Coadministration of Histone Deacetylase Inhibitors and Perifosine Synergistically Induces Apoptosis in Human Leukemia Cells through Akt and ERK1/2 Inactivation and the Generation of Ceramide and Reactive Oxygen Species

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Abstract

Interactions between histone deacetylase inhibitors (HDACIs) and the alkyl-lysophospholipid perifosine were examined in human leukemia cells. Coadministration of sodium butyrate, suberoylanilide hydroxamic acid (SAHA), or trichostatin with perifosine synergistically induced mitochondrial dysfunction (cytochrome c and apoptosis-inducing factor release), caspase-3 and -8 activation, apoptosis, and a marked decrease in cell growth in U937 as well as HL-60 and Jurkat leukemia cells. These events were associated with inactivation of extracellular signal-regulated kinase (ERK) 1/2 and Akt, p46 c-Jun-NH2-kinase (JNK) activation, and a pronounced increase in generation of ceramide and reactive oxygen species (ROS). They were also associated with up-regulation of Bax and a marked conformational change in Bax accompanied by membrane translocation. Ectopic expression of Bcl-2 delayed but was ultimately ineffective in preventing perifosine/HDACI-mediated apoptosis. Enforced expression of constitutively active mitogen-activated protein kinase kinase (MEK) 1 or myristoylated Akt blocked HDACI/perifosine-mediated ceramide production and cell death, suggesting that MEK/ERK and Akt inactivation play a primary role in these phenomena. However, inhibition of JNK activation (e.g., by the JNK inhibitor SP600125) did not attenuate sodium butyrate/perifosine-induced apoptosis. In addition, the free radical scavenger N-acetylcysteine attenuated ROS generation and apoptosis mediated by combined treatment. Finally, the acidic sphingomyelinase inhibitor desipramine attenuated HDACI/perifosine-mediated ceramide and ROS production as well as cell death. Together, these findings indicate that coadministration of HDACIs with perifosine in human leukemia cells leads to Akt and MEK/ERK disruption, a marked increase in ceramide and ROS production, and a striking increase in mitochondrial injury and apoptosis. They also raise the possibility that combining these agents may represent a novel antileukemic strategy.

Introduction

Alkyl-lysophospholipids represent a novel class of antitumor agents that target cell membranes and induce tumor cell apoptosis while sparing their normal counterparts (1, 2). Alkyl-lysophospholipids include several clinically relevant compounds including edelfosine (Et-18-OCH3; ref. 3), miltefosine (HePC; ref. 4), and perifosine, also known as D-21266 (5). Perifosine has shown promising preclinical activity and is currently undergoing phase II clinical evaluation (6). The mechanisms by which perifosine exerts its antineoplastic activity remain unclear. However, recent studies indicate that perifosine induces cell cycle arrest as a consequence of induction of p21WAF1 accumulation (7, 8) through a mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK)–dependent and p33-independent mechanism (7, 9). Perifosine also induces tumor cell apoptosis in association with inactivation of the phosphatidylinositol 3-kinase/Akt (10) and activation of the stress-activated protein kinase/c-Jun-NH2-kinase (JNK) pathways (2).

Histone deacetylase inhibitors (HDACIs) represent a structurally diverse group of compounds that inhibit the deacetylation of histones, permitting the chromatin scaffolding to assume a more relaxed, open conformation, which generally promotes gene transcription. HDACIs induce differentiation but also apoptosis in neoplastic cells through multiple mechanisms. Recent evidence suggest that HDACIs also enhance acetylation of nonhistone proteins (11), including the heat shock protein Hsp90, leading to the dissociation of client proteins and subsequent degradation by the proteasome system (12). This mechanism has been implicated in down-regulation of Raf-1 and inactivation of MEK/ERK in cells treated with the HDACI depsipeptide (12). Several studies have also showed that the lethal effects of HDACIs may involve generation of reactive oxygen species (ROS; refs. 13, 14) and ceramide (15).

The complex effects that both HDACIs and perifosine exert on survival signaling pathways, particularly ERK and Akt (9, 10, 16), led us to hypothesize that these agents might cooperate to trigger leukemic cell apoptosis. Here we report that HDACIs interact in a highly synergistic manner with perifosine to induce Bax conformational change, caspase activation, and apoptosis in myeloid and lymphoid leukemia cells (U937, HL-60, and Jurkat). Furthermore, these events are related functionally to multiple perturbations in cell signaling pathways, including inactivation of the antiapoptotic Akt and MEK/ERK pathways, production of the proapoptotic molecule ceramide, and generation of ROS. Together, these findings suggest that combined treatment with HDACIs and alkyl-lysophospholipids such as perifosine warrants attention in leukemia and possibly other hematologic malignancies.
Materials and Methods

Cells. The human leukemia U937, HL-60, and Jurkat cells (American Type Culture Collection, Manassas, VA) were cultured as previously reported (17). U937 cells stably overexpressing constitutively active Akt or MEK1 and their empty vector counterpart (pUSEamp) were generated as follows. Constitutively active forms of Akt (Myk-tagged myristoylated Akt) and MEK1 (hemagglutinin-tagged MEK1 S218D/S222D) were obtained from Upstate Biotechnology (Lake Placid, NY) and transfected into U937 cells using the Amaxa nucleofector (Koeln, Germany) as recommended by the manufacturer. Stable single cell clones were selected in the presence of 400 μg/mL of geneticin. Thereafter, cells from each clone were analyzed for myc-Akt and hemagglutinin-MEK1 expression by Western blot as described below. U937 cells stably overexpressing Bcl-2 (clone D9) were used as previously described (18).

