The second schedule involved two administrations of 40 mg/kg with a 48-hour interval; 10-Gy irradiation was applied immediately after the second administration. Two doses of 40 mg/kg perifosine led to a substantial growth delay of 8 days. Again, a radiation dose of 10 Gy induced a substantial growth delay (21 days). Two administrations of perifosine combined with 10-Gy irradiation led to complete remission of the KB tumor in six of seven animals (Fig. 3B). The combined therapy of 10-Gy irradiation and perifosine was more effective than a single irradiation dose of 13 Gy, which ultimately resulted in regrowth of the tumors in six of eight animals (Fig. 3B). This corresponds with an enhancement factor of at least 1.3. The third schedule involved a split dose radiation consisting of
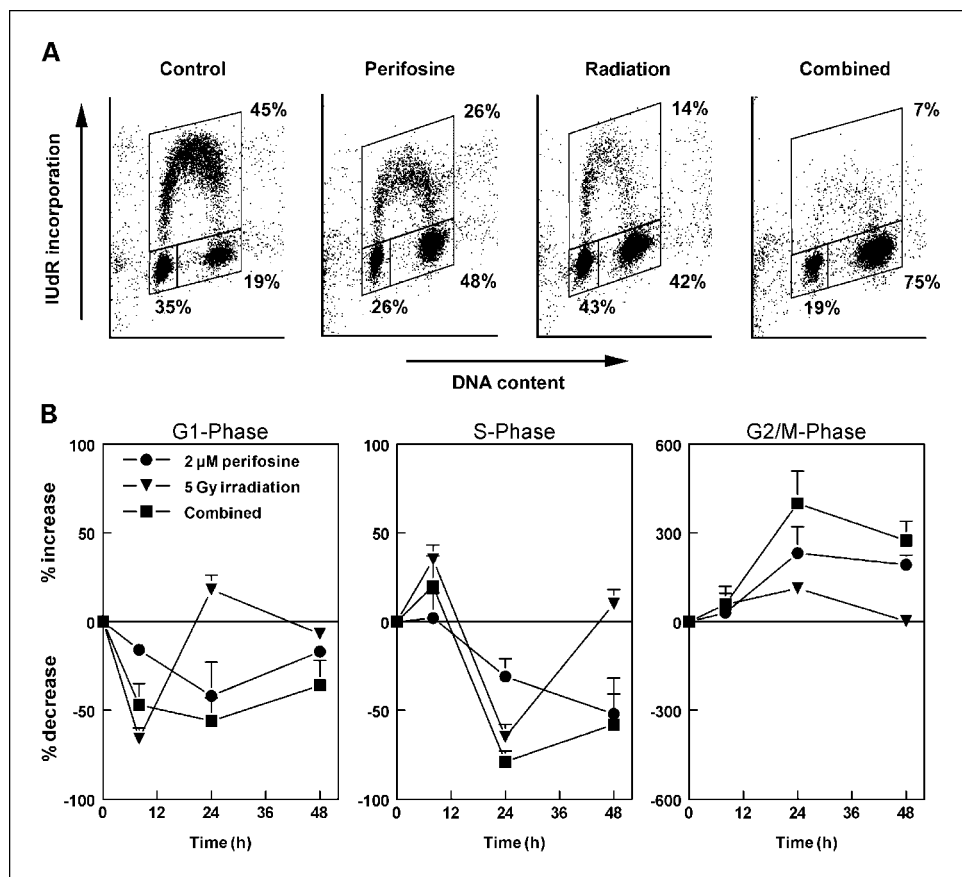

Fig. 2. Analysis of cell cycle progression after treatment with either 2 μmol/L perifosine, 5 Gy radiation, or the combination. *A*, representative cell populations collected 24 hours after treatment. Cells were labeled with IUdR for determination of S-phase fraction (*top region*, positive for IUdR) and stained with propidium iodine for distinguishing G$_1$ (*bottom left region*) from G$_2$-M cells (*bottom right region*). *B*, time course representation of cell cycle progression of KB cells after the different treatment schedules. Cell cycle changes are represented relative to untreated cells. *Points*, means; *bars*, SD.

