Histone deacetylase inhibitors induce G2-checkpoint arrest and apoptosis in cisplatinum-resistant ovarian cancer cells associated with overexpression of the Bcl-2–related protein Bad

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Abstract
Trichostatin A produces predominantly G1 cell-cycle blockade and differentiation of the cisplatinum-sensitive A2780 ovarian cancer cell line. Given the propensity of ovarian tumors to become resistant to cisplatinum, often leading to cross-resistance to other agents, we have extended these observations by examining how the emergence of resistant phenotypes in A2780 cells affects the actions of histone deacetylase (HDAC) inhibitors. Trichostatin A exposure (100 ng/mL, 24 hours) induced ultrastructural differentiation of the “intrinsically” cisplatinum-resistant A2780-9M subline, with the reappearance of intercellular junctions and lumina containing primitive microvilli. Similar trichostatin A exposure in the acquired resistance A2780CP cells produced minimal differentiation consisting of occasional weak intercellular junctions. Independently of the differences in trichostatin A–induced differentiation, in both resistant sublines trichostatin A produced a similar reduction in cell viability, by >90%, within 5 days of treatment. Diminished viability in both A2780-9M and CP cells was associated with the absence of cell cycle arrest in G1, resulting in predominant G2–checkpoint arrest accompanied by a 10- to 20-fold increase in Annexin V binding and the reemergence of apoptosis. Similar cell cycle arrests and apoptosis were also observed using other HDAC inhibitors and in other resistant ovarian cancer cell lines (OVCAR-3 and SK-OV-3). Trichostatin A–induced apoptosis in resistant cells is in sharp contrast to its effects on the parental cisplatinum-sensitive A2780 and normal MRC-5 fibroblast cell lines (predominant cycle arrest in G1 with no detectable apoptosis). Western immunoblot analysis indicated trichostatin A triggers apoptosis in resistant ovarian cancer cells via p53-independent activation of the intrinsic “mitochondrial” pathway, commensurate with induction of the Bcl-2–related protein Bad. These results suggest cisplatinum resistance alters the effects of HDAC inhibition through a shift in cell cycle arrest from the G1 to the G2 checkpoint and reactivation of the intrinsic mitochondrial apoptotic cascade. [Mol Cancer Ther 2005;4(4):603–11]

Introduction
The use of platinum-based combination chemotherapy remains the standard treatment for ovarian epithelial cancers, with an initial clinical response rate of ~70% (1). However, for advanced stage ovarian tumors (stages III–IV) the 5-year survival remains a dismal 10% (2), primarily due to the fact that both primary and recurrent tumors often develop resistance to cisplatinum. Further complicating the matter, resistance to platinum-based compounds often leads to resistance to a broad cross-section of other functionally unrelated chemotherapeutic agents (multidrug resistance; ref. 3).

Multiple mechanisms have been described that contribute to the ability of cells to resist the actions of chemotherapeutic agents (4), including increased DNA damage tolerance, due to increased DNA damage repair, induction of survival factors, and alterations in apoptotic signaling. In ovarian cancers, cisplatinum resistance is often associated with diminished apoptotic signaling, involving the silencing of genes for cell cycle regulation (p21WAF1/Cip1, p16, and Rb; ref. 5), DNA damage assessment (p53; ref. 6), and DNA mismatch repair (7).

Gene silencing, associated with tumorigenesis and chemotherapeutic resistance, has recently emerged as an area of intense investigation due to recent advances in our understanding of the link between modifications of chromatin structure and the transcriptional activity of genes (8). Studies have shown that gene silencing is often associated with hypoacetylation of histone proteins through the aberrant actions of the histone deacetylase (HDAC) enzymes. Given the close association between histone acetylation and the transcriptional silencing of genes associated with oncogenesis, inhibitors of HDAC enzymes are emerging as a potentially important new class of anticancer agents (9). To date, a number of studies have

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shown specific actions of various HDAC inhibitors on the reexpression of silenced genes involved in cell-cycle arrest (10), DNA repair/damage assessment (11), and apoptosis (12), pathways that are also associated with cisplatinum resistance.