Reagents. Perifosine was provided by the Cancer Treatment and Evaluation Program, National Cancer Institute (Bethesda, MD). Sodium butyrate was obtained from Calbiochem (San Diego, CA). SAHA and SP600125 were purchased from Alexis Corp. (San Diego, CA). Desipramine was purchased from Sigma (St. Louis, MO). All reagents were formulated as recommended by their suppliers.

Cell Growth and Viability. Cell growth and viability were assessed using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) compound. Briefly, after exposure of cells to the indicated drugs for various intervals, cells were seeded into 96-well plates (100 μL/well) in the presence of 20 μL of MTS solution (Promega, Madison, WI). Cells were incubated for an additional 4 hours, after which absorbance, reflecting reduction of MTS by viable cells, was determined at 490 nm using a microplate reader. Values were expressed as a percentage relative to those obtained in untreated controls.

Assessment of Apoptosis. Apoptotic cells were routinely identified by Annexin V-FITC staining as previously described (17).

Protein Extraction and Western Immunoblotting. Protein extraction and Western blot were done as previously described in detail (17). Primary antibodies used were p21\(^{VAF1/CDP1}\), p22\(^{AMP}\), and MEK1 (Transduction Laboratories, Lexington, KY); cytochrome c, caspase-3, polyol Bax, and Mc-I (PharMingen, San Diego, CA); caspase-8 (Alexis Corp, San Diego, CA); poly(ADP-ribose) polymerase (Biomol Research Laboratories, Plymouth Meeting, PA); Bid, phospho-MEK1/2 (Ser217/221), phospho-Akt (Ser473), phospho-GSK3\(\alpha/\beta\), phospho-FKHR, phospho-cdc2, Akt, p38, Bcl-xL, XIAP, total and acetylated histones H3 and H4 (Cell Signaling Technology, Beverly, MA); Bcl-2 (Dako, Carpinteria, CA); total and phospho-ERK1/2 (Tyr204), total and phospho-JNK (Thr183/Tyr185), phospho-p38 (Tyr182), MEK2, Bim, 14-3-3, Raf-1, hemagglutinin, myc, and apoptosis-inducing factor (Santa Cruz Biotechnology, Santa Cruz, CA); and Bak and o-tubulin (Calbiochem).

Bax Conformational Change. Cells were lysed in buffer containing 150 mmol/L NaCl, 10 mmol/L HEPES (pH 7.4), antiproteases, and 1% CHAPS (19). Protein lysates (500 μg) were subjected to immunoprecipitation using anti-cytosolic monoclonal antibody 6A7 (Sigma) that recognizes only Bax protein that has undergone conformational change (20). Immunoprecipitates were then subjected to Western blot analysis with anti-Bax polyclonal antibody (PharMingen).

Subcellular Fractionation. Leukemic cells (4 × 10\(^6\)) were lysed using digitonin buffer after which cytosolic and membrane fractions were separated by centrifugation as previously described (17). The purity of cytosolic and membrane fractions was routinely confirmed by Western blot using antibody against cytochrome c oxidase subunit IV (COX4), which resides strictly in the inner mitochondrial membrane. Bax detected in the membrane fraction is presumed to represent mitochondrial Bax.

Ceramide Production. After drug treatment, cellular lipids were extracted and mass amounts of ceramide were measured by the diacylglycerol kinase enzymatic method as previously described (21). In parallel, total phospholipids present in cellular lipid extracts used for ceramide analysis were quantified as previously described (15).

Determination of Reactive Oxygen Species. ROS production was monitored with carboxy-H\(_2\)DCFDA (Molecular Probes, Eugene, OR). After treatment, cells were incubated with 10 μg/mL carboxy-H\(_2\)DCFDA for 30 minutes, after which they were washed with PBS, and the percentage of cells displaying increased dye uptake, reflecting an increase in ROS levels, determined using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Statistical Analysis. The significance of differences between experimental conditions was determined using the Student’s t test for unpaired observations. Analysis of synergy was done by median dose effect analysis using commercially available software (CalcuSyn, Biosoft, Ferguson, MO; ref. 22).

