Coadministration of the Heat Shock Protein 90 Antagonist 17-Allylaminomethyl-17-demethoxygeldanamycin with Suberoylanilide Hydroxamic Acid or Sodium Butyrate Synergistically Induces Apoptosis in Human Leukemia Cells

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ABSTRACT

Interactions between the histone deacetylase inhibitors (HDACIs) suberoylanilide hydroxamic acid (SAHA) and sodium butyrate (SB) and the heat shock protein (Hsp) 90 antagonist 17-allylaminomethyl-17-demethoxygeldanamycin (17-AAG) have been examined in human leukemia cells (U937). Coadministration of marginally toxic concentrations of 17-AAG with sublethal concentrations of SB or SAHA resulted in highly synergistic induction of mitochondrial damage (i.e., cytochrome c release), caspase-3 and -8 activation, and apoptosis. Similar interactions were noted in human promyelocytic (HL-60) and lymphoblastic (Jurkat) leukemia cells. These events were accompanied by multiple perturbations in signal transduction, cell cycle, and survival-related pathways, including early down-regulation of Raf-1, inactivation of extracellular signal-regulated kinase (ERK) 1/2 and mitogen-activated protein/ERK kinase (MEK) 1/2, diminished expression of phospho-Akt, and late activation of c-Jun-NH2-terminal kinase, but no changes in expression of phospho-p38 mitogen-activated protein kinase. Coadministration of 17-AAG blocked SAHAMEDiated induction of the cyclin-dependent kinase inhibitor p21CIP1 and resulted in reduced expression of p27KIP1 and p34cdc2. 17-AAG/SAHA-treated cells also displayed down-regulation of the antiapoptotic protein McI-1 and evidence of Bel-2 cleavage. Enforced expression of doxycycline-inducible p21CIP1 or constitutively active MEK1 significantly diminished 17-AAG/SAHA-mediated lethality, indicating that interference with ERK activation and p21CIP1 induction play important functional roles in the lethal effects of this regimen. In contrast, enforced expression of constitutively active Akt failed to exert cytoprotective actions. Together, these findings indicate that coadministration of SAHA or SB with the Hsp90 antagonist 17-AAG in human leukemia cells leads to multiple perturbations in signaling, cell cycle, and survival pathways that culminate in mitochondrial injury and apoptosis. They also raise the possibility that combining such agents with Hsp90 antagonists may represent a novel antileukemic strategy.

INTRODUCTION

Hsp90\(^5\) represents a member of a class of proteins referred to as molecular chaperones. Hsp90 and other family members control the intracellular trafficking and folding of diverse cellular proteins, particularly those involved in signal transduction, cell cycle regulation, and survival decisions (1, 2). In the absence of Hsp90, or when the function of Hsp90 is disrupted, multiple client proteins are targeted for ubiquitination and proteosomal degradation (3, 4). The latter include c-Raf, ErbB-2, steroid receptors, Bcr/Abl, Akt, epidermal growth factor, cyclin B1, and cyclin D1, among numerous others (5–8). Recently, attention has been directed at the development of pharmacological Hsp90 inhibitors as chemotherapeutic agents. Such efforts have focused on the ansamycin antibiotics, including geldanamycin and its closely related analogue, 17-AAG (9, 10). Interactions between these drugs and the ansamycin-binding pocket of the Hsp90 NH2 terminus can interfere with binding of Hsp90 to client proteins and may also prevent release of the mature protein (11, 12). In view of evidence of activity in preclinical systems (13, 14), clinical trials involving 17-AAG have been initiated in humans (15). Aside from their intrinsic capacity to induce tumor cell death (14), Hsp90 antagonists such as 17-AAG may also potentiate, in a sequence-dependent manner, the lethal effects of conventional cytotoxic drugs such as paclitaxel (16). Given the documented activity of 17-AAG against Bcr/Abl+ leukemia cells (6), the possibility exists that Hsp90 antagonists might play a role in chronic myelogenous leukemia and related hematopoietic malignancies (6, 17).

HDACIs represent a structurally diverse group of compounds that inhibit the deacetylation of histones and, in so doing, permit the chromatin scaffolding to assume a more relaxed state (18–20). This in turn leads to transcriptional activation of diverse genes, particularly those involved in cellular differentiation (21, 22). Of these, the CDKI p21CIP1 may play a particularly important role (23). HDACIs also induce apoptosis in neoplastic cells through a process that involves, at least in some cell types, generation of reactive oxygen species (24). Whereas the factors determining whether HDACIs induce differentiation versus apoptosis in tumor cells remain to be elucidated, it is tempting to implicate p21CIP1. For example, blockade of p21CIP1 expression by a construct encoding p21CIP1 antisense mRNA results in a marked increase in HDACI-mediated lethality in U937 cells (25). In addition, the CDKI flavopiridol abrogates HDACI-mediated p21CIP1 induction, a phenomenon also associated with a dramatic increase in cell death (26). Currently, a number of HDACIs are undergoing clinical evaluation, including butyrate (27), SAHA (28), MS-275 (29), and depsipeptide (30), among others.