Table 1. *In vivo* antitumor effect of perifosine, radiation, and the combination

Treatment	No radiation	2 × 5 Gy	1 × 10 Gy	1 × 13 Gy
No perifosine	0	18.7 ± 6.8 (*n* = 8)	21.3 ± 4.7 (*n* = 7)	33.9 ± 10.2 (*n* = 8)
1 × 40 mg/kg	1.6 ± 3.9 (*n* = 8)	ND	20.2 ± 7.3 (*n* = 5)	ND
2 × 40 mg/kg	8.3 ± 9.5 (*n* = 9)	ND	∞	ND
3 × 40 mg/kg	12.3 ± 6.6 (*n* = 9)	∞	ND	ND

NOTE: Normalized tumor growth delay is expressed as the number of days necessary to reach 250% of the initial tumor volume minus the number of days necessary to reach 250% of the initial tumor volume for untreated animals (±SD). ∞, complete and sustained tumor regression.
Abbreviation: ND, not determined.

two fractions of 5 Gy on days 2 and 4. Perifosine was administered in thee doses of 40 mg/kg on days 0, 2, and 4. As expected, both perifosine treatment and radiotherapy as single modalities led to a substantial growth delay (12 and 19 days, respectively). Again, combined treatment led to complete tumor regression, which sustained for at least 90 days (Fig. 3C).

Toxicity after oral perifosine treatment and local tumor irradiation. Body weight of animals treated according to the treatment schedules described in the previous paragraph was monitored and used as an index for systemic toxicity. In all three experiments, no significant weight loss due to local tumor irradiation was observed. A single dose of 40 mg/kg perifosine resulted in a slight but reversible weight loss, which sustained for 10 days. Increased weight loss was observed after combined treatment; however, this was reversible, and initial body weight

was regained within 3 weeks (Fig. 3D). Although two oral doses of 40 mg/kg resulted in a reduction in body weight of 6% at day 4 after start of treatment, the initial body weight was regained after 2 weeks. When this dose schedule was combined with 10-Gy irradiation at day 2, weight loss up to 8% on day 7 was observed. Again, this toxicity was reversible and lasted 18 days (Fig. 3E). Administration of 40 mg/kg perifosine at days 0, 2, and 4 did not result in increased toxicity compared with 40 mg/kg perifosine administered only at days 0 and 2. Maximum weight loss was encountered when three doses of perifosine were applied with a split dose of 2 × 5-Gy irradiation. However, this did not exceed 10% of initial body weight and lasted for <3 weeks (Fig. 3F).

Histopathologic analysis. KB tumors were excised at different time intervals (4-6 days) after treatment and stained using a cleaved caspase-3–specific antibody. Because untreated