Results

Coadministration of Histone Deacetylase Inhibitors and Perifosine Results in a Striking Increase in Growth Arrest, Mitochondrial Injury, and Apoptosis in Human Leukemia Cells. To characterize interactions between HDACIs and perifosine in U937 cells, dose-response studies were done (Fig. 1). Perifosine and sodium butyrate were minimally toxic by Annexin V analysis when administered alone at concentrations as high as 3 μmol/L and 1.5 mmol/L respectively (Fig. 1A and B). However, coexposure of cells to 1 mmol/L sodium butyrate and 2 μmol/L perifosine markedly increased apoptosis (64%), and at 2.5 to 3 μmol/L perifosine, the large majority of cells were apoptotic (∼82%). Similarly, when cells were exposed to 2.5 μmol/L perifosine and increasing concentrations of sodium butyrate, a significant increase in apoptosis was observed at 0.5 μmol/L sodium butyrate, and the majority of cells were apoptotic at sodium butyrate concentration ≥1 μmol/L (Fig. 1B). Time course analysis revealed that whereas 1 μmol/L sodium butyrate or 2.5 μmol/L perifosine administered individually were minimally toxic over a 48-hour treatment interval, combined treatment resulted in a clear increase in apoptosis (e.g., 32%) by 16 hours, and a very substantial increase in lethality after 24 and 48 hours (74% and 86%, respectively; Fig. 1C).

Median dose effect analysis of apoptosis induction by sodium butyrate and perifosine administered at a fixed ratio (400:1) over a range of concentrations (0.5-1.4 mmol/L and 1.25-3.5 μmol/L, respectively) yielded combination index values considerably <1.0, corresponding to a highly synergistic interaction (Fig. 1D).

Attempts also were made to extend these findings to other HDACIs and other human leukemia cells. A 24-hour exposure to 1 μmol/L SAHA or 100 nmol/L trichostatin was minimally toxic to U937 cells (Fig. 1E). However, after combination with a subtoxic concentration of perifosine (2.5 μmol/L), a pronounced increase in lethality occurred. Similarly, exposure of HL-60 and Jurkat cells to sodium butyrate (1 and 0.75 mmol/L, respectively) with perifosine (2 and 3 μmol/L, respectively) resulted in a marked increase in apoptosis (Fig. 1F). Lastly, MTS assays, which reflect both cell proliferation and viability (Fig. 1G) revealed that coexposure of U937 cells to perifosine and sodium butyrate, trichostatin, or SAHA for 24 hours resulted in a marked inhibition of cell growth (73%, 68%, and 50%, respectively) and 75% to 90% by 72 hours, whereas individual treatment had only modest effects.

To determine whether perifosine might modify sodium butyrate–mediated histone acetylation in U937 cells, the extent of histone H3 and H4 acetylation was monitored. Exposure to sodium butyrate alone (1 mmol/L; 8 or 16 hours) resulted in a marked increase in levels of acetylated histone H3 and H4, whereas total levels were unchanged (Fig. 2A). Moreover, perifosine itself had no effect on histone acetylation, nor did it modify the actions of sodium butyrate.
Western blot analysis (Fig. 2B) revealed that coadministration of sodium butyrate and perifosine (24 hours) resulted in a marked increase in pro-caspase-3 and -8 processing, poly(ADP-ribose) polymerase degradation, and release of cytochrome c and apoptosis-inducing factor into the cytosolic fraction. In contrast, sodium butyrate and perifosine administered alone were largely ineffective.

Cotreatment of U937 Cells with Sodium Butyrate and Perifosine Results in Bak Up-regulation, Bid Cleavage, and a Marked Bax Conformational Change and Translocation to the Mitochondrial Fraction. In view of the role of expression and subcellular localization of Bcl-2 family proteins in apoptosis regulation (23, 24), expression of these proteins was monitored in U937 cells treated for 20 hours with sodium butyrate (1 mmol/L) ± perifosine (2.5 μmol/L; Fig. 3A). No major changes in expression of Bax, Bcl-2, Bcl-xL, Bim, or XIAP proteins were noted with any treatment. However, expression of the pro-apoptotic protein Bak was increased in response to sodium butyrate alone and in combination with perifosine. Finally, cells exposed to both sodium butyrate and perifosine exhibited a marked decrease in Bid protein levels, presumably reflecting cleavage/activation. Earlier examination of protein expression (i.e., 16 hours) revealed a modest increase in Bak expression but no significant change in Bid levels. However, levels of Mcl-1, an antiapoptotic protein that plays a key role in the survival of malignant hematopoietic cells (25), were slightly diminished in perifosine-treated cells but substantially reduced in cells exposed to sodium butyrate/perifosine (Fig. 3A).
addition, combined (but not individual) treatment induced cleavage of the 14-3-3 protein (Fig. 3C), an event associated with Bax conformational change (29).

To determine whether Bcl-2 could protect cells from the lethal effects of the sodium butyrate/perifosine regimen, transfectant cells (U937/Bcl-2) ectopically expressing Bcl-2 as shown by Western blot (Fig. 3D, inset) were used. U937/Bcl-2 cells were substantially protected from sodium butyrate/perifosine lethality after 24 hours exposure (Fig. 3D), suggesting that sodium butyrate/perifosine–induced apoptosis involves activation of the intrinsic, mitochondrial pathway, an event opposed by Bcl-2 (23). However, the extent of protection progressively declined over the ensuing 24 to 48 hours, consistent with reports that Bcl-2 can delay rather than prevent apoptosis (30).