Presently, no information is available concerning the impact, if any, that disruption of Hsp90 function might have on the response of neoplastic cells to agents such as SAHA or SB. To address this issue, we have examined the effects of coadministration of these agents with 17-AAG in human myeloid and lymphoid leukemia cells in relation to induction of cell death. Here we report that 17-AAG interacts in a highly synergistic manner with SAHA or SB to induce mitochondrial damage, caspase activation, and apoptosis in several human leukemia cell types. These events are associated with down-regulation of the Raf/MEK/ERK pathway and abrogation of p21CIP1 induction. These findings suggest that combined treatment with SAHA or SB and Hsp90 antagonists such as 17-AAG may represent a novel therapeutic approach in leukemia and possibly other hematological malignancies.
MATERIALS AND METHODS

Cells. The human leukemia U937 (31), HL-60 (32), and Jurkat (33) cell lines were obtained from American Type Culture Collection (Manassas, VA), cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), and maintained at 37°C in a fully humidified atmosphere containing 5% CO₂. All experiments were performed on logarithmically growing low-passage (∼20) cells. Tet-On Jurkat cell lines inductively expressing a constitutively active AKT (Myc-tagged myristoylated AKT), constitutively active MEK1 (HA-tagged MEK1 S218D/S222D), and p21(CIP1/WAF1) have previously been extensively described (34).

Reagents. 17-AAG was generously provided by the Cancer Treatment and Evaluation Program, National Cancer Institute (Bethesda, MD). SAHA was purchased from Calbiochem (San Diego, CA). SB and TSA were obtained from Calbiochem (San Diego, CA). Doxycycline was purchased from Sigma (St. Louis, MO). All reagents were prepared and used as recommended by their suppliers. In general, concentrations of 17-AAG, SAHA, and SB were used that exerted relatively little toxicity (i.e., 15% apoptosis) over the relevant interval when administered alone.

Assessment of Apoptosis. Apoptotic cells were identified by several different techniques. Annexin V-FITC Staining. Briefly, at the end of the exposure interval, 10⁶ cells were collected, washed in cold PBS, and then resuspended in binding buffer [10 mm HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂] containing fluorescein-labeled annexin V (PharMingen, San Diego, CA) and PI (5 μg/ml). Samples were incubated for 15 min and then analyzed by flow cytometer (Becton Dickinson FACScan).

In some cases, apoptosis was also assessed by TUNEL assay or by morphological assessment of Wright Giemsa-stained cytospin preparations as described previously in detail (35, 36). In all cases, results obtained with annexin V/PI or TUNEL analysis closely paralleled those obtained by morphological assessment of apoptosis.

MTS Assay. Effects of various drug exposures on cell proliferation/viability were also monitored by the MTS assay (36), which reflects reduction of the dye MTS into a colored formazan product in the metabolically active cells. In this assay, diminished MTS reduction can stem from a decrease in cell number (reduced proliferation) as well as apoptotic and nonapoptotic forms of cell death.

Protein Extraction and Western Immunoblotting. Protein extraction and Western blot were performed as described in detail (36). The primary antibodies used in this study were as follows: p21(WAF1/CIP1), p27(Kip1), and MEK1 were purchased from Transduction Laboratories (Lexington, KY). α-Tubulin and cyclin B1 were purchased from Oncogene Resarch Products (Boston, MA). Cytochrome c, caspase-3, caspase-8, cyclin A, cyclin D1, Bax, Hsp27, Mcl-1, and underphosphorylated retinoblastoma were provided by PharMingen. PARP was obtained from Biomel Research Laboratories (Plymouth Meeting, PA). cdc2, phospho-cdc2, ERK1/2, phospho-MEK1/2, and XIAP were obtained from Cell Signaling Technology (Beverly, MA). Bcl-xL, phospho-ERK1/2, phospho-JNK, phospho-p38, phospho-Akt, Raf-1, myc tag, HA tag, Hsp70, and Hsp90 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bcl-2 was provided by DAKO (Carpinteria, CA). For all studies, blot were subsequently reprobed (after stripping) with antibody to tubulin (α) to document equivalent loading and transfer of protein.