Given the propensity of ovarian cancer cells to develop resistance to cisplatinum, it is important to understand how resistant phenotypes affect the actions of HDAC inhibitors. Recent data indicate the actions of HDAC inhibitors in several tumor cell types can be blocked, or altered, by overexpression of antiapoptotic factors associated with cisplatinum resistance, such as Bcl-2 (13, 14), NF-κB (15), and the phosphatidylinositol-3 kinase/Akt kinase survival pathway (16), which are often overexpressed in cisplatinum-resistant ovarian cancer cells (17, 18).

Previously we reported that trichostatin A induced G1 arrest and “epithelial-like” transformations of cisplatinum-sensitive A2780 ovarian cancer cells, commensurately with changes in the expression of genes involved in cell-cycle regulation and differentiation (19). In the current studies, we examined two cisplatinum-resistant A2780 sublines to determine the effect of the emergence of resistant phenotypes on HDAC inhibition. Our results indicate that both “intrinsic” and “acquired” resistance in A2780 sublines alter the actions of HDAC inhibitors, resulting in predominant G2-checkpoint cell cycle arrest and subsequent loss of cell viability due to reactivation of apoptotic cascades. Apoptosis was restricted to resistant cell lines and was not affected by concurrent HDAC inhibitor–induced differentiation. Apoptosis also occurred independent of p53 expression through reactivation of the intrinsic “mitochondrial” Bcl-2/Bax pathway, commensurate with increased expression of the proapoptotic Bcl-2–related protein Bad.

Materials and Methods
Cell Culture and Reagents
The human ovarian cancer cell line A2780 and its cisplatinum-resistant subline A2780CP were obtained from the European Collection of Cell Cultures (Salisbury, United Kingdom). SK-OV-3, OVCAR-3, and MRC-5 cells were from the American Type Culture Collection (Manassas, VA). A2780-9M cells were derived from the parental A2780 line, in our laboratory, by maintaining the cells in continuous culture for 9 months. Cell lines were cultured as follows: A2780, A2780CP, and A2780-9M ovarian cancer cells in RPMI 1640; SK-OV-3 ovarian cancer cells in McCoy’s 5a with 1.5 mmol/L L-glutamine; OVCAR-3 ovarian cancer cells in RPMI 1640 with 2 mmol/L L-glutamine + 0.001 mg/mL insulin; and normal fetal lung fibroblast MRC-5 cells in Eagle’s MEM with Earle’s balanced salts. All media were supplemented with 10% fetal bovine serum, with the exception of the OVCAR-3 medium, where 20% fetal bovine serum was added. Cultures were grown at 37°C in a humid 95% air/5% CO2 chamber and were periodically tested to ensure they remained free of mycoplasma infection during the course of the experiments. Trichostatin A and sodium butyrate were obtained from Sigma (St. Louis, MO); hexamethylene bisacetamide was from Calbiochem (La Jolla, CA); and CI-994 was generously provided by Pfizer (Groton, CT).

Trichostatin A Treatment
Twenty-four hours before trichostatin A addition, cells were subcultured onto fresh 100-mm tissue culture plates. The following day, the media was replaced with media containing 100 ng/mL trichostatin A for 24 hours. After 24 hours, the trichostatin A was removed by changing the media and the cells were returned to the incubator for the remainder of the experimental period.

H&E Staining
Cells grown on microscope slides (Lab-Tek, Naperville, IL) were washed twice with PBS, fixed with 4% paraformaldehyde (5 minutes, room temperature), dehydrated through a series of ethanol washes, and stained with H&E.

Cell Viability
Cells grown on culture plates were treated with trichostatin A at day 0 as described above. On subsequent days, cells were trypsinized from the culture plates, diluted with PBS, incubated with 1 volume of a 0.4% trypan blue solution for 15 minutes at room temperature, and an aliquot examined for viable cells using a hemocytometer as previously described (19).

Electron Microscopy
Untreated and trichostatin A–treated cells were fixed and embedded in polymer capsules for electron microscopy as previously described (19). The polymerized cell pellets were subsequently sectioned on an Ultratome (LKB, New York, NY), affixed to 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined on a JEM1010 JEOL electron microscope.

Immunohistochemistry
Immunostaining of MLH1 (1:100 dilution, BD Pharmagen, San Diego, CA) was done on cells grown on Lab-Tek chamber slides as previously described (19) using a DakoAutoStainer (DAKO, Carpinteria, CA) with 3,3’-diaminobenzidine as the substrate.