Coexposure of U937 Cells to Sodium Butyrate and Perifosine Results in Inactivation of the MEK/ERK and Activation of JNK Pathways. In view of evidence that the mitogen-activated protein kinases (MAPK) ERK, JNK, and p38 MAPK play a critical role in cell fate (31, 32), the effects of sodium butyrate and perifosine on the expression and activation of MAPKs was examined. Consistent with previous reports (18, 33), exposure of U937 cells to 1 mmol/L sodium butyrate for 20 hours resulted in a marked decrease in the phosphorylation (activation) of MEK1/2 and ERK1/2 (Fig. 4A). In contrast, 2.5 μmol/L perifosine induced a clearly discernible (pMEK1/2) or modest (pERK1/2) increase in phosphorylation of these proteins. However, cells exposed to sodium butyrate + perifosine displayed a pronounced reduction in MEK1/2 and ERK1/2 phosphorylation relative to cells treated with perifosine alone. Furthermore, a striking increase in phosphorylation of p46 JNK was observed in cells treated with sodium butyrate and perifosine together, but not individually. Inactivation of MEK/ERK in perifosine/sodium butyrate–treated cells was observed at early intervals before the onset of extensive apoptosis (e.g., 8 and 16 hours), whereas a slight increase in the phosphorylation of p46 JNK was detected only after 16 hours of combined treatment (data not shown). Little change in the phosphorylation status of p38 MAPK was observed in cells exposed to agents individually or in combination, nor in protein levels of MEK1/2, ERK1/2, p46 JNK, or p38 MAPK under any conditions. Thus, concomitant administration of sodium butyrate resulted in inactivation of the cytoprotective MEK/ERK pathway in human leukemia cells exposed to perifosine, accompanied by a delayed activation of the stress-related JNK pathway.

Previous studies indicate that perifosine alone induces JNK activation and prevents ERK activation by serum in U937 cells (2), whereas it enhances activation of ERK in human keratinocyte HaCaT cells (9). Detailed perifosine dose response studies revealed that concentrations as low as 0.5 μmol/L enhanced ERK1/2 activation, an effect even more pronounced at 1 to 4 μmol/L concentrations (Fig. 4B). However, 8 μmol/L perifosine resulted in a decrease in ERK1/2 phosphorylation. In contrast, an increase in p46 JNK phosphorylation was noted in cells exposed to 4 μmol/L perifosine, and further increases were observed at higher drug concentrations (e.g., 8 μmol/L). No major changes in ERK1/2 or p46 JNK protein levels were detected at any of the concentrations examined. Thus, coadministration of subtoxic concentrations of perifosine and sodium butyrate mimicked the actions of considerably higher concentrations of perifosine with respect to the pattern of MAPK activation status.

Inactivation of MEK/ERK, but not Activation of p46 JNK, Plays a Functional Role in Apoptosis Induced by the Sodium Butyrate/Perifosine Regimen. To determine whether inactivation
of MEK/ERK plays a functional role in sodium butyrate/perifosine-induced apoptosis, U937 cells were stably transduced with a constitutively active MEK1 construct. Two separate clones, MEK-CA6 and MEK-CA22, displayed high and low levels, respectively, of constitutively active MEK1 (Fig. 4C). In contrast to wild-type cells (Fig. 4A), the decrease in phosphorylation of ERK in either sodium butyrate- or sodium butyrate/perifosine-treated MEK-CA6 cells (Fig. 4D) or in MEK-CA22 cells (data not shown) was minimal. Interestingly, an increase in JNK activation in sodium butyrate/perifosine-treated MEK-CA6 cells was not apparent (Fig. 4D). Significantly, apoptosis after combined treatment with perifosine and sodium butyrate or SAHA was substantially attenuated in both MEK-CA6 and MEK-CA22 cells, compared with their empty vector counterparts pUSEamp (P < 0.001 in each case; Fig. 4E). Notably, the mutant cells displayed much higher level of ERK phosphorylation/activation than pUSEamp cells (Fig. 4E, inset). Furthermore, activation of MEK/ERK in Jurkat cells inductively expressing a constitutively active MEK1 construct under doxycycline control (18) also resulted in significant decreases in sodium butyrate/perifosine-mediated lethality (data not shown). Together, these findings argue that diminished ERK1/2 activation in sodium butyrate/perifosine-treated leukemia cells plays a significant functional role in sodium butyrate/perifosine lethality.

Pretreatment of U937 cells with the JNK inhibitor SP600125 (5 μmol/L) largely abrogated sodium butyrate/perifosine-mediated c-Jun phosphorylation but did not significantly diminish sodium butyrate/perifosine-mediated apoptosis (data not shown), arguing against a functional role of JNK in sodium butyrate/perifosine-mediated cell death.