Cytchrome c Release. Cytochrome c release into the cytosolic fraction was monitored as described previously (36). Cells (4 × 10⁶) were washed with PBS and resuspended in 100 μl of assay buffer (75 mM NaCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 1 mM EDTA, 250 mM sucrose, and 700 μg/ml digitonin). The cells were incubated on ice for 2 min and pelleted by centrifugation. The supernatant was added to 2× Laemmli buffer and boiled for 5 min. Proteins were separated by SDS-PAGE, and cytochrome c released into the cytosol was evaluated by Western blot as described above.

Statistical Analysis. The significance of differences between experimental conditions was determined using Student's t test for unpaired observations. Synergistic interactions were evaluated using median dose effect analysis using a commercially available software package (Calcusyn; Biosoft, Ferguson, MO; Ref. 37). In this system, the CI value for each fraction affected corresponds to a synergistic interaction.

RESULTS

To characterize interactions between 17-AAG and various HDACIs (e.g., SAHA, SB, or TSA), U937 mononcytic leukemia cells were exposed to either 1.5 μM SAHA, 1 mM SB, or 100 nM TSA and 500 nM 17-AAG, alone and in combination for 28 h, after which the extent of apoptosis was assessed by TUNEL assay (Fig. 1A). As illustrated by the photomicrographs in Fig. 1A, administration of each of the agents alone only marginally increased the percentage of TUNEL-positive cells. However, when SAHA, SB, or TSA was combined with 17-AAG, the extent of apoptosis, reflected by the appearance of TUNEL-positive cells, increased dramatically. Concordant results were obtained when apoptosis was assessed by flow cytometric analysis of annexin V/PI uptake and by Wright-Giemsa-stained cytospin preparation (data not shown). Parallel studies were performed using the MTS assay, which reflects both inhibition of cell proliferation and viability (Fig. 2A). Administration of SAHA, SB, or TSA alone did not affect MTS reduction, whereas 17-AAG by itself was associated with approximately a 40% decline. However, combined treatment with 17-AAG and either SAHA, SB, or TSA resulted in a very substantial decrease in MTS reduction (i.e., 80–90%). Western blot analysis (Fig. 1C) revealed that administration of SAHA and 17-AAG in combination resulted in a marked increase in procaspase-3 and -8 processing, PARP degradation, and release of cytochrome c into the cytosolic cell fraction, whereas the agents administered individually exerted minimal effects. These findings are consistent with an increase in apoptosis in SAHA/17-AAG-treated cells. Median dose effect analysis (Fig. 1D) of apoptotic interactions (monitored by

Fig. 1. 17-AAG interacts synergistically with HDACIs to induce mitochondrial damage, caspase activation, and apoptosis in U937 cells. A. U937 cells were treated with either 1.5 μM SAHA, 1 mM SB, or 100 nM TSA and 500 nM 17-AAG, alone and in combination for 28 h, after which the extent of apoptosis was assessed by TUNEL assay as described in “Materials and Methods.” B, U937 cells were treated as described above for 24 h, after which MTS reduction was evaluated after an additional 4 h. C, cells were treated with SAHA ± 17-AAG as described above, and then whole cell lysates were obtained, the proteins were separated by SDS-PAGE, and Western blot analysis was used to monitor expression of caspase-3, caspase-8, and PARP. Alternately, mitochondria-free cytosolic fractions were obtained as described in “Materials and Methods” and subjected to Western blot analysis as described above to monitor release of cytochrome c. Each lane was loaded with 25 μg of protein; the blots were subsequently stripped and reprobed with antibodies to tubulin (α) to document equivalent loading and transfer. The result of a representative study is shown; two additional experiments yielded equivalent results. D, median dose effect analysis of apoptosis induction by SAHA and 17-AAG. U937 cells were exposed to varying concentrations of SAHA and 17-AAG in a fixed ratio (3:1), after which apoptosis was monitored at 28 h by annexin V/PI analysis. CI values were determined for each fraction affected using a commercially available software program as described in “Materials and Methods.” CI values < 1.0 correspond to a synergistic interaction.
fig. 2. SAHA and 17-AAG induce apoptosis in U937 cells in a time- and dose-dependent manner as well as in other human leukemia cell types. A, U937 cells were exposed to 17-AAG (500 nM) ± 1.5 μM SAHA for the indicated interval, after which the percentage of apoptotic cells was determined by flow cytometric analysis of annexin V-stained samples as described in “Materials and Methods.” B, U937 cells were exposed for 28 h to the designated concentration of 17-AAG alone (C) or in conjunction with 1.5 μM SAHA (•), after which the percentage of apoptotic cells was determined by annexin V analysis as described above. C, cells were exposed to the designated concentration of SAHA alone (C) or in combination with 500 nM 17-AAG (•), after which apoptosis was determined as described above. Values represent the means for three separate experiments performed in triplicate ± SD. D, HL-60 promyelocytic and Jurkat lymphoblastic leukemia cells were exposed to SAHA (0.75 μM) or SB (0.75 μM) alone or in combination with 17-AAG (500 nM) for 48 h, after which the extent of apoptosis was monitored by annexin V analysis as described above. Values represent the means ± SD for three separate experiments performed in triplicate.

