Perifosine, a Novel Alkylphospholipid, Induces p21WAF1 Expression in Squamous Carcinoma Cells through a p53-independent Pathway, Leading to Loss in Cyclin-dependent Kinase Activity and Cell Cycle Arrest

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ABSTRACT

Alkylphospholipids (ALKs) are a novel class of antineoplastic compounds that display potent antiproliferative activity against several in vitro and in vivo human tumor models. However, the mechanism by which these agents exert this desired effect is still unclear. In this study, we investigated the effect of perifosine, a p.o.-bioavailable ALK, on the cell cycle kinetics of immortalized keratinocytes (HaCaT) as well as head and neck squamous carcinoma cells. All cells were sensitive to the antiproliferative properties of perifosine with an IC50 of ~0.6–8.9 μM. Cell cycle arrest at the G1-S and G2-M boundaries was observed in H112, HN30, and HaCaT cells independent of p53 function, and this effect was preceded by loss in cdc2 and cyclin-dependent kinase (cdk) 2 activity. Analysis of cdks complexes in vitro demonstrated that perifosine, up to 20 μM, did not directly interfere with these enzymes. However, aphidicolin-synchronized H112 cells released in the presence of perifosine (10 μM) demonstrated increased expression of total p21WAF1 and increase association of p21WAF1 with cyclin-cdks complexes resulting in reduced cdc2 activity. HCT116 isogenic cell lines were used to assess the role of p21WAF1 induction by perifosine. This compound (20 μM) induced both G1-S and G2-M cell cycle arrest, together with p21WAF1 expression in both p53 wild-type and p53−/− clones. By contrast, p21−/− variants demonstrated no p21WAF1 induction or cell cycle arrest. Similar results were obtained with other ALK congeners (miltefosine and edelfosine). These data, therefore, indicate that perifosine blocks cell cycle progression of head and neck squamous carcinoma cells at G1-S and G2-M by inducing p21WAF1, irrespective of p53 function, and may be exploited clinically because the majority of human malignancies harbor p53 mutations.

INTRODUCTION

The mechanisms of epigenetic alterations in human carcinogenesis that affect intrinsic cellular pathways, such as those involved in proliferation, differentiation, and apoptosis, are poorly understood and likely to hinder the development of relevant therapeutic approaches (1, 2). Similarly, the molecular pathogenesis of HNSCC is not clearly delineated and thus presents a significant challenge for prevention, treatment, and management of this disease (3, 4). Furthermore, advanced HNSCC portends a very poor prognosis with standard therapies (5). It follows that new treatment approaches, including the use of novel anticancer agents that may modulate many of these abnormal cellular pathways, are clearly needed for treating HNSCC. In this regard, ALKs and the structurally related ether lipids represent a new class of potential cancer therapeutics, and the minimal chemical structure is associated with antiproliferative properties (6, 7). Most ALKs are structural analogues of the naturally occurring platelet-activator factors, which are metabolically stable because of resistance to phospholipase activities (7). Examples of this class of compounds include Et-180CH3 (edelfosine), HePC (miltefosine), and octade-cylopipridine (perifosine; Fig. 1A; Refs. 7–9).

Miltefosine, the first ALK to be tested in human clinical trials, demonstrated severe gastrointestinal toxicity when given p.o. (10, 11), but a topical formulation of this agent has been approved for clinical use in Europe to treat metastatic breast carcinomas to the skin (12, 13). Perifosine resulted from a search to develop a tolerable oral form of ALK (8). Phase I trials of this compound are currently being evaluated in Europe and the United States (14). Although ALKs have been studied in detail, the exact mechanism of their antiproliferative properties remains unclear. Recent data suggest that modulation of cell surface receptors, inositol metabolism, phospholipases, protein kinase C, and other mitogenic pathways may all be involved (15–17) and can be summarized as a “modulation of signaling pathways” (18).

Perifosine has been characterized for its antiproliferative effect in a number of tumor cell lines (8). For instance, those derived from breast, colon, prostate, and larynx demonstrated reasonable sensitivity (8). More remarkably, cell lines derived from certain HNSCCs, for instance KB and Hep-2 (larynx) and SAS (tongue), were found to be very sensitive to the effects of perifosine (8). We have reported previously on a panel of HNSCC cell lines derived from primary and secondary cancer lesions of different clinical stages (T2–T4), which have been extensively characterized for alterations of the major tumor suppressor genes (p53 and p16INK4a) and components of the cell cycle (19–21). Therefore, by using this model system of HNSCC, we evaluated in detail the antiproliferative action of perifosine.