Flow Cytometry
Cell cycle analysis was done using a modified Vindelov propidium iodide DNA staining procedure as previously described (19). Samples of untreated and treated cells were run on a Coulter Epix XL flow cytometer and evaluated using the Phoenix flow DNA modeling system to determine the percentage of cells in G1, S, and G2-M phases.

Mitotic Index
Cells grown on microscope slides (Lab-Tek) were treated with trichostatin A (24 hours), washed in PBS, fixed, and stained with 10 μg/mL 4’,6-diamidino-2-phenylindole in PBS containing 5 μg/mL RNase A. For each sample, five fields were randomly counted (~250 cells) by fluorescence microscopy, and the percentage of cells undergoing mitosis was scored blindly.

Apoptosis Assay
Annexin V binding was measured by flow cytometry (20) using the APOTEST-FITC kit (DAKO). Briefly, cells were trypsinized from the culture dishes, centrifuged at 300 × g,
and washed twice with ice-cold PBS. The final cell pellet was resuspended in ice-cold binding buffer at 10^5 to 10^6 cells/mL. Ninety-six microliters of cell suspension were incubated with 1 μL of Annexin V-FITC and 2.5 μL of propidium iodide (250 μg/mL, stock) and incubated on ice in the dark for 10 minutes. Following the incubation, samples were diluted with binding buffer to 250 μL and run on a Coulter Epix XL flow cytometer.

**Western Immunoblotting**

Untreated and trichostatin A–treated (100 ng/mL, 24 hours) cells attached to 100-mm culture dishes were washed twice with ice-cold PBS, followed by addition of 1 mL lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.1% SDS, 100 μg/mL phenylmethylsulfonyl fluoride, 1% NP40, and 0.5% sodium deoxycholate) for 2 minutes on ice. Twenty micrograms of total protein, diluted 1:1 in Laemmli loading buffer, were run on 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes as described (21). To ensure qualitative transfer of all proteins, the leftover gel was silver stained and the protein-bound nitrocellulose membranes were stained using the Reversible Protein Detection Assay (Sigma). Western blotting was carried out essentially as described (22). Briefly, nonspecific protein binding was blocked by preincubation of membranes in TBST containing 5% nonfat dried milk for 1 hour at room temperature, followed by incubation with primary antibodies at 4°C overnight. The following day, the blots were washed and the bound antibodies detected by incubating for 1 hour at room temperature with either alkaline phosphatase– or horse-radish peroxidase–conjugated anti-mouse or anti-rabbit secondary antibodies (1:5,000 dilution, Promega, Madison WI), followed by color development, using either Western Blue alkaline phosphatase substrate (Promega) or the chemiluminescence detection system enhanced chemiluminescence advanced (Amersham, Piscataway, NJ), and detection on Hyperfilm (Amersham). Polyclonal antibodies against Bcl-2 (1:500), Bcl-xL (1:300), Bax (1:1,000), p53 (1:500), glyceraldehyde-3-phosphate dehydrogenase (1:1,000), and monoclonal antibodies against MDM-2 (1:500) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies to Bad (1:300), MDM-2 (Ser166, 1:1,000), and monoclonal antibodies against poly(ADP-ribose) polymerase (1:1,000) were obtained from Cell Signaling Technology (Beverly, MA). Relative band intensities were determined using a Gel Documentation System with Gel Expert software from Nucleotech (Westport, CT).

**Results**

To examine what effects cisplatinum-resistant phenotypes have on the action of HDAC inhibitors, we used the previously described cisplatinum-sensitive A2780 epithelial derived ovarian cancer cell line and its acquired resistance A2780CP subline (23). In addition, we also placed A2780 cells into long-term culture (>60 passages) to develop a drug-naïve “intrinsically” resistant subline (A2780-9M). The use of long-term culture of A2780 cells to develop an intrinsic resistance subline was based on several lines of reasoning (see Discussion). Foremost was an observation in our laboratory that hMLH1 expression (Fig. 1A), the loss of
which has previously been linked to cisplatinum resistance in A2780CP (24), diminished with increasing passage of the parental A2780 cells. As evident in the figure, the parental A2780 cells have abundant hMLH1 expression (>95% of the cells) whereas the high-passage A2780-9M subline lacked expression. As a control, we also confirmed the absence of hMLH1 expression in the A2780CP subline, where the loss of hMLH1 expression has previously been directly linked to cisplatinum resistance, as shown by the resensitization of A2780CP cells to cisplatinum when transfected with the hMLH1 gene (7). Western immunoblotting confirmed the loss of hMLH1 protein in the 9M and CP cell lines, as compared with the parental A2780 cell (data not shown).