**Exposure of U937 Cells to Sodium Butyrate and Perifosine Is Associated with Inactivation of Akt Pathway.** Consistent with previous reports (8, 10), Western blot analysis (Fig. 5A) showed that exposure of U937 cells to perifosine (2.5 μmol/L) for 16 to 20 hours resulted in a marked decrease in Akt phosphorylation, whereas sodium butyrate (1 mmol/L) by itself resulted in only a slight decrease. However, Akt activation was essentially abrogated when cells were cotreated with perifosine and sodium butyrate. Enhanced Akt dephosphorylation in cells exposed to sodium butyrate/perifosine (20 hours) could be explained, at least in part, by reductions in total Akt protein levels. A more detailed time course study revealed that Akt down-regulation and inactivation occurred as early as 4 hours, whereas poly(ADP-ribose) polymerase cleavage was detected only by 16 hours (Fig. 5A), suggesting that factors other than caspase activation may be involved in Akt inactivation. In addition, combined exposure of cells to perifosine and sodium butyrate resulted in a modest but discernible decline in the phosphorylation of several downstream Akt targets including FKHR, AFX, and GSK3α/β (Fig. 5A). The expression of several cell cycle regulatory proteins was also examined. As previously reported (17, 18), exposure of U937 cells to sodium butyrate (1 mmol/L) for 16 to 20 hours induced a marked accumulation of p21^{CIP1} protein. However,
perifosine (2.5 μmol/L) by itself did not induce p21CIP1, nor did it significantly modify p21CIP1 induction by sodium butyrate. Higher concentrations of perifosine alone (≥4 μmol/L) did in fact induce p21CIP1 expression (data not shown), consistent with earlier reports (7). Decreased expression of p27KIP1 was observed in cells exposed to sodium butyrate/perifosine (Fig. 5A) due in part to caspase-mediated degradation (data not shown). Furthermore, consistent with earlier reports (17, 18), sodium butyrate treatment resulted in a marked increase in expression of underphosphorylated pRb, accompanied by partial cleavage of the underphosphorylated species. However, whereas perifosine alone (2.5 μmol/L) failed to modify pRb phosphorylation, combination with sodium butyrate produced extensive cleavage of underphosphorylated pRb. Lastly, combined treatment did not modify p34cdc2 phosphorylation status. Thus, combined exposure of leukemia cells to perifosine and sodium butyrate was associated with the pronounced inactivation of Akt, diminished phosphorylation of its downstream targets, and degradation of p27kip1 and pRb.

Akt Inactivation Plays a Functional Role in Histone Deacetylase Inhibitor/Perifosine–Mediated Apoptosis in U937 Cells. Previous results indicate that perifosine lethality proceeds through an Akt-dependent mechanism (10). To determine whether diminished Akt phosphorylation/activation in HDACI/perifosine-treated cells contributed to the enhanced lethality of this regimen, U937 cells were stably transfected with a construct expressing constitutively active (myristoylated) Akt. As shown in Fig. 5B, two separate clones, Akt-CA6 and Akt-CA22, of U937 cells expressing constitutively active MEK and the empty vector (pUSEamp) and subjected to Western blot analysis using anti-hemagglutinin (HA) antibody, D, MEK-CA6 cells were exposed to perifosine (2.5 μmol/L), sodium butyrate (1 mmol/L), or the combination of perifosine and sodium butyrate for 24 hours after which cells lysates were prepared and expression of HA-MEK and phosphorylation of ERK1/2 and JNK were monitored by Western blot analysis. E, MEK-CA6, MEK-CA22, and pUSEamp cells were exposed to perifosine (2.5 μmol/L) and/or HDACIs (sodium butyrate, 1 mmol/L, and SAHA, 1.5 μmol/L) for 24 hours after which the extent of apoptosis was determined by Annexin V analysis. Points, means of three separate experiments; bars, SD. *, P < 0.001 in each case (significantly lower than values for empty vector pUSEamp cells). Western blot was also used to monitor ERK1/2 phosphorylation levels in perifosine/sodium butyrate-treated transfectant cells (inset).
after combined treatment with perifosine and sodium butyrate or SAHA was significantly attenuated in both Akt-CA4 and Akt-CA6 cells compared with their empty vector counterpart pUSEamp (P < 0.001 in each case; Fig. 5D). Interestingly, constitutive activation of Akt failed to protect U937 cells from the lethal effects of higher concentrations of sodium butyrate or SAHA administered alone (e.g., 4 mmol/L and 5 μmol/L, respectively; P > 0.05 for each clone).

In contrast to sodium butyrate effects, perifosine lethality was markedly diminished in cells expressing the constitutively active Akt (Akt-CA4 and Akt-CA6) relative to controls (pUSEamp; Fig. 5E). Together, these findings suggest that disruption of Akt signaling plays a significant functional role in both perifosine lethality as well as synergistic antileukemic interactions with HDACIs.