In this study, we report that in vitro, perifosine is growth inhibitory in a representative panel of HNSCC cells, resulting in the blockade of cells in G1-S and G2-M. This effect is caused by modulation of cdk activity by the up-regulation and increased association of p21WAF1 with cdk/cyclin complexes, occurring in a p53-independent manner. We provide evidence that p21WAF1 up-regulation is required for perifosine-induced cell cycle arrest, because p21WAF1−/− cells were found to be insensitive to this effect of the drug.

MATERIALS AND METHODS

Cell Culture. Culture conditions of cell lines established from HNSCC are described elsewhere (21). Briefly, cells were maintained on a layer of lethally irradiated Swiss 3T3 fibroblasts in DMEM supplemented with 10% fetal bovine serum and 0.4 μg/ml hydrocortisone at 37°C in 95% air/5% CO2, and prior to subculturing or experimental procedures, feeder cells were removed as described (21). HaCaT cells (immortal epidermal keratinocytes) were maintained without a feeder layer support, and culture conditions were as described above. HCT116 cells of various genetic backgrounds (p53−/−, p53−/−, and p21WAF1−/−) were a kind gift from Dr. Bert Vogelstein (Johns Hopkins, Baltimore, MD) and maintained as described above and in the presence of 350 μg/ml genetin (Sigma Chemical Co., St. Louis, MO).

Drugs. ASTA Medica AG (Germany) and Aventis Pharmaceuticals (Bridgewater, NJ) provided perifosine and flavopiridol, respectively, to the Development Therapeutics Program, National Cancer Institute. For in vitro studies, perifosine was reconstituted in PBS at a stock concentration of 100 mM.
and further diluted in PBS to the working concentration (0.1–30 μM) for experimental procedures.

Assessment of Thymidine Incorporation in Perifosine-treated HNSCC Cells. Cell proliferation studies by measuring the uptake of [3H]thymidine was performed as described (21). Briefly, HNSCC and HaCaT cells (1–2 × 10^4/well) were grown overnight in 24-well plates and exposed to either perifosine (0.1–30 μM) or PBS (control). After treatment (24–48 h), cells were pulsed with [3H]thymidine (1 μCi/well) for 4–6 h, fixed (5% trichloroacetic acid), and solubilized (0.5 M NaOH) before scintillation counting. Experiments were performed in triplicates.

Cell Cycle Analysis. Analysis of cellular DNA content by flow cytometry was performed as described (21). Briefly, perifosine (0.1–30 μM) or control-treated cells (HNSCC, HaCaT, HCT116, and isogenic variants) were harvested after 24 h, washed briefly in ice-cold PBS, and fixed in 70% ethanol. DNA was stained by incubating the cells in PBS containing propidium iodide (50 μg/ml) and RNase A (1 mg/ml) for 30 min at 37°C. Fluorescence was measured and analyzed using FACS Caliber (Becton Dickinson Immunocytometry Systems, San Jose, CA) and ModFit (Verity Software, Topsham, ME), respectively. For time-dependent analysis, cells treated with perifosine (10 μM) were harvested at the indicated time (0–24 h) and processed as described.

Analysis of cdk Activity in Perifosine-treated HNSCC Cells. Assessment of in vitro cdc2 and cdk2 activity were as reported (21, 22). Briefly, exponentially growing HNSCC and HaCaT cells were exposed to perifosine (10 μM) or PBS for 24 h and subsequently lysed as described above, and 500 μg of total cellular protein were used to immunoprecipitate active cdc2 and cdk2 complexes. After capturing with gammabind G Sepharose and subsequent washes, the active immune complexes were assessed for activity in the presence of increasing concentrations of perifosine (0.1–30 μM) or flavopiridol (300 nM) in the kinase assay buffer containing [γ-32p]ATP (3000 Ci/mmol) and 0.2 mg/ml histone H1, 25 μM ATP. Reactions were incubated at 37°C for 30 min and terminated by the addition of SDS-gel loading buffer, resolved in SDS-PAGE, and dried gels were subjected to autoradiography and phosphorimaging.