Fig. 3. *In vivo* efficacy and toxicity of treatment with perifosine, radiation, and combined schedules. BALB/c nude mice bearing KB xenografts with a mean diameter of ∼ 6 mm were treated with either perifosine orally, radiation, or a combination. *A-C,* animals treated with single or multiple doses of perifosine and a single or split dose of γ-radiation, as indicated. Tumor size was measured at least three times a week (quantification of treatment efficacy, number of animals/group are summarized in Table 1). *D-F,* treatment-induced toxicity, expressed as changes in body weight, after treatment as indicated.
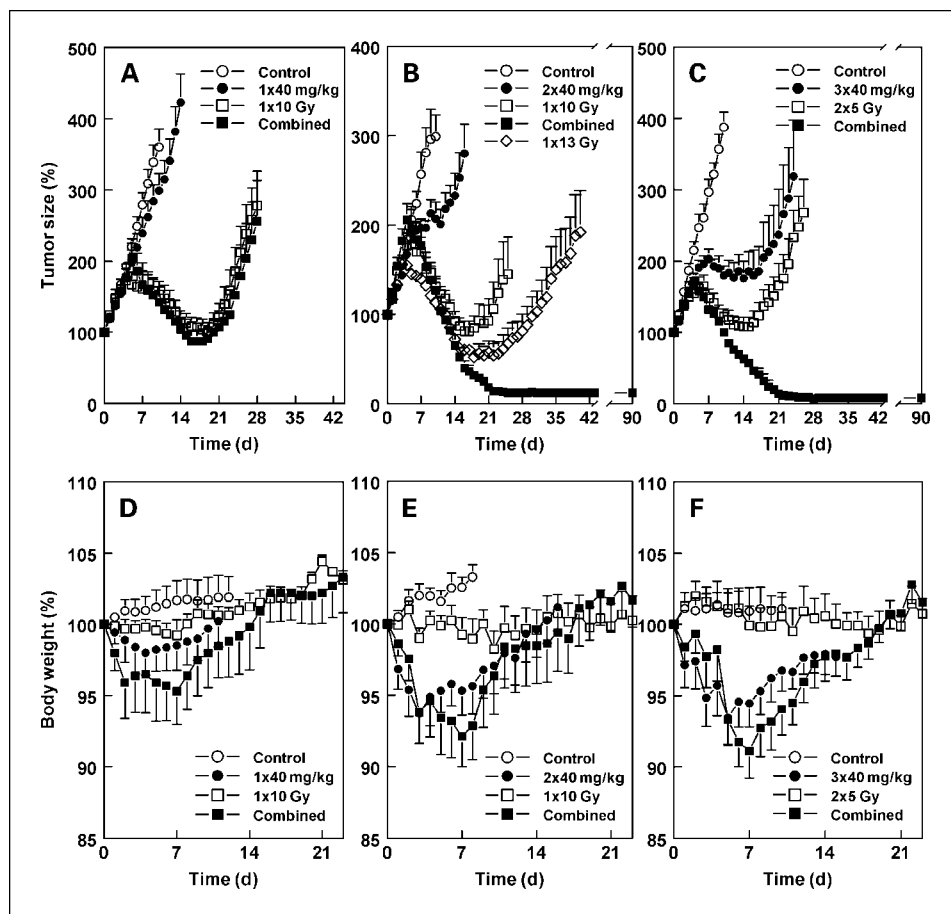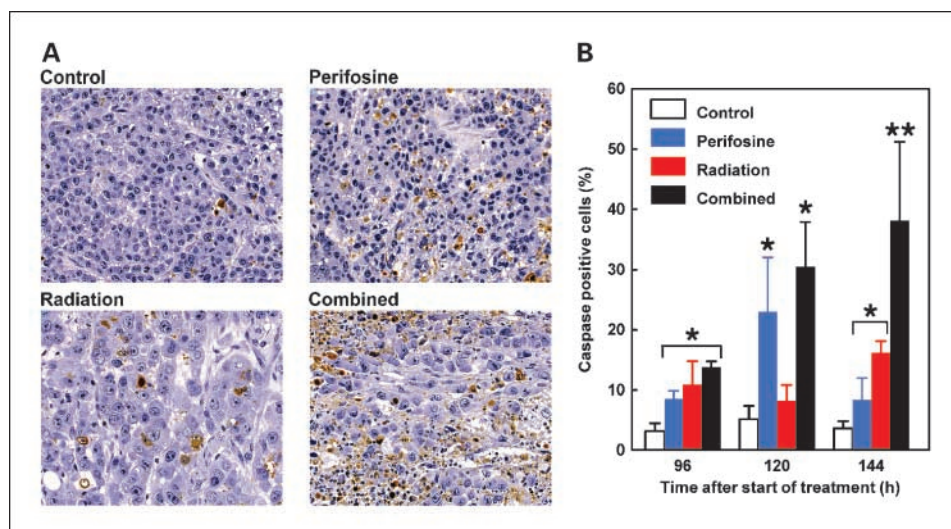
Fig. 4. Histopathologic analysis of KB xenografts. Tumor-bearing animals received 40 mg/kg perifosine at 0 and 48 hours; animals were irradiated at 48 hours. At 96, 120, and 144 hours after start of treatment, tumors were excised and stained for cleaved caspase-3. *A,* representative sections of tumors from mice, 120 hours after either no treatment (control), treated with perifosine, radiation, or the combination. Magnification, ×40. *B,* quantification of the fraction of apoptotic cells present in tumor sections, harvested at various time points after start of treatment with perifosine, radiation, or a combination. *, $P < 0.05$, for separate treatments compared with controls; **, $P < 0.03$, for combined treatment compared with separate treatments (one-tailed Student's t test).