A Marked Increase in Ceramide Generation Plays a Functional Role in Sodium Butyrate/Perifosine-Mediated Apoptosis. Recent evidence suggests that the lethal effects of both HDACIs and certain alkyl-lyosphospholipids (e.g., miltefosine) involve ceramide production (15, 34). To address this issue, the acidic sphingomyelinase inhibitor desipramine, which diminishes ceramide production (35), was used. Exposure of cells to perifosine (2.5 μmol/L), sodium butyrate (1 mmol/L), or SAHA (1.5 μmol/L) individually modestly increased ceramide production, whereas combined treatment with perifosine and either sodium butyrate or SAHA resulted in substantially greater increases (Fig. 6A). Furthermore, pretreatment of U937 cells with 25 μmol/L desipramine resulted in a significant decrease in sodium butyrate/perifosine- and SAHA/perifosine-induced ceramide production (P < 0.01 in each case). Interestingly, this was associated with significant decreases in cell death (P < 0.01 in each case; Fig. 6B), which roughly paralleled reductions in ceramide production. In contrast, GW4869 (a neutral sphingomyelinase inhibitor) and fumonisin B1 (a ceramide synthase inhibitor) had no effect on the lethality of these regimens (P > 0.05 in each case; data not shown). Treatment of cells with desipramine did not, however, prevent perifosine/sodium butyrate-mediated inactivation of Akt or ERK1/2 (Fig. 6B, inset), suggesting that perifosine/...
sodium butyrate lethality proceeds, at least in part, through acidic sphingomyelinase-mediated ceramide generation, and that such events may occur downstream of Akt and ERK inactivation.

**Histone Deacetylase Inhibitor/Perifosine–Mediated Ceramide Production Requires Both ERK and Akt Inactivation.** To assess the hierarchy between ERK and Akt inactivation and ceramide generation, U937 cells stably expressing either constitutively active MEK1 or Akt were used. Exposing empty vector cells (pUSEamp) individually to sodium butyrate, SAHA, or perifosine resulted in 1.5- to 2-fold increases in ceramide levels, whereas exposure of U937 cells to HDACI/perifosine is associated with a marked increase in ceramide production, which leads to a dramatic increase in ROS production. A, U937 cells were exposed to the HDACIs SAHA (1.5 μmol/L) or sodium butyrate (1 mmol/L) and perifosine (2.5 μmol/L) alone or in combination for 20 hours in the presence or absence of desipramine after which cells were harvested and ceramide level was determined as described in Materials and Methods. Alternatively, the extent of apoptosis was determined by 24 hours of exposure using Annexin V as described in Materials and Methods (B). Columns, means of at least three separate experiments; bars, SD *, P < 0.01 (significantly lower than values obtained for cells treated with sodium butyrate/perifosine or SAHA/perifosine in the absence of desipramine). Western blot was also used to monitor the effect of desipramine on sodium butyrate/perifosine–mediated ERK and Akt dephosphorylation/inactivation (inset). C, MEK-CA6, Akt-CA6, and pUSEamp cells were exposed to perifosine (2.5 μmol/L) and HDACIs (sodium butyrate, 1 mmol/L, and SAHA, 1.5 μmol/L) alone or in combination for 20 hours after which cells were harvested and ceramide levels were determined as described in Materials and Methods. Columns, means of at least three separate experiments; bars, SD, * P < 0.01 (significantly lower than values obtained for pUSEamp cells). D, U937 cells were exposed to the HDACIs SAHA (1.5 μmol/L) or sodium butyrate (1 mmol/L) and perifosine (2.5 μmol/L) alone or in combination for 24 hours after which ROS production was determined as described in Materials and Methods. Columns, means of at least three separate experiments; bars, SD, * P < 0.01 (significantly higher than values obtained for cells exposed to perifosine, sodium butyrate, or SAHA administered individually). E and F, U937 cells were coexposed to the HDACIs SAHA (1.5 μmol/L) or sodium butyrate (1 mmol/L) and perifosine (2.5 μmol/L) for 24 hours in the presence or absence of N-acetyl-L-cysteine (NAC; 15 mmol/L) and desipramine after which extent of apoptosis (E) and ROS production (F) were determined as described in Materials and Methods. Columns, means of at least three separate experiments; bars, SD *, P < 0.01 (significantly lower than values obtained for cells exposed to sodium butyrate/perifosine or SAHA/perifosine in the absence of N-acetyl-L-cysteine and desipramine).
combined treatment with perifosine and sodium butyrate or SAHA resulted in 6.5- or 4-fold increases, comparable to results obtained in U937 wild-type cells (Fig. 6C). In marked contrast, no increases in ceramide generation (compared with individual drug exposure) were observed in MEK-CA6 and Akt-CA6 cells after combined treatment. Similar results were observed in two additional clones (data not shown). Collectively, these findings argue that inactivation of both ERK and Akt are required for and operate upstream of HDACI/perifosine-mediated increases in ceramide generation.