Annexin V/PI positivity (between 17-AAG and SAHA over a range of concentrations yielded CI values considerably less than 1.0, corresponding to a high degree of synergism.

A time-course analysis of cell death in drug-treated U937 cells is shown in Fig. 2A. Whereas the extent of apoptosis in 17-AAG/SAHA-treated cells, reflected by annexin V positivity, was minimal after 16 h of drug exposure, very substantial lethality was noted by 28 h. Although treatment with 500 nM 17-AAG alone for 48 h was clearly toxic to these cells, the extent of lethality was still very significantly less than that observed in cells exposed to both agents (P < 0.002). Exposure to SAHA alone (1.5 μM) minimally induced apoptosis over the entire 48-h treatment interval. A 17-AAG dose-response study revealed that a minimally toxic SAHA concentration (1.5 μM) significantly increased the lethality of 17-AAG administered at concentrations as low as 250 nM but that effects were greatest for 17-AAG concentrations of ≥500 nM (Fig. 2B). Conversely, SAHA concentrations of ≥1 μM were most effective in potentiating 17-AAG lethality (Fig. 2C).

Attempts were then made to determine whether potentiation of 17-AAG-induced apoptosis could be extended to include other human leukemia cell lines. As shown in Fig. 2D, coadministration (48 h) of a subtoxic concentration of SB (0.75 μM) with a marginally toxic concentration of 17-AAG (500 nM) resulted in a very dramatic increase in apoptosis (i.e., ∼55%) in HL-60 promyelocytic leukemia cells. Similarly, synergistic interactions between 17-AAG and SAHA were observed in these cells (Fig. 2D). Comparable results were obtained when interactions between SAHA or SB and 17-AAG were examined in Jurkat human lymphoblastic leukemia cells, although it should be noted that these cells were considerably less sensitive to 17-AAG than their U937 or HL-60 cell counterparts.

To determine what effect, if any, coadministration of 17-AAG might have on HDACi-mediated maturation, expression of CD11b, a myelomonocytic maturation marker, was monitored in U937 cells exposed to 1.5 μM SAHA ± 500 nM 17-AAG for 48 h. Whereas SAHA alone resulted in ∼37% CD11b positivity (versus 6% in controls), and 17-AAG by itself was ineffective (4% positive), coadministration of these agents reduced the percentage of CD11b+ cells to ∼20% (P < 0.05 versus SAHA alone; data not shown). These findings indicate that potentiation of HDACi-mediated apoptosis in human leukemia cells is accompanied by a reduction in cellular maturation.

The effects of combined treatment of U937 cells with 17-AAG and SAHA were then explored with respect to the expression of several cell cycle-related proteins. Exposure to SAHA robustly induced p21Cip1 after 16 h, and effects were even more marked after 28 h (Fig. 3A). However, coadministration of 17-AAG, which by itself failed to modify p21Cip1 expression, essentially abrogated SAHA-mediated p21Cip1 up-regulation. No changes in p27Kip1 levels were noted at 16 h under any conditions, although reduced expression was observed at 28 h in cells exposed to 17-AAG or 17-AAG + SAHA. Reductions in levels of phosphorylated p34cdc2 were observed at both intervals in cells exposed to 17-AAG ± SAHA, although this phenomenon could be attributed, at least in part, to reductions in total p34cdc2 levels. Expression of cyclin A and cyclin D1 was largely unperturbed after exposure of cells to 17-AAG ± SAHA. In contrast, cyclin B1 levels were reduced at 28 h in cells exposed to SAHA ± 17-AAG. Lastly, treatment of cells with SAHA alone resulted in underphosphorylation of pRb, whereas exposure of cells to 17-AAG and particularly the combination of 17-AAG + SAHA resulted in marked cleavage of the underphosphorylated pRb species. Thus, treatment of cells with SAHA and 17-AAG resulted in changes in expression of multiple cell cycle proteins previously related to apoptosis, most notably interference with p21Cip1 induction.

Effects of the drug combination were then examined with respect to expression of various Hsps. Exposure of cells to 17-AAG ± SAHA did not modify expression of Hsp90 at either 16 or 28 h (Fig. 3B). Treatment with 17-AAG alone resulted in an increase in Hsp27 expression, an effect that was diminished by coadministration of

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SAHA. Cells exposed to 17-AAG ± SAHA exhibited an increase in Hsp70 expression at 16 h, along with evidence of protein degradation, which persisted for the ensuing 12 h. By 28 h, protein expression had returned to basal levels.