The loss of hMLH1 expression is related to the emergence of a cisplatinum-resistant phenotype (Fig. 1B). As shown in the photomicrographs, exposing the parental A2780 cells to 5 μmol/L cisplatinum resulted in >95% loss of cell viability within 72 hours. Similar cisplatinum exposure in either the A2780-9M or CP cells had no measurable effect on cell viability, with the A2780-9M and A2780CP cells continuing to grow and divide normally, analogous to what has previously been shown by others in A2780CP cells (23).

In the parental A2780 cell line we have shown that HDAC inhibition produced both morphologic and ultrastructural changes commensurate with epithelial-like differentiation (19). In the current studies (Fig. 2), trichostatin A induced morphologic transformation of the A2780-9M cells similar to those previously observed in the parental A2780 cell line (19). Trichostatin A treatment of A2780 and A2780-9M cultures altered their cellular morphology from the primarily small, round, undifferentiated clusters of cells with minimal cytoplasm observed in the control cultures (Fig. 2, top) to monolayer cultures with a significant increase in the cytoplasmic content and emergence of a stellate-like morphology containing multiple cytoplasmic projections (Fig. 2, bottom). In contrast, trichostatin A exposure in the acquired resistance A2780CP cells produced minimal morphologic changes, restricted to a small percentage of cells (<10%) developing an elongated fibroblast-like appearance, whereas the vast majority of cells continued to exist as undifferentiated clusters.

Ultrastructural examinations by electron microscopy (Fig. 3) were undertaken to determine whether structures consistent with epithelial-like differentiation, previously observed in the parental A2780 cells (19), are also reinduced in trichostatin A–treated resistant cells. Electron microscopic analysis of untreated cells from all three cultures indicated cytoplasmic membranes of adjacent cells abut without forming intercellular junctions. Occasional, poorly formed, intercellular luminal spaces were present but were devoid of microvilli. Following treatment with trichostatin A, abundant intercellular junctional fusions, resembling zona occludens, were clearly present between adjacent cells in both the A2780 and A2780-9M cultures, an indication of epithelial-like differentiation. Frequent well-defined intercellular luminal spaces were also present in the trichostatin A–treated cultures, often containing epithelial-like surface microvilli. In contrast, consistent with the minimal morphologic transformation observed in trichostatin A–treated A2780CP cells (Fig. 2), minimal ultrastructural differentiation was observed in the CP cells, with only occasional weak junctional fusions, poorly formed luminal spaces, and no identifiable microvilli. Finally, immunohistochemical staining of control and trichostatin A–treated cells indicated that ultrastructural differentiation of the A2780-9M cells was accompanied by an increase in staining for cytokeratin proteins (data not shown), similar to what we previously reported in the A2780 cells (19), whereas the A2780CP cells showed no discernable increase in cytokeratin expression.

In addition to the ultrastructural epithelial-like differentiation identified above, trichostatin A also induced a discernible reduction in cell proliferation, readily shown in time-course studies (Fig. 4). Untreated cultures from all three cell lines exhibited very active proliferation (doubling...
time, ~24 hours). Exposure to trichostatin A produced a complete cessation of cell proliferation in all three cell lines within 24 hours. Following trichostatin A, A2780 cells remained static for 3 days with no observable loss of cell viability, followed by renewed cellular proliferation by day 5. In contrast, trichostatin A exposure of either cisplatinum-resistant A2780-9M or CP subline initiated a significant (P < 0.05) loss of cell viability, beginning on day 2 and continuing through day 5, resulting in a reduction in cell numbers to <8% of day 0. To determine whether the changes in morphology and viability observed following trichostatin A (Figs. 2 and 4) are a generalized effect of HDAC inhibition, we repeated these experiments using additional HDAC inhibitors: CI-994 (10 μmol/L), hexamethylene bisacetamide (1 mg/mL), and sodium butyrate (1 mmol/L). Our results indicated that treating A2780, A2780-9M, or A2780CP cells with other HDAC inhibitors produced changes in morphology and cell viability indistinguishable from those presented above using trichostatin A (data not shown).