**Exposure of U937 Cells to Histone Deacetylase Inhibitor/Perifosine Is Associated with a Dramatic Increase in ROS.** Recent evidence suggests that the HDACI lethality, either alone or in combination with other agents, involves ROS generation (13, 14, 36). Furthermore, alkylphospholipids such as edelfosine have also been shown to enhance ROS generation (37). Exposure of U937 cells to perifosine (2.5 μmol/L), sodium butyrate (1 mmol/L), or SAHA (1.5 μmol/L) for 16 and 24 hours resulted in only a moderate increase in ROS production (i.e., <10% over control values); however, combined treatment with perifosine and sodium butyrate resulted in a marked increase in ROS production in 63% of cells (Fig. 6D). Similar results were observed when perifosine was combined with SAHA. In addition, pretreatment of cells with the free radical scavenger N-acetyl-l-cysteine (15 mmol/L) attenuated sodium butyrate/perifosine-mediated lethality (Fig. 6E), whereas significantly, albeit partially, reducing ROS generation (Fig. 6F). Moreover, pretreatment of cells with desipramine, which resulted in a decrease in HDACI/perifosine-mediated lethality (Fig. 6B) as well as ceramide generation (Fig. 6A), also significantly attenuated ROS production (Fig. 6F). Collectively, these findings suggest that HDACI/perifosine-mediated lethality in human leukemia cells proceeds through a ceramide-dependent process operating upstream of ROS generation and downstream of ERK and Akt inactivation.

**Discussion**

The present findings indicate that coadministration of clinically relevant HDACIs and perifosine results in a striking increase in mitochondrial injury, caspase activation, and apoptosis in human leukemia cells. These events are associated with multiple perturbations of diverse cell signaling pathways including inactivation of Akt and MEK/ERK, activation of JNK, an increase in ceramide and ROS production, and a marked conformational change in Bax accompanied by translocation to the mitochondrial membrane. Such findings are consistent with the results of several recent reports indicating that a variety of agents that perturb signal transduction pathways, including proteasome, CDK, and phosphatidylinositol 3-kinase inhibitors are able to lower the threshold for HDACI-mediated lethality, culminating in marked anti-leukemic synergism (18, 36, 38).

It has been shown that a conformational change in Bax is required for its proapoptotic activity, manifested by the translocation of cytochrome c, Samc/DIABLO, and apoptosis-inducing factor from the mitochondria to the cytosol (20, 26–28). The present studies showed that combined exposure of U937 cells to sodium butyrate and perifosine resulted in a marked increase in Bax conformational change, accompanied by extensive translocation of Bax to the mitochondria. Recent studies suggest that 14-3-3 proteins interact physically with Bax and inhibit its conformational change and translocation to the mitochondria through a Bax phosphorylation-independent but partially caspase-dependent process (29).

Notably, cleavage of 14-3-3 proteins was observed only in cells exposed to sodium butyrate and perifosine together but not individually. It is therefore tempting to speculate that cleavage of 14-3-3 protein in sodium butyrate/perifosine-treated cells promotes a conformational change in Bax as well as mitochondrial translocation, contributing to the pronounced increase in apoptosis.

The Akt pathway plays an important antiapoptotic role (39); furthermore, disruption of the phosphatidylinositol 3-kinase/Akt cascade has been implicated in perifosine-mediated lethality in various tumor cells, including the epithelial carcinoma cell lines A431 and HeLa cells (8, 10). Consistent with these results, exposure to perifosine inactivated Akt and reduced phosphorylation of several of its downstream targets (e.g., FKHR) in human leukemia cells; moreover, enforced expression of Akt significantly diminished perifosine-mediated lethality in these cells. Interestingly, constitutive activation of Akt failed to attenuate HDACI-mediated lethality despite dramatically reducing apoptosis induced by perifosine or the perifosine/sodium butyrate regimen. In contrast, potentiation of HDACI-mediated antileukemic activity by the Hsp90 antagonist 17-AAG or the phosphatidylinositol 3-kinase inhibitor LY294002 proceeds through Akt-independent processes (18, 33). Together, these findings indicate that cytoprotective role of the Akt pathway in regulating the lethality of HDACI-containing regimens is highly context dependent and varies with the agents used.

Although down-regulation of the Akt pathway has been implicated in perifosine lethality (10), the precise mechanism by which this phenomenon occurs is not known. Here, exposure of human leukemia cells to marginally toxic concentrations of perifosine resulted in a moderate but significant increase in levels of ceramide (∼1.5- to 2-fold), a proapoptotic lipid second messenger (40). Induction of ceramide generation by perifosine has not previously been reported, although ceramide production has been implicated in alkyl-lysophospholipid miltefosine-mediated apoptosis in the human keratinocyte HaCaT cells (34). Similarly, minimally toxic concentrations of HDACIs also led to modest increases in ceramide production, in accord with a recent report (15). Significantly, coadministration of perifosine and HDACIs led to a very pronounced increase (∼7-fold) in ceramide levels and marked inactivation of Akt, accompanied by a dramatic increase in cell death. The ability of the sphingomyelinase inhibitor desipramine to diminish HDACI/perifosine-mediated ceramide production and apoptosis indicate that the lethality of this regimen involves activation of acidic sphingomyelinase and enhanced ceramide production. Moreover, the inability of the ceramide synthase inhibitor fumonisin B1 to attenuate HDACI/perifosine-mediated lethality is consistent with its failure to block alkylphosphocholine-mediated apoptosis in Bcr/Abl+ human leukemia cells (41).