RESULTS

Effect of Perifosine on Proliferation of HNSCC Cells. We examined a representative panel of cell lines derived from naturally occurring HNSCCs (HN4, HN8, HN12, HN19, and HN30) and HaCaT cells for sensitivity to increasing concentrations of perifosine. Proliferation was assessed by the incorporation of [3H]thymidine into cellular DNA. As illustrated in Fig. 1B, exposure to perifosine (0.1–30 μM) for 24 h resulted in a dose-dependent inhibition of [3H]thymidine uptake in all cell lines tested. The IC_{50}s (50% inhibitory concentration) for growth were between 0.6 and 8.9 μM, reaching IC_{50}s of ~10 μM. On the basis of this observation, we chose to use two malignant HNSCC phenotypes, HN12 and HN30, and immortalized HaCaT cells for subsequent experiments. The distinct biological features reported previously, for instance the insensitivity of HN30 to γ-irradiation and to certain anticancer agents (21) and the remarkable tumorigenicity in vivo of HN12 cells (21), suggest that they may represent useful models for HNSCC.

Effect of Perifosine on Cell Cycle Progression at G1/S and G2/M in HNSCC Cells. Although the ALKs are known to be antiproliferative, the exact mechanism by which they induce this effect is still unknown. We sought to investigate whether perifosine may be targeting the cell cycle regulatory mechanisms of HNSCC cells by initially determining the DNA content of HNSCC cells exposed to perifosine using FACS analysis. Dose-response analysis was performed where HNSCC cells were exposed to increasing concentrations of perifosine (0.1–30 μM) for 24 h and subsequently processed for cell cycle analysis. As illustrated in Fig. 2A, in HN12 cells a
minimal sensitivity to 0.1–1 μM perifosine was observed when compared with control. Surprisingly, a consistent increase in \( G_2 -M \) along with a concomitant decline in the S-phase fraction was notable in all cell types tested (HN12, HN30, and HaCaT) at concentrations \( \geq 3 \mu M \) perifosine, with maximal effects observed with 10 μM. On the basis of these data, we chose 10 μM, unless specified otherwise, as an effective concentration (IC\(_{50}\)) for further characterization of this anticancer agent. For time-dependent studies, perifosine (10 μM)-treated HN12 cells were harvested at the indicated times (0–24 h) and processed for cell cycle analysis. As shown in Fig. 2B, a clear effect on cell cycle progression was observed as early as 4 h after treatment, with an increased accumulation of cells at \( G_2 -M \). With prolonged exposure (4–12 h), a progressive accumulation of cells in \( G_2 -M \) (69% compared with baseline of 39%) is observed with a decline in those cells in S-phase (10% compared with a baseline of 19%). This effect became even more apparent at longer time points (12–24 h). The data indicate that perifosine (10 μM) is able to cause an accumulation of cells at \( G_1 \) and \( G_2 -M \) phases of the cell cycle. Similar observations were made for HaCaT and HN30 cells (data not shown).

**Perifosine-induced \( G_2 -M \) Accumulation of HNSCC Cells Is Attributable to an Increase in \( G_2 \) and Not \( M \)-Phase DNA Content.** Because our previous data had indicated an accumulation of HNSCC in \( G_2 -M \) when exposed to perifosine, we sought to determine whether these cells arrest in \( G_2 \) or \( M \). For this, we measured the mitotic index of cells after 10 μM perifosine treatment to assess whether the mitotic apparatus may have been perturbed, thus explaining the increase in \( G_2 -M \) DNA content. As a positive control for increased mitotic index, we used nocodazole. As illustrated in Fig. 3 (right panel), most (>50%) HaCaT and HN12 cells treated with nocodazole (0.5 μg/ml) demonstrated loss of nuclear membrane and chromosome condensation, features that are typical of cell cycle arrest in metaphase when caused by microtubule disruptors. In contrast, most (>90%) HNSCC cells exposed to either vehicle (Fig. 3, left panel) or 10 μM perifosine (Fig. 3, middle panel) showed intact nuclear membrane without an increase in mitotic figures. Similar observations were seen with HN30 cells (data not shown). Thus, increased \( G_2 -M \) DNA content provoked by perifosine in HNSCC cells results from arrest of cells in \( G_2 \) (interphase) with loss of entry into mitosis.