Flow cytometry was used to examine the relationship between HDAC inhibitor–induced changes in cell proliferation/viability and alterations in the cell cycle (Fig. 5). The DNA content histograms of untreated A2780, A2780-9M, and A2780CP cell lines were essentially identical, with majority of the cells in G1 (62.2%, 64.1%, and 61.1%, respectively), an elevated S-phase fraction (25.9%, 27%, and 29.5%), consistent with the previously observed rapid cell doubling times, and a minimal G2-M fraction (11.9%, 8.9%, and 9.4%). Following trichostatin A, there was a noticeable shift in the DNA content histograms of the A2780 cells as compared with the resistant A2780-9M and CP cells. In A2780 cells, trichostatin A produced complete G1-S and G2-M blockades, as evident by the lack of change in G1-phase cells (62.2% untreated versus 62.5% trichostatin A), a significant reduction (P < 0.05) in S-phase cells (25.9% untreated versus 0.5% trichostatin A) to levels normally seen in nondividing cells (<1%), and a concurrent increase in the G2-M-phase fraction to 36.9%, consistent with cells already in S phase, at the time of trichostatin A addition, transitioning through DNA replication to arrest in G2-M. In A2780-9M and CP cells, trichostatin A also produced a significant reduction (P < 0.05) in the S-phase fraction to 6.4% and 5.4%, respectively; however, the percentage of...
cells in S phase remained elevated in comparison with nondividing or trichostatin A–treated A2780 cells. The residual elevated S phase in trichostatin A–treated 9M and CP cells results in a significant (P < 0.05) reduction in G1–phase cells to 36% and 34.1%, respectively, and thus, at best, there is only partial G1-S block. The reduction in cells in G1 and S phases, in the resistant cultures, was offset by a nearly 7-fold increase in G2-M phase to 57.6% and 60.4%, respectively, indicating predominant cell cycle arrest in resistant cells occurs in G2-M. Further analysis of trichostatin A–treated A2780, A2780-9M, and A2780CP cells, to determine their mitotic index, indicated less than 1% of the cells were actively undergoing mitosis, suggesting that the cells found in G2-M are arrested at the DNA damage G2 checkpoint. Similar time course and cell cycle analysis studies were also done on two other intrinsically cisplatinum-resistant ovarian cancer cell lines, OVCAR-3 (25) and SK-OV-3 (26). Trichostatin A–treated OVCAR-3 and SK-OV-3 cells exhibited a similar loss of cell viability to that described above in the 9M and CP cells, associated with the complete absence of G1 arrest (S phase; 22.3% control versus 16.6% trichostatin A for OVCAR-3 and 23.6% control versus 17.9% trichostatin A for SK-OV-3) leading to predominant G2-checkpoint arrest. In contrast, trichostatin A exposure in a normal, dividing, fibroblast cell line (MRC-5) produced G1 arrest (S phase; 19.7% control versus 0.5% trichostatin A) with no loss of cell viability, analogous to our observations in the parental A2780 cell line (data not shown).

Cell cycle arrest at the G2 checkpoint is often associated with DNA damage–induced repair and/or apoptosis. Therefore, we examined the A2780 and A2780-9M and CP cultures for evidence of trichostatin A–induced apoptosis using Annexin V binding (Fig. 6). In A2780 cells, trichostatin A produced no increase in Annexin V binding at either 24 or 48 hours, consistent with the absence of any detectable loss of cell viability in these cultures. In both cisplatinum-resistant sublines, trichostatin A induced a significant (P < 0.001) 10– to 20-fold increase in Annexin V binding in 30% to 40% of cells within the first 24 hours, and in 75% to 90% of cells by 48 hours, consistent with the previously observed loss of cell viability in these cultures. Increased Annexin V binding was also observed using the other HDAC inhibitors described above as well as in trichostatin A–treated OVCAR-3 and SK-OV-3 cultures (data not shown).