To determine whether enhanced apoptosis in 17-AAG/HDACI-treated U937 cells could be related to alterations in expression of Bcl-2 family members, particularly those whose expression is known to be modified by Hsp90 antagonists, Western analysis was performed. No changes were noted in levels of Bax, XIAP, and Bcl-xL after treatment of cells with SAHA ± 17-AAG (Fig. 3C). On the other hand, Mcl-1 expression was very modestly reduced in cells exposed to 17-AAG alone or the combination of 17-AAG + SAHA at both the 16- and 28-h intervals. In addition, whereas levels of the Bcl-2 protein did not change appreciably with any treatment, a putatively proapoptotic Bcl-2 cleavage product (38) could be faintly discerned in cells exposed to the combination of SAHA + 17-AAG for 28 h.

More detailed time course studies were then performed to assess the effects of SAHA ± 17-AAG on various signal pathways implicated in apoptosis regulation. Treatment of U937 cells with 17-AAG for 6 h resulted in reduced expression of Raf-1 (Fig. 4), consistent with previous reports (5). However, Raf-1 down-regulation was even more pronounced in cells coexposed to SAHA at this early interval. At intervals of ≥16 h, Raf-1 protein was undetectable in cells exposed to 17-AAG alone or to 17-AAG + SAHA. As anticipated, levels of phosphorylated (activated) MEK1/2, a major target of Raf-1 (39), were clearly diminished after a 2-h exposure to 17-AAG or 17-AAG + SAHA and were undetectable at intervals of ≥16 h. Interestingly, treatment of cells with SAHA alone for ≥16 h resulted in a very pronounced reduction in levels of phospho-MEK1/2. Essentially parallel results were obtained when expression of phospho-ERK1/2, the major MEK1/2 target (39), was monitored. Levels of ERK1/2 remained unperturbed for all treatment conditions, as did levels of total MEK over the initial 6-h treatment interval. However, total MEK expression declined in cells exposed to 17-AAG ± SAHA after 16–28 h of drug exposure. Thus, coexpression of U937 cells with 17-AAG + SAHA was associated with early and very extensive down-regulation of the Raf-1/MEK/ERK pathway, although SAHA alone effectively antagonized MEK1/2 and ERK1/2 activation at later intervals. Similar down-regulation of Raf/MEK/ERK expression/activation was observed in Jurkat cells exposed to 17-AAG + SAHA (data not shown).

In contrast to these findings, exposure of cells to 17-AAG ± SAHA exerted minimal effects on activation of the stress-related kinases JNK and p38 mitogen-activated protein kinase at early intervals, although marked JNK phosphorylation was noted in cells treated for 28 h with 17-AAG + SAHA (Fig. 4). In addition, modest but discernible reductions in levels of phospho-Akt were observed in cells exposed to 17-AAG ± SAHA at exposure intervals of ≥6 h. Thus, coadministration of SAHA and 17-AAG was associated with early inactivation of the cytoprotective Raf/MEK/ERK and Akt pathways and late activation of the stress-related JNK pathway.

To assess the functional significance of some of these perturbations in 17-AAG/SAHA-mediated lethality, Jurkat cells inducibly expressing a constitutively active MEK1 under the control of doxycycline were either left untreated or treated for 24 h with 2 μg/ml doxycycline, after which they were exposed to the combination of 5 μM 17-AAG ± 1 μM SAHA for an additional 48 h. At the end of this interval, cells were separated into two pools. One pool was lysed and analyzed for HA-MEK1 and phospho-ERK1/2 expression by Western blot (Fig. 5A), whereas the other pool was used to determine the percentage of apoptotic cells using the annexin V-FITC assay as described in Materials and Methods. For A, each lane was loaded with 25 μg of protein; blots were subsequently reprobed with antibody directed against α-tubulin (tub) to control for equal loading and transfer of proteins. Two additional experiments yielded similar results. For B, values represent the means ± SD for four experiments. * significantly less than the values obtained for cells exposed to SAHA/17-AAG in the absence of doxycycline, P < 0.05.

Fig. 4. Coadministration of 17-AAG and SAHA diminishes levels of Raf-1, phospho-MEK, and phospho-ERK while increasing JNK activation in U937 cells. Logarithmically growing U937 cells were exposed to 500 nM 17-AAG ± 1.5 μM SAHA alone or in combination for indicated intervals, after which protein lysates were prepared and subjected to Western blot analysis using antibodies recognizing only the phosphorylated (activated) forms of MEK (Ser218/222), ERK (p44/42 Tyr204/202), p38 (Tyr182/185), and Akt (Ser473). In addition, antibodies recognizing total Raf-1, MEK1, and ERK1/2 proteins were also used. Each lane was loaded with 25 μg of protein; blots were subsequently reprobed with antibody directed against α-tubulin to control for equal loading and transfer of proteins. Two additional experiments yielded similar results.