**Perifosine Indirectly Inhibits cdc2, cdk2, and cdk4/6 Activity in HNSCC Cells.** Because perifosine affected cell cycle progression at the \( G_1 -S \) and \( G_2 -M \) transition, we next sought to investigate whether cdks, responsible for cell cycle control, were targets for inactivation.
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A: In Vivo

To assess the putative effect of perifosine on cdks, HNSCC cells were treated with increasing concentrations of perifosine (0–10 μM) for 24 h, and their corresponding lysates were immunoprecipitated with specific antisera that recognized cdc2 and cdk2 for in vitro kinase reactions. As shown in Fig. 4A, treatment of HN12 cells with perifosine resulted in a dose-dependent reduction in the activity of both cdc2 and cdk2. To ensure that these observations were not attributable to loading differences, immunoprecipitates (5 μl) were assessed by Western blotting using appropriate antibodies, and as shown (Fig. 4A), drug treatment did not result in altered cdk protein levels. Quantitation of relative activities of all cdks by phosphorimaging indicated (Fig. 4A, lower panel) a reduction of approximately 50 and 80–90% with 5 and 10 μM perifosine, respectively. Similar results were observed for HaCaT and HN30 cells (data not shown). These findings suggest that the loss in cdc2 and cdk2 activity may explain the effects of perifosine in G1-S and G2-M cell cycle progression.

To further investigate whether this effect results in a decreased activity of cdks in vivo, we took advantage of the availability of polyclonal antisera to pRb, recognizing the protein form that is phosphorylated at threonine 356 and serine 780 residues, which represent specific phosphoacceptor sites for cdk2 and cdk4/6, respectively. Cells treated with perifosine (10 μM) or PBS as described above for the indicated time (0–24 h) were lysed and immunoblotted with the indicated antibodies. As shown in Fig. 4B, exposure to perifosine (12–24 h) results in a decrease in pRb phosphorylation on threonine 356 and serine 780, suggesting that perifosine diminishes effectively the in vivo activity of G1 cdks (cdk2 and cdk4/6).

To determine whether perifosine could modulate the activity of cdks by direct interaction with the catalytic subunit of these kinases, lysates obtained from exponentially growing HN30 cells were immunoprecipitated for in vitro kinase reactions, and properly activated cdc2 and cdk2 were incubated with increasing concentrations of perifosine in the kinase reaction. As a positive control, cdc2 and cdk2 complexes were incubated with flavopiridol (300 nM), a known direct inhibitor of cdks (22–25). As shown in Fig. 4C, no effect was observed on cdc2 and cdk2 kinase activity up to 20 μM perifosine, whereas 300 nM flavopiridol demonstrated a characteristic inhibition of cdk activity under similar experimental conditions (Fig. 4C, last lane). In contrast, as described in Fig. 4A, kinase complexes obtained from HN30 cells previously exposed to perifosine (10 μM) demonstrated reduced intrinsic activity (data not shown). Thus, the effect of perifosine on cdk activity in intact cells may occur by the modulation of upstream signals important for cdk activation.

Perifosine Arrests Cells at the G2-M Transition by Up-Regulation of p21WAF1 and Loss of cdc2 Activity in HNSCC Cells. Because perifosine treatment results in arrest and accumulation of cells in G2, we sought to assess the kinetics of cdc2 kinase activity after drug exposure in more detail. As an approach, G1-S-synchronized HN12 cells were released in the presence or absence of 10 μM perifosine and harvested (0–12 h) for the assessment of cdc2 activity as described above. As shown in Fig. 5A, HN12 cells demonstrated minimal cdc2 activity upon release from the aphidicolin block (0–3 h), which is indicative of cells in S-phase. Further cell cycle progression (6–9 h) results in significant elevation of this activity, which peaked at 12 h after release. This increased cdc2 activity represents the required activation of the kinase necessary for G2-M progression (26). However, cells released in the presence of perifosine initially showed a minimal increase in cdc2 activity (3–6 h, see below) but further elevations were prevented (9–12 h). Thus, the data indicate that the accumulation of cells with a G2 DNA content by perifosine treatment may result from a loss in cdc2 activity. As a positive control, G1-S-synchronized HN12 cells released in the presence of nocodazole, a known mitotic blocker, resulted in a characteristic activation of cdc2 by 12 h (27). Parallel lysates were analyzed by Western blot analysis and, as indicated in Fig. 5B, showed no differences in cdc2 expression that may explain the loss of activity of this kinase by perifosine. Similarly, cyclin A levels were mostly unaltered and, as expected, those of cyclin B1 were initially absent at the G1-S transition (0 h) but showed a gradual increase in its expression, reaching a maximal level at 12 h that is crucial for cdc2 activation (26). Of note, cyclin B1 expression appears to be induced earlier after perifosine treatment (Fig. 5B, third lane). This elevation may explain the apparent increase in cdc2 activity observed in Fig. 5A. At 9 h after release, perifosine-treated cells demonstrated unaltered cdc2, cyclin A, or cyclin B1 expression, despite lack of cdc2 activity. However, by 12 h, a slight decline in cyclin B1 was observed, which is unlikely to
explain fully the total loss in cdc2 activity observed 9–12 h after treatment.