To begin to understand the mechanisms involved in HDAC inhibitor–induced apoptotic cell death, we analyzed the expression patterns of the tumor suppressor protein p53 (Fig. 7A), a well-recognized mediator of DNA damage–induced G2-checkpoint arrest and apoptosis (27). Both the A2780 and A2780-9M cultures exhibited similar expression of p53, which were unaffected by treatment with trichostatin A. Untreated A2780CP cells also express p53; however, in CP cells trichostatin A treatment produced a dramatic reduction in p53 expression by >90%. Loss of p53 protein in trichostatin A–treated A2780CP cells was not associated with an increase in the p53 inactivating/sequestering protein, MDM-2, or with changes in the ubiquitin ligase activity of MDM-2, as measured by phosphorylation of MDM-2 at Ser166. Additionally, the loss of p53 protein in A2780CP cells occurred rapidly, within 18 to 24 hours of treatment, before any detectable loss in cell viability (see Fig. 4). The loss of p53 also occurred before any significant apoptosis-associated protein degradation, as seen by the fact that poly(ADP-ribose) polymerase, a known substrate for caspase 3, showed minimal (<10%) proteolytic cleavage in these cells.

Because DNA damage–induced apoptosis occurs via the intrinsic mitochondrial pathway, involving members of the Bcl-2 family (28), we also examined our cultures for the expression of several pro- and antiapoptotic Bcl-2 family members (Fig. 7B). The expression of the antiapoptotic Bcl-2 and Bcl-xL proteins remained essentially unchanged in all three cell lines, with only a modest 2-fold increase in Bcl-xL detected in trichostatin A–treated A2780-9M cells. The proapoptotic Bax protein, which was elevated 2.5– to 3-fold in untreated A2780-9M and CP cells as compared with the parental A2780 cell line, was unaffected by trichostatin A. In contrast, expression of the...
proapoptotic BH3-only Bad protein, which was negligible in extracts from untreated cells of all three cultures, increased dramatically in trichostatin A–treated A2780-9M and CP cells, as compared with either untreated cells or control and trichostatin A–treated, nonapoptotic, A2780 cells. Thus, trichostatin A–induced expression of Bad in A2780-9M and CP cells may provide a potential mechanism for trichostatin A–triggered apoptosis by the intrinsic mitochondrial pathway.

Discussion
In the current studies, we compared the actions of HDAC inhibitors on ovarian cancer cell lines with acquired (A2780CP) and intrinsic (A2780-9M, OVCAR-3 and SK-OV-3) resistance to cisplatinum. Development of the intrinsically resistant A2780-9M subline in our laboratory was prompted by our observations that hMLH1 expression diminished with increasing A2780 cell passage. Loss of hMLH1 expression has previously been linked to cisplatinum resistance in A2780CP cells (7) and ovarian tumors (29). The emergence of a resistant phenotype (A2780-9M), following extended culture, was also consistent with data indicating A2780 cultures may contain a small (<10%) drug-naïve subpopulation with defective hMLH1-associated cisplatinum resistance (30).

Exposing cisplatinum-resistant cells to inhibitors of HDAC (trichostatin A, CI-994, hexamethylene bisacetamide, and sodium butyrate) induced epithelial-like ultrastructural changes and/or cell cycle arrest at the G2 checkpoint, followed by apoptotic cell death. That HDAC inhibitors are capable of inducing apoptosis in cisplatinum-resistant ovarian cancer cells was an unanticipated finding, given the previous data that the phosphatidylinositol-3 kinase/Akt kinase survival pathway is overexpressed in both A2780CP and OVCAR-3 cells (17) and the several recent reports that phosphatidylinositol-3 kinase/Akt kinase activity inhibits HDAC inhibitor–mediated apoptosis (15, 16, 31). Apoptosis in resistant A2780-9M and CP sublines also contrasts markedly with our current and previous data (19) that trichostatin A can induce differentiation of the parental cisplatinum-sensitive A2780 cells in the absence of detectable apoptosis.

In the present studies, HDAC inhibitor–induced apoptosis in cisplatinum-resistant cell lines was associated with G2 arrest, as compared with the predominant G1 arrest observed in nonapoptotic A2780 and MRC-5 cells. Studies in U937 leukemic cells also showed HDAC inhibition induced G1 arrest and differentiation, which was associated with the absence of detectable apoptosis (32). This observation was subsequently confirmed by showing that preventing G1 arrest impaired HDAC-mediated differentiation...
and triggered apoptosis (33). A similar shift, from HDAC inhibitor–induced differentiation to apoptosis, has also been observed following the disruption of G1 arrest by the cyclin-dependent kinase inhibitor flavopiridol (34).