Fig. 5. Enforced activation of MEK/ERK pathway significantly protects cells from 17-AAG/SAHA-mediated apoptosis. Jurkat cells inducibly expressing constitutively active HA-tagged MEK1 under the control of doxycycline were either left untreated or treated for 24 h with 2 μg/ml doxycycline, after which they were exposed to the combination of 5 μM 17-AAG ± 1 μM SAHA for an additional 48 h. At the end of this interval, cells were separated into two pools. One pool was lysed and analyzed for HA-MEK1 and phospho-ERK1/2 expression by Western blot (A), whereas the other pool was used to determine the percentage of apoptotic cells using the annexin V-FITC assay as described in Materials and Methods. For A, each lane was loaded with 25 μg of protein; blots were subsequently reprobed with antibody directed against α-tubulin (tub) to control for equal loading and transfer of proteins. Two additional experiments yielded similar results. For B, values represent the means ± SD for four experiments. * significantly less than the values obtained for cells exposed to SAHA/17-AAG in the absence of doxycycline, P < 0.05.
AAG/SAHA-treated cells. As shown in Fig. 5B, cells exposed to 17-AAG + SAHA exhibited a partial but statistically significant reduction in apoptosis in the presence of doxycycline ($P < 0.005$). Similar results were obtained in a second doxycycline-inducible clone (MT6; data not shown). These findings suggest that diminished activation of ERK in 17-AAG/SAHA-treated leukemic cells contributes to the lethality of this drug combination.

To assess what functional role $p21^{CIP1}$ might play in 17-AAG/SAHA-mediated lethality, a $p21^{CIP1}$ inducible system was used. Jurkat cells exposed to SAHA + 17-AAG in the absence of doxycycline failed to display $p21^{CIP1}$ induction, whereas addition of doxycycline to the medium resulted in robust $p21^{CIP1}$ expression in both treated and untreated cells (Fig. 6A). Unexpectedly, treatment of cells with 17-AAG/SAHA resulted in an increase in $p21^{CIP1}$ expression in cells ectopically expressing this protein, raising the possibility that 17-AAG/SAHA may in some way stabilize ectopically expressed $p21^{CIP1}$ protein. In any case, enforced expression of $p21^{CIP1}$ modestly but significantly diminished apoptosis in SAHA/17-AAG-treated cells ($P < 0.02$ versus values obtained in cells exposed to drugs in the absence of doxycycline; Fig. 6B). A modest but statistically significant reduction in apoptosis was also noted in cells ectopically expressing $p21^{CIP1}$ after exposure to the combination of 17-AAG + SB ($P < 0.05$; Fig. 6B). These findings raise the possibility that dysregulation of $p21^{CIP1}$ in SAHA/17-AAG-treated cells may contribute functionally, at least in part, to the cytotoxicity of this regimen.

Previous studies have suggested that the lethal effects of 17-AAG proceed through a PI3K/Akt-dependent mechanism (40). To determine whether the modest reduction in Akt activation in SAHA/17-AAG-treated cells contributed to the enhanced lethality of this regimen, a Jurkat cell line inducibly expressing a constitutively active (myristoylated) Akt was used. As shown in Fig. 7A, treatment of Jurkat cells with 17-AAG + SAHA resulted in a small but discernible reduction in expression of phospho-Akt. When cells expressing the inducible Akt were exposed to SAHA/17-AAG regimen in the presence of doxycycline, a striking increase in phospho-Akt expression was noted. However, addition of doxycycline to the medium failed to reduce the extent of 17-AAG/SAHA-mediated apoptosis, despite markedly enhancing Akt activation ($P > 0.05$; Fig. 7B). Together, these findings argue against the possibility that disruption of Akt signaling plays a role in synergistic antileukemic interactions between HDACIs and 17-AAG.