Recently, it has become clear that the endogenous cdk inhibitors p21WAF1 and p27KIP1 may have a role in the G2-M transition by inhibiting cdc2 (28). To test the possibility that perifosine could alter the expression of these inhibitors, immunoblots were performed using antisera to p21WAF1 and p27KIP1 using the same lysates as described above. Although the expression of p27KIP1 remained unaltered in control cells, a minimal increase in this protein was observed beyond 6 h of drug treatment. In contrast, p21WAF1 levels, although unaffected by the aphidicolin block, were significantly induced when cells were released in the presence of perifosine (3–12 h). Of interest, increased expression of p21WAF1 in HN12 cells occurred despite a lack of functional p53 (29). Together, the data suggest that perifosine, unexpectedly, modulates p21WAF1 expression in the absence of functional p53, and this elevation (along with minimal increase in p27KIP1) could lead to the loss of cdk activity/cell cycle arrest induced by this agent.

**Increased p21WAF1 Expression and Its Association with Cdk Complexes in Perifosine-treated HNSCC Cells Results in Loss of Kinase Activity.** To further characterize the effects of perifosine on p21WAF1 induction and to determine whether increased levels of this protein associate with the cdk complexes, we initially compared the effects of perifosine to γ-irradiation, an established DNA-damaging agent that provokes accumulation of cell in G2 phase in HNSCC cells (21). As observed elsewhere, treatment of HaCaT cells with γ-irradiation provokes accumulation of cells in G2 phase because of loss in cdc2 activity (21). As shown in Fig. 6A, γ-irradiation was unable to induce p21WAF1. In contrast, treatment of HaCaT cells with perifosine provoked a rapid (3-h) induction of p21WAF1, followed by a decline in cyclin B1 by 24 h. As stated elsewhere (19, 20), these cells lack normal p53 function; thus, the p21WAF1 induction observed occurs, again, through a p53-independent pathway. Furthermore, steady-state expression levels of cdc2, cdk2, and β-actin were unaltered by either treatment. Similar effects were observed in the other three remaining HNSCC cells (data not shown).

We next determined whether induction of p21WAF1 by perifosine results in increased association with cdk complexes. For this approach, cdc2, cdk2, and p21WAF1 were immunoprecipitated from HaCaT cells treated with perifosine (10 μM) and immunoblotted for the indicated proteins with appropriate antibodies. Fig. 6B shows that perifosine treatment (12–24 h) resulted in increased association of p21WAF1 with the cdc2 complex. However, no differences in cyclin A association with cdc2 was observed with perifosine treatment, whereas levels of immunoprecipitated cdc2 proteins were unaltered. Similarly, cdk2 immunoprecipitates demonstrated a clear increase in p21WAF1 association. A limited increase in p27KIP1 association was also observed, but no differences were noted in the relative levels of immunoprecipitated cdk2 protein upon treatment. Finally, p21WAF1 immunoprecipitates demonstrated that the total amount of protein immunoprecipitated increased (12–24 h), reflecting the induction of p21WAF1 provoked by perifosine. Interestingly, the association of p21WAF1 with either cdc2 or cyclin A was dramatically elevated. The data indicate that p21WAF1 associates with cdc2 and cyclin A upon treatment of cells with perifosine. The observed association could lead to a loss in cdc2 activation at an earlier point in the G2 phase, thus preventing the later activation of cdc2/cyclin B1. Collectively, the data suggest that the loss in cdk activity provoked by perifosine at either G1-S or G2-M may result from the induction and increased association of p21WAF1 to cyclin/cdk partners, which would explain a delay in cell cycle progression from G2 to M and potentially in G1 phase as well.