KB tumors grow rapidly and display central areas of necrosis, these analyses were done on the peripheral rim of vital tumor tissue. Compared with untreated tumors, an increase in the number of apoptotic cells was found after radiation or perifosine (Fig. 4A). The most prominent apoptotic response was observed after combined treatment with radiation and perifosine. Furthermore, enlarged nuclei were clearly visible in the radiation-treated and, to a lesser extent, combined-treated tumors. In untreated tumors, the percentage of apoptosis varied between $3.2 \pm 1.3\%$ to $5.2 \pm 2.2\%$ (Fig. 4B). Radiation (1×10 Gy) induced a significant increase in tumor apoptosis ranging from $11.0 \pm 3.8\%$ on day 4 to $16.2 \pm 1.9\%$ on day 6. Tumors treated with perifosine only (2×40 mg/kg) also showed an increase in the amount of apoptosis, which was maximal at 5 days posttreatment ($23.0 \pm 9.0\%$; $P < 0.05$). The largest increase in the apoptotic response resulted from the combined radiation plus perifosine treatment. The percentage of apoptosis increased progressively from $13.8 \pm 1.0\%$ at 4 days to $30.5 \pm 7.4.1\%$ at 5 days and $38.2 \pm 13.1\%$ at 6 days. These numbers were also significantly higher than the amount of apoptosis induced by both treatments separately ($P < 0.03$ at 6 days).

Tumor and normal tissue pharmacokinetics. To quantify tumor and normal tissue distribution after the various schedules, one to three doses of perifosine, together with tracer amounts of [^{14}C]perifosine, were administered to tumor-bearing animals. A single dose of 40 mg/kg perifosine resulted in plasma levels of 5 μg/mL at 48 hours after administration, which declined thereafter. A second 40 mg/kg dose at 48 hours resulted in further increased plasma levels of 9 μg/mL at 96 hours and 7 μg/mL at 144 hours after the second administration. Finally, plasma levels exceeded 10 μg/mL after a third administration at 96 hours (Fig. 5A). Steady-state tumor concentrations of around 75 μg/g were measured from 48 until 144 hours after a single administration. A second administration increased maximum tumor levels to ∼150 μg/g at 96 to 144 hours. A third dose of 40 mg/kg at 96 hours resulted in tumor levels reaching ∼200 μg/g at 144 hours after start of treatment (Fig. 5B).

The area under the concentration-time curve was calculated from the concentration-time curves of the major organs (Fig. 5C). Perifosine was found in all organs, but the highest concentration was measured in tumor tissue and the small intestine. Higher drug accumulation was found after a second oral administration, whereas a third administration resulted in only a limited further increase in area under the concentration-time curve. To exclude an effect of radiation on drug uptake in the KB carcinoma, one group of animals was irradiated with 10 Gy, immediately followed by a first dose of 40 mg/kg perifosine and a second dose at 48 hours after irradiation; 144 hours after irradiation, similar tumor concentrations (170 μg/g) were measured compared with tumors of animals that received perifosine without radiation (data not shown).

Discussion

In this study, we show *in vitro* and *in vivo* enhancement of radiation-induced cell death by the alkylphospholipid perifosine in the KB squamous cell carcinoma. *In vitro*, perifosine reduced clonogenic survival, enhanced apoptosis, and blocked cell cycle progression after irradiation. *In vivo*, radiation or perifosine as single modality induced a dose-dependent tumor growth delay. However, multiple doses of perifosine combined with single or split dose irradiation resulted in complete and sustained remission of KB tumor xenografts.

To our knowledge, this is the first study in which the oral alkylphosphocholine analogue perifosine is shown to increase radioresponsiveness *in vivo*. Previous efficacy studies in animals with perifosine as single agent have already showed tumoristatic effects after long-term or high-dose administration of the drug (7). Based on mechanistic insights collected over recent years by our group and others (13, 18, 21, 26), we considered the likelihood that perifosine would increase the cytotoxic effect of radiation *in vivo*.