Several recent studies have shown that HDACIs attenuate Raf-1 expression and MEK/ERK activation (16), possibly by interfering with Hsp90 function (12). Although coadministration of sodium butyrate did not abrogate ERK activation in cells exposed to a low, minimally toxic concentration of perifosine, it did reduce activation to levels well below those observed in cells exposed to perifosine alone, suggesting that diminution in ERK activation by HDACIs promotes perifosine lethality by blocking a cytoprotective response. The ability of constitutively active MEK to block perifosine/HDACIs-mediated lethality is concordant with this hypothesis.

Consistent with evidence that perifosine activates the proapoptotic, stress-related JNK pathway in U937 cells exposed to alkyl-lysophospholipids (2), we found that perifosine activated JNK in a dose-dependent manner and that coadministration of HDACIs with...
perifosine resulted in a pronounced increase in JNK activation. The balance between activation of the stress-related JNK and the cytotoxic ERK pathway regulates cell survival decisions (31); moreover, JNK has been implicated in ceramide lethality in human leukemia cells (42). However, in contrast to the lethal effects of alkyl-lysophospholipid alone, in which interruption of the JNK pathway attenuated lethality, JNK inhibition by SP600125 failed to block HDACI/perifosine-mediated lethality. One possible explanation for these disparate results is that under conditions in which the cytotoxic Akt and ERK pathways are disrupted (e.g., by the combination of perifosine and HDACIs), Akt activation may be dispensable for lethality.

Enhanced lethality of the HDACI/perifosine regimen was associated with an increase in ROS production and was significantly attenuated by the free radical scavenger N-acetyl-L-cysteine, implicating oxidative damage in HDACI/perifosine lethality. Interestingly, desipramine, which diminished HDACI/perifosine-mediated ceramide production lethality, also reduced ROS generation, supporting a model in which ceramide signals downstream to trigger oxidative injury and cell death. The hierarchical relationship between ceramide and ROS generation may vary with the cell type and inciting stimulus. For example, although ceramide generates ROS (43), increases in ROS formation can increase ceramide levels in certain cells (e.g., lung epithelial cells; ref. 44). Significantly, enforced activation of Akt or ERK blocked HDACI/perifosine-mediated ceramide generation. Previous studies have shown that ceramide and ROS can down-regulate Akt through a caspase-dependent process (45). Alternatively, ceramide blocks 3-phosphoinositide binding to the pleckstrin homology domain of Akt (46). However, pretreatment of cells with desipramine, which markedly diminished HDACI/perifosine-mediated ceramide production, did not prevent inactivation of either Akt or ERK. Moreover, coadministration of HDACIs and perifosine failed to increase ceramide levels in cells expressing constitutively active Akt or MEK/ERK. Together, these findings show that inactivation of both MEK/ERK and Akt is required for HDACI/perifosine-induced ceramide production. Although the ability of Akt to block ceramide production has previously been described (47), the capacity of ERK to attenuate ceramide production has not. Collectively, these findings support a model in which antileukemic synergy between perifosine and HDACIs involves inactivation of MEK/ERK (by HDACIs) and Akt (by perifosine), leading in turn to a marked increase in ceramide production via the acidic sphingomyelinase pathway, culminating in oxidative injury and cell death.

The factors regulating HDACI-mediated apoptosis in neoplastic cells may involve diverse processes, including generation of ROS (13, 14). Bid activation (13), down-regulation of Hsp90 function (12), activation of stress-related pathways (16), and cell cycle disturbances (48), among others. Furthermore, agents that disrupt signaling and cell cycle pathways (e.g., the CDK inhibitor flavopiridol) promote HDACI-mediated lethality in leukemic cells (38). The present findings indicate that perifosine represents a promising candidate for this strategy. Specifically, they indicate that coexposure of leukemic cells to perifosine and HDACIs results in Akt and ERK inactivation, leading to a pronounced increase in the acidic sphingomyelinase-dependent generation of ceramide and ROS, and a striking increase in mitochondrial dysfunction (e.g., induction of Bak, Bax conformational change, and mitochondrial translocation). Given the recent introduction of HDACIs (49) and perifosine (6) into the clinical arena, further attempts to explore this novel strategy seem warranted.

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Coadministration of Histone Deacetylase Inhibitors and Perifosine Synergistically Induces Apoptosis in Human Leukemia Cells through Akt and ERK1/2 Inactivation and the Generation of Ceramide and Reactive Oxygen Species

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