**Assessment of Perifosine on the Cell Cycle Progression in Isogenic HCT116 Colon Carcinoma Cells.** To assess the contribution of p21WAF1 to the cell cycle effects of perifosine, we took advantage of the availability of isogenic HCT116 colon carcinoma variant cells (p53+/−, p53−/−, and p21WAF1+/−; Ref. 30). As demonstrated in Fig. 7, A and B, perifosine is able to block cell cycle progression at G1-S and G2-M in the p53+/− variant HCT116 cells. Furthermore, perifosine caused the fraction of cells in G2-M phase to increase dramatically from 17 to 67%, whereas those in S-phase declined from 29 to 10%. This effect of perifosine in p53−/− cells was associated with an induction in expression of p21WAF1 (Fig. 7C). Furthermore, p53 protein levels were also induced. To evaluate whether the cell cycle arrest and the induction of p21WAF1 was dependent on p53 function, we tested the effect of perifosine in the p53−/− HCT116 variant cells. As demonstrated (Fig. 7), G2-M cell fraction increased from 19 to 72% upon perifosine treatment,
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ALKs and the structurally related ether lipids represent a novel class of antiapoptotic agents derived from lysophospholipids, which display cytostatic effects in preclinical studies (8, 17, 18). This family includes the original ALK, ET-18-OCH₃ (edelfosine), and the structurally more simple hexadecylphosphocholine (miltefosine) and octadecylpipiridine (perifosine; Refs. 8, 18). Miltefosine showed antitumor activity in early clinical trials, despite significant gastrointestinal toxicity (10, 13). This compound was recently approved in Europe for patients with cutaneous metastasis (13). Of interest, miltefosine demonstrated activity in visceral leishmaniasis (32). Additionally, clinical trials with edelfosine have showed promising results as a purging agent in patients undergoing bone marrow transplantation (33). To allow the systemic administration of ALK, an analogue of miltefosine, perifosine, was synthesized (8), and several clinical trials with oral perifosine are undergoing in Europe and in the United States (14). This class of compounds shares several interesting features and include differential cytotoxicity of malignant versus normal cells as observed in endothelial cells (16) and in human lymphocytes and mouse embryonic fibroblasts. Furthermore, ALKs have synergistic cytotoxic properties with cyclophosphamide, cisplatin, and γ-irradiation (16, 18, 34) and can induce differentiation in a number of leukemia models (35). Although the exact mechanism of action of ALK is unknown, it is thought that these molecules can modulate signal transduction pathways at the membrane level by modulating protein kinase C and phospholipase B, among others (15, 17, 36).

Although members of the ALK family are able to induce apoptosis in combination with γ-irradiation (16), the role of ALKs on cell cycle progression is still unknown, which prompted us to investigate the cell cycle effects of perifosine, the only p.o.-acting ALK in clinical trials.

Initially, we determined the antiproliferative properties of perifosine in HNSCC. Potent antiproliferative effects were observed in this panel of cell lines with IC₅₀ values in the 1–10 μM range, irrespective of the presence of a functional p53. Similar activity of perifosine was observed in other keratinocyte models (9). To examine the antiproliferative effect of perifosine in more detail, cell cycle progression of HNSCC lines exposed to perifosine was determined. Surprisingly, accumulation of cells with G₁ and G₂-M DNA content was observed in all cell types tested. Time-dependent studies showed that 4 h of exposure to perifosine is sufficient to promote the arrest of cells with G₁ and G₂-M DNA content and relative loss in S-phase population. To determine more precisely where in G₁-M perifosine may be arresting cells, mitotic index analyses were conducted and showed that perifosine arrests cells at G₂ with interphase nuclei, in contrast to nocodazole, a known microtubule inhibitor, which arrested cells at the M phase.