In the present studies, we tested the combination of perifosine and radiation in the human KB tumor grown *in vitro* and as xenograft in nude mice. To exclude any contribution of a perifosine-related metabolite to the cytotoxic effect, we tested the stability of the compound, both *in vitro* (not shown) and *in vivo* (29). No significant degradation (<4%) of perifosine was measured.

From the *in vitro* experiments, we can conclude that perifosine affects cellular sensitivity to radiation, and that this

Fig. 5. Tumor and normal tissue pharmacokinetics of three escalating doses of perifosine. Mice bearing KB tumor xenografts were administered 40 mg/kg perifosine/[^{14}C]perifosine orally on day 0 (1 ×40 mg/kg), days 0 and 2 (2 × 40 mg/kg), or days 0, 2, and 4 (3 × 40 mg/kg). On days 2, 4, and 6, plasma, organs, and tumors were collected, and drug exposure was determined. *A,* time-dependent plasma concentrations after oral administration of perifosine. *B,* time-dependent i.t. drug concentrations. *C,* perifosine disposition in tumor and normal tissue. Area under the curve (*AUC*) up to 144 hours, after one, two, or three doses at 0, 48, and 96 hours, respectively. *Points/columns,* means (4-5 animals/group); *bars,* SE.
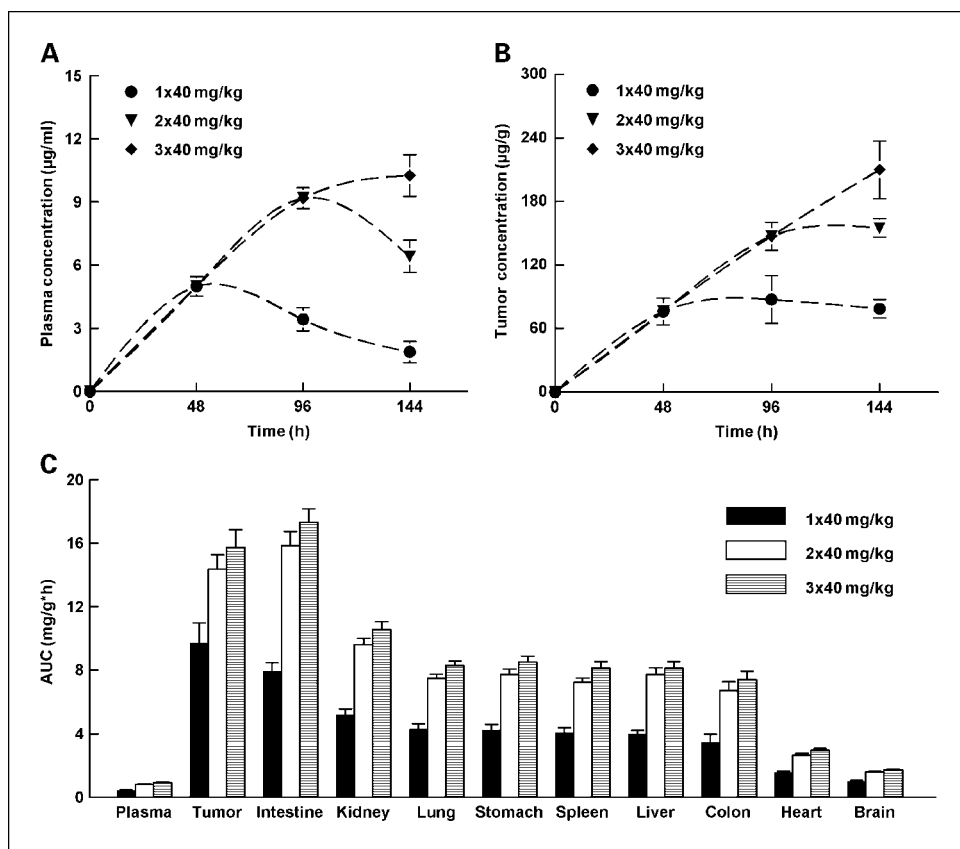
interaction results in increased cytotoxicity as measured by both short-term and long-term assays. A more than additive apoptotic response was observed when radiation was combined with low concentrations of perifosine. The slow kinetics of apoptosis induction in these cells, associated with late (>24 hours) caspase-3 activation, are consistent with a post-mitotic or delayed type of apoptosis (34).