### DISCUSSION

The present findings indicate that combined exposure of human leukemia cells to SAHA or SB in conjunction with the Hsp90 antagonist 17-AAG results in a striking increase in mitochondrial injury, caspase activation, and apoptosis. By modifying chromatin structure, HDACIs activate a variety of genes, including $p21^{CIP1}$, associated with cell cycle arrest and, in some cases, differentiation (21, 23, 41, 42). However, under certain circumstances (i.e., when they are administered at high concentrations), HDACIs can also induce apoptosis in neoplastic cells (22, 43). Although the factors that determine whether HDACIs trigger differentiation versus apoptosis remain to be...
elucidated, induction of p21^{CIP1} (23) and generation of reactive oxygen species as well as induction of Bid cleavage (24) have been implicated in this decision. In this regard, it may be relevant that 17-AAG, by disrupting the chaperone function of Hsp90, induces perturbations in the intracellular disposition and expression of multiple proteins involved in cell cycle regulation, signal transduction, and maturation (5–8). One possibility is that 17-AAG, by interfering with one or more such pathways, disrupts the “normal” differentiation process in HDACI-treated leukemic cells, thereby causing them to engage the cell death program. An alternative possibility is that combined exposure of cells to these agents induces conflicting signals, which in turn trigger mitochondrial injury and apoptosis. Finally, it is known that HDACIs may perturb the acetylation status of many proteins other than or in addition to histones, including Hsp90, which may lead to degradation of various survival-related signal transduction proteins (44). Furthermore, it has recently been shown that the hydroxamic acid HDACI LAQ824 induces acetylation of Hsp90 in K562 chronic myelogenous leukemia cells, resulting in degradation of the Hsp90 client protein Bcr/Abl accompanied by apoptosis (45). Thus, the possibility that certain HDACIs might further disrupt Hsp90 chaperone function in cells exposed to 17-AAG, thereby potentiating cell death, appears plausible. It is important not to generalize the present results to include HDACIs and all cell types in light of evidence that in some cases, 17-AAG may in fact block HDACI-mediated lethality. For example, Huang et al. (46) reported that geldanamycin blocked TSA-mediated apoptosis as well as histone H4 acetylation in COS cells. These results stand in contrast to the present findings demonstrating that 17-AAG markedly increased TSA-induced apoptosis and loss of viability in U937 cells (Fig. 1, A and B). The basis for these disparate results is not clear, but may reflect (a) different effects of geldanamycin versus 17-AAG, (b) differential responses of monkey kidney cells transformed with an origin-defective mutant of SV40 (e.g., COS-7) versus human myeloid leukemia cells (U937), or (c) a combination of these factors. Whether and to what extent the interactions described herein can be extended to other novel HDACIs and other neoplastic cell types remain to be established.

The ability of 17-AAG to attenuate HDACI-mediated induction of p21^{CIP1} was unanticipated and, to the best of our knowledge, has not been described previously. Because p21^{CIP1} is a downstream target of ERK (47), it is tempting to speculate that 17-AAG/HDACI-mediated interruption of the Raf-1/MEK/ERK pathway (vidae infra) may contribute to this phenomenon. The present findings are consistent with the results of earlier studies demonstrating that p21^{CIP1} blocks HDACI-mediated apoptosis. For example, interference with p21^{CIP1} induction by an antisense construct (25) or by the transcriptional repressor and CDK1 flavopiridol (26) blocks HDACI-induced maturation in human leukemia cells and reciprocally promotes apoptosis. Conversely, induction of p21^{CIP1} has been shown to block azelaic acid bishydroxamic (ABHA)-mediated apoptosis in colon carcinoma cells (HCT116; Ref. 48). In accord with these findings, enforced expression of p21^{CIP1} partially but significantly diminished the cytotoxicity of both the 17-AAG/SAHA and 17-AAG/SB regimes. Although the mechanism by which p21^{CIP1} opposes apoptosis is not known with certainty, binding to and interference with the activation of caspase-3 have been invoked (49). It is important to note that whereas enforced expression of p21^{CIP1} significantly attenuated SAHA/17-AAG lethality, the degree of protection was quite modest, indicating that factors other than or in addition to dysregulation of p21^{CIP1} are involved in antileukemic synergism.

In view of evidence that Hsp90 is a key regulator of the Raf-1/MEK/ERK pathway (5), it is noteworthy that SAHA/17-AAG regimens induced marked down-regulation and inactivation of the cytoplasmic MEK/ERK cascade. Specifically, exposure of U937 cells to 17-AAG + SAHA accelerated the reduction in Raf-1 expression at early intervals (e.g., ≤6 h). Combined exposure of cells to SAHA and 17-AAG also resulted in the marked inactivation of MEK1/2 and ERK1/2, although the effects were not demonstrably greater than those observed in cells exposed to 17-AAG alone. This suggests that additional factors in all likelihood contribute to the striking synergism between these agents. In any case, the observation that enforced expression of constitutively active MEK significantly, albeit partially, protected leukemic cells from SAHA/17-AAG-mediated lethality argues strongly that disruption of this pathway plays an important role in potentiation of apoptosis. Interestingly, exposure to SAHA alone for longer intervals (i.e., ≥16 h) also resulted in inactivation of MEK and ERK, analogous to effects observed in lung cancer cells exposed to the HDACI depsipeptide (44). Thus, 17-AAG and SAHA may cooperate to disable the Raf/MEK/ERK cascade. However, SAHA administered alone exerted minimal lethality, indicating that MEK/ERK inactivation, by itself, is insufficient to trigger the apoptotic program. On the other hand, the possibility that disruption of Raf-1 signaling may represent a very potent cell death stimulus when it is combined with additional perturbations in other cell cycle and apoptotic regulatory cascades appears plausible.