The progression of the cell cycle is governed by the cyclical activation of serine-threonine kinases, cdks, that are formed by the catalytic subunit (cdk), positive cofactors (cyclins), and negative cofactors, endogenous cdk inhibitors (26, 37, 38). Modulation of cdks by direct interaction with the catalytic subunit, or indirectly by modulating the upstream cofactors necessary for cdk activation or by the up-regulation of endogenous cdk inhibitor, can lead to loss in cdk activity with arrest in cell cycle progression (24, 25, 37, 39). To determine whether perifosine has the capacity to modulate cdk activity, HNSCCs were exposed to perifosine, and cdk2 and cdk7 kinase activity was assessed by immunocomplex kinase reactions. Loss in cdk activity was demonstrated with perifosine. However, differences observed in the rate of loss of activity between the two kinases may reflect, in part, increased sensitivity of cdk2 to the inhibitory effects of

**Fig. 6.** The G₂ arrest provoked by perifosine is associated with induction of p2₁(WAF₁) and its increased association with cdk/cyclin complexes. A, lysates were prepared from HaCaT cells exposed to γ-irradiation (20 Gy) and perifosine (10 μM), and 25-μg aliquots were resolved in polyacrylamide-SDS gels for Western blots with the indicated antibodies and detected by enhanced chemiluminescence. B, lysates from perifosine (10 μM)-treated HaCaT cells were immunoprecipitated (IP) for cdc2, cdk2, and p2₁(WAF₁), resolved in denaturing polyacrylamide gels, and immunoblotted for the indicated proteins using appropriate antibodies. The data shown are representative of three independent experiments.

with a loss in S-phase (from 41 to 11%), and induction of p2₁(WAF₁), despite lack of p53. Thus, p53 function is dispensable for the cell cycle effects of perifosine and for the induction of p2₁(WAF₁), which is in line with our previous observations in “p53-defective” HaCaT and HN12 cells (19, 20, 31). To establish conclusively the role of p2₁(WAF₁) in the cell cycle effects of perifosine, we also tested the HCT116 p2₁(WAF₁⁻/⁻) variants. As observed in Fig. 7, perifosine failed to induce cell cycle arrest in these cells, which was associated with the lack of p2₁(WAF₁) induction despite normal expression of p53 in these cells. To determine whether the effects on cell cycle progression are shared by two other known ALKs, edelfosine and miltefosine, we exposed HCT116 cell lines to these two compounds. Prominent G₁-S and G₂-M arrest was observed along with p2₁(WAF₁) increase in the p53⁻/⁻ and p53⁻/- variants (data not shown). Furthermore, when HCT116 p2₁⁻/⁻ cells were exposed to these compounds, similar to perifosine, no cell cycle effects were observed (data not shown). Collectively, we can conclude that ALKs, including perifosine, may block cell cycle progression by the p53-independent induction of p2₁(WAF₁).

**DISCUSSION**

**Fig. 7.** Time-dependent studies showed that 4 h of exposure to perifosine is sufficient to promote the arrest of cells with G₁ and G₂-M DNA content and relative loss in S-phase population.
perifosine, resulting from raised intrinsic kinase activity attributable to a high number of asynchronous cells traversing the G1 and S phases (>80%) at any given time. Additionally, this difference could result from a complex interplay of rate of induction of p21WAF1 (at least 6 h), the molar concentration of active cdk2 or cdc2 kinases, and the time required for completion of S-phase. Furthermore, to confirm the loss of cdk activity in this cellular system, we measured the phospho-rylation status of the protein product of the Rb tumor suppressor gene (pRB), a known endogenous substrate for cdks, by site specific phospho-specific antisera. Indeed, loss in phosphorylation at specific cdk2 and cdk4/cdk6 sites were observed with perifosine, suggesting that the drug has the capacity to inhibit both G1 and G2 cdks, when assessed by either in vitro kinase assays or loss in Rb phosphorylation status.