In a clonogenic survival assay, prolonged exposure to perifosine induced marked radiosensitization. This is in line with reports describing a treatment duration-dependent cytotoxicity by alkylphospholipids (35, 36). Furthermore, radiosensitization by perifosine is suggested to be dependent on intense and prolonged protein kinase B/Akt inhibition (37). Along similar lines, prolonged inhibition of RAS-mediated survival pathways has been identified as a strategy to radiosensitize tumor cells (38–41).

Cell cycle–disrupting agents are considered attractive candidates to combine with radiotherapy (42). One possible mechanism by which perifosine exerts its radiosensitizing effect might be by redistribution of cells in a radiosensitive cell cycle (43). However, although pretreatment of KB cells with perifosine led to a relative accumulation in the sensitive G$_2$-M phase, we did not find a significant reduction in clonogenic survival after irradiation under these conditions (data not shown). Furthermore, these prominent cell cycle effects were observed at concentrations far exceeding those at which radiosensitization was found. Therefore, a major role of cell cycle redistribution in perifosine-induced radiosensitization is unlikely.

The steep dose-response relationship of perifosine in this tumor model *in vitro* was also evident *in vivo* and seemed crucial for the radiosensitizing effect. A single dose of 40 mg/kg

was ineffective by itself and did not enhance radiation-induced tumor growth delay. Multiple (two to three) administrations, however, resulted in significant tumor growth delay and, when combined with radiation, to complete tumor eradication.

The mechanism by which perifosine exerts its antitumor effect *in vivo*, either as single agent or in combination with radiation, remains uncertain. Based on our *in vitro* data, both apoptotic and nonapoptotic cell death contribute to the observed response. Furthermore, our histopathologic analyses show a significant increase in apoptosis after treatment with perifosine or radiation. The largest increase in the amount of apoptosis was observed after combined therapy. Taken together, these data support a significant role of apoptotic cell death in the antitumor effect of the combination treatment.

Tumor response was correlated with the degree of perifosine accumulation in tumor tissue. In fact, the amount of drug uptake by tumor cells after perifosine treatment as single or combined modality could well be the determining factor for treatment outcome. We measured plasma and tumor levels at 48 hours after the last oral administration, because perifosine uptake by the KB tumor has been shown to reach a plateau after this time interval (29). Because this plateau was maintained for at least 168 hours after administration, a prolonged tumor exposure is likely. Following a single oral dose of 40 mg/kg perifosine, mean maximum tumor levels of 87 µg/g were measured. This schedule was ineffective in enhancing the radiation response. Multiple perifosine administrations causing tumor growth delay and, in combination with radiation, induced tumor regression, resulted in tumor levels ranging from 125 µg/g up to almost 300 µg/g. All these concentrations exceeded the levels of other alkylphosphocholines, such as miltefosine, octadecylphosphocholine, and

erucylphosphocholine, measured in *N*-nitrosomethyl-urea–induced tumors in rats after oral administration of tumor growth–inhibiting doses (44). Importantly, the maximal perifosine plasma concentration measured in radiation-enhancing treatment schedules corresponded with clinically achievable plasma levels. In patients with advanced cancer, both steady-state levels during treatment with a loading dose/maintenance schedule (9) and peak plasma levels measured in patients receiving 200 mg/d (20) were in this range.

In conclusion, our data show that perifosine increases radiosensitivity *in vitro* and enhances tumor response to radiation. Multiple doses of perifosine were more effective than a single dose and, when given in combination with radiation, led to complete and sustained tumor regression. These studies also show that the tumor response after combined treatment is mediated, at least partly, by induction of apoptosis. Based on these findings, perifosine is an attractive candidate for evaluation as a radiosensitizer in clinical studies.

Acknowledgments

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