Recent studies in breast cancer cells have implicated disruption of the PI3K/Akt pathway in 17-AAG-mediated lethality (40), and down-regulation of Akt (i.e., by the PI3K inhibitor LY294002) has been associated with enhanced susceptibility of colon tumor cells to butyrate-induced apoptosis (50). Such results raise the possibility that the inactivation of Akt in cells exposed to SAHA and 17-AAG contributes to synergism between these agents. However, in contrast to enforced induction of p21^{CIP1} or constitutively active MEK1/2, enforced expression of constitutively active Akt failed to diminish SAHA/17-AAG-mediated lethality. Such a finding argues against the involvement of Akt inactivation in synergistic interactions between 17-AAG and SAHA, at least in human leukemia cells. In this regard, recent evidence from our laboratory suggests that synergistic induction of apoptosis by HDACIs such as SAHA and LY294002 in human leukemia cells proceeds through an Akt-independent mechanism (34). Cells exposed to SAHA and 17-AAG displayed several other perturbations in the expression of cell cycle-, signaling-, and survival-related proteins that may have contributed to lethality of this regimen. For example, exposure of cells to 17-AAG ± SAHA resulted in reduced expression of the CDKI p27^{KIP1}, which is known to exert antiapoptotic actions (51). Combined treatment with SAHA and 17-AAG also resulted in late down-regulation of the antiapoptotic protein Mcl-1 and the generation of a Bcl-2 cleavage fragment. Recent studies have emphasized the potentially important role of Mcl-1 expression in the survival of malignant hematopoietic cells (52), and proapoptotic actions of the Bcl-2 cleavage product have been described previously (38). Finally, cells exposed to both 17-AAG and SAHA exhibited a late increase in JNK phosphorylation (activation), which, in conjuncion with interruption of the Raf/MEK/ERK cascade, signifies a shift away from cytoprotective and toward stress-related signaling (53). Although several of these perturbations may represent secondary phenomena, they could nevertheless serve to amplify effects of the initial apoptotic stimulus. Given the pleiotropic nature of 17-AAG actions (54), it would not be surprising if mechanisms underlying synergistic interactions between HDACIs and Hsp90 antagonist were multifactorial in nature.

Recently, considerable attention has been directed at the concept of combining novel, molecularly targeted compounds such as Iressa, STI571, bryostatin 1, flavopiridol, and UCN-01 with established cytotoxic agents (55–61). In this context, there is evidence that Hsp90 antagonists such as 17-AAG may also lower the threshold for cyto-
toxic drug-induced lethality (16, 62). However, evidence is accumu-
lating that combining signal transduction with cell cycle inhibitors or
combining each of these agents with others of its class constitutes a
very potent apoptotic stimulus in neoplastic cells. Examples of this
strategy include coadministration of (a) UCN-01 with MEK1/2 inhib-
itors (63); (b) STI571 with flavopiridol (64), MEK1/2 inhibitors (35),
or PI3K inhibitors (65); and (c) hereceptin with flavopiridol (66). The
present findings suggest that HDACs, which function as cell cycle
inhibitors, differentiation inducers, and modulators of signal trans-
duction pathways (22), may represent particularly attractive candi-
dates for such combination approaches. The discovery of synergistic anti-
leukemic interactions between HDACs and CDKis (26), PI3K inhib-
itors (50), and now Hsp90 antagonists provides further support for this
notion. It should be recognized that the threshold for induction of
apoptosis in continuously cultured cell lines such as Jurkat may be
considerably lower than that of primary human leukemic myeloblasts,
noteably in cells obtained from heavily pretreated patients. Nev-
evertheless, given the ongoing clinical development of HDACs, includ-
ing SAHA (67), as well as the recent initiation of Phase I and II trials of
Hsp90 inhibitors such as 17-AAG (15), further investigation of interac-
tions between Hsp90 antagonists and clinically relevant HDACs appear
warranted, particularly in hematologic malignancies.

Accordingly, studies are currently under way.

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Coadministration of the Heat Shock Protein 90 Antagonist 17-Allylamino-17-demethoxygeldanamycin with Suberoylanilide Hydroxamic Acid or Sodium Butyrate Synergistically Induces Apoptosis in Human Leukemia Cells

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