To further examine the effects of perifosine on cdks, aphidicolin-synchronized cells were released in the presence of perifosine. We found that perifosine prevented the activation of cdc2 necessary for the G1-M transition. Of note, examination of the molecular complexes containing cdc2 revealed that the loss of cdc2 activity was clearly preceded by up-regulation of p21WAF1. p21WAF1 belongs to the cip/kip family of endogenous cdk inhibitors (p21, p27, and p57), which regulates directly the activity of cdks, and was initially identified as a mediator of p53-induced growth arrest (40–43). This family of endogenous inhibitors can prevent the activation of cdks. Moreover, even activated cyclin/cdk complexes are readily inhibited by cip family members (44). Although initially thought to play a unique role in G1-S transition, it has become apparent recently that at least p21WAF1 and p27KIP1 also have a clear role in the G2-M transition (28, 45). Furthermore, biochemical and genetic studies demonstrated that p21WAF1 family members at low concentrations may promote the assembly and activation of D-type cyclin kinase, whereas at higher concentrations, they are able to suppress cdk activity (45, 46). Besides cell cycle control, p21WAF1 has reported roles in transcription, DNA repair, differentiation, and apoptosis (47–50). A major transcriptional regulator of p21WAF1 is the tumor suppressor gene p53, which binds to specific p53-binding sites within the p21WAF1 promoter (41), although additional p53-independent mechanisms have also been described (51–53).

To assess the biological importance for the up-regulation of p21WAF1 induced by perifosine, we compared the effects of a known DNA-damaging agent, γ-irradiation, in HaCaT cells, an immortalized keratinocyte cell line with nonfunctional p53 (31). Although both treatments provoked the arrest of cells in G2-M, only perifosine up-regulated p21WAF1. Thus, the induction observed is not dependent on a functional p53. To determine whether the increased p21WAF1 is indeed associated with cdk complexes, HNSCC cells exposed to perifosine demonstrated a clear increased association between p21WAF1 and both G1 and G2 cyclin/cdk complexes. p21WAF1 was
also associated with cyclin A/cdk complexes. This association may explain the lack of cdk2 activation observed in aphidicolin-synchronized cells upon perifosine treatment, because cyclin A, the predominant cyclin in S-phase, initially binds and activates cdk2 in this phase, and subsequently, cyclin A associates with cdk2 in late S and early G2 phases (26, 38). Thus, induced p21WAF1 may prevent the proper activation of cdc2/cyclin A, leading to a block in early G2 phase, and may represent a good candidate to explain the observed loss of cdk activity elicited by the treatment with perifosine.

To determine the exact role of p21WAF1 in the cell cycle effects of perifosine, we used the HCT116 cell lines isogenic for wild-type, p53−/−, and p21WAF1−/− genotypes (30). As expected, wild-type cells exposed to perifosine demonstrated clear evidence of G1−S and G2−M arrest. Again, this effect was accompanied by induction of p21WAF1. When the p53−/− cell lines were exposed to perifosine, nearly identical effects to those displayed in wild-type cells were observed, reinforcing the notion that the p21WAF1 up-regulation is not dependent on p53 function, and that the cell cycle effects of perifosine are not attributable to p53 modulation. Finally, cells devoid of p21WAF1 but with intact p53 failed to arrest upon perifosine treatment. Together, these data provide the first evidence that perifosine can arrest cells G2−M transition and delay in the progression of G1 by up-regulating p21WAF1 in a p53-independent fashion, thus leading to a loss in cdk activity. The actual mechanism whereby perifosine may modulate the expression of p21WAF1 is still unclear and under current investigation. Furthermore, the detailed mechanism of effects in G1 will require analysis of cdk2 activation in background controlled for Rb expression.

In summary, our data indicate that the ALK perifosine promotes cell cycle arrest at either G1−S or G2−M because of a p53-independent up-regulation of p21WAF1. On the basis of these findings, we present a working model (Fig. 8) by which ALKs may modulate cdk2 and block cell cycle progression. In this model, ALKs induce p21WAF1 expression by still unknown p53-independent mechanisms, and accumulated p21WAF1 protein leads to a loss in cdk activity by associating with cdk/cyclin complexes, resulting in the accumulation of cells in G1 and G2−M, even in tumor types with abnormal p53 gene status. The predicted capacity to inhibit cdk2/cyclin E will require experimental confirmation in appropriate models. This novel effect preventing cell cycle progression, along with the known apoptotic properties of ALKs, suggests that these anticancer agents may represent good candidates for further evaluation in the treatment and/or prevention of a variety of human neoplasms.

REFERENCES


Perifosine, a Novel Alkylphospholipid, Induces $p21^{WAF1}$ Expression in Squamous Carcinoma Cells through a p53-independent Pathway, Leading to Loss in Cyclin-dependent Kinase Activity and Cell Cycle Arrest

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