Because several studies have shown that disruption of HDAC inhibitor–induced G1 arrest prevents differentiation, the suggestion has been made that HDAC inhibitor–induced differentiation is dependent on cell cycle arrest in G1. Furthermore, the apparent relationship between G1 arrest and apoptosis has also led to speculation that differentiation and apoptosis might be mutually exclusive actions of HDAC inhibition (35). The current studies challenge both these assertions, as shown by the fact that trichostatin A–induced differentiation of the intrinsically resistant A2780-9M cells occurs in the absence of significant G1 arrest, and also occurs concurrently with trichostatin A–triggered apoptosis, a clear indication that the differentiation and apoptotic actions of HDAC inhibition are not mutually exclusive.

Identifying the biochemical pathways responsible for HDAC inhibition–triggered apoptosis is an area of intensive investigation. In normal cells, a complex DNA-damage surveillance system, involving G1-S and G2-M checkpoint arrests, interrupts cell cycle progression to allow time for DNA repair (36). Disruption of these DNA damage checkpoints is often associated with both the development of tumors and the emergence of chemotherapeutic resistance (37). In all four cisplatinum-resistant ovarian cell lines, trichostatin A–induced apoptosis was associated with a failure of the cells to arrest in G1, as compared with the predominant G1 arrest observed in the nonapoptotic A2780 and MRC-5 cells. Thus, in cisplatinum-resistant ovarian cancer cells, the absence of G1-S-checkpoint arrest may be an important requirement for HDAC inhibitor–induced apoptosis. A link between failure to arrest in G1 and apoptosis in our cisplatinum-resistant cells is consistent with a report in CEM cells (38), where HDAC inhibition–induced apoptosis was restricted to cells with a 4n DNA content (G2-M phase) and could be prevented by inducing G1 arrest through overexpression of cell cycle inhibitors.

The p53 protein plays a crucial role in regulating DNA damage repair, cell cycle arrest, and apoptosis (39). A recent report indicates that HDAC inhibitor–induced apoptosis is mediated by p53 (40). However, in the present studies, trichostatin A–triggered apoptosis in ovarian cancer cells does not require p53. Trichostatin A treatment abolished p53 expression in A2780CP cells before the onset of apoptosis, and SK-OV-3 cells, which contain a deletion of the p53 gene (41), were also sensitive to trichostatin A–induced apoptosis. Although the present studies do not allow us to determine the precise mechanism responsible for trichostatin A–mediated suppression of p53 in A2780CP cells, the absence of identifiable changes in the negative regulator of p53, the MDM-2 protein (42), or the ubiquitin ligase activity of MDM-2, via phosphorylation of Ser166 (43), may be an indication that trichostatin A alters the transcriptional activity of the p53 gene.

As previously alluded to, the G1-S and G2-M checkpoints are crucial periods in the cell cycle when DNA damage is recognized and repaired, and failing that, apoptotic pathways are triggered. In normal cells apoptosis resulting from genotoxic stress/DNA damage is mediated by the Bcl-2 family of proteins via the “intrinsic or mitochondrial apoptotic pathways” (44). The Bcl-2 family is divided into two main groups: prosurvival members, such as Bcl-2 and Bcl-xL, and proapoptotic members, such as Bax, Bak, Bad, and Bok. In A2780-9M and CP cells, trichostatin A induced apoptosis commensurate with increased expression of the BH3-only protein Bad. Bad proteins promote apoptosis through their ability to bind Bcl-2 and Bcl-xL and displace sequestered Bax (45), and thus, HDAC inhibitor–induced apoptosis in the 9M and CP cells may also be related to the elevated basal levels of Bax protein observed in these cells.

In conclusion, cisplatinum resistance in ovarian cancer cells, either intrinsic or acquired, does not produce cross-resistance to HDAC inhibitors. Instead, the emergence of resistant phenotypes in ovarian cancer cells alters the actions of HDAC inhibitors, resulting in a shift in cell cycle arrest from the G1-S to the G2-M checkpoint and the apoptosis-associated elevated expression of the BH3-only protein Bad.

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