Ornithine decarboxylase activity in tumor cell lines correlates with sensitivity to cell death induced by histone deacetylase inhibitors

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Abstract
Inhibitors of histone deacetylases (HDAC) show significant promise as targeted anticancer agents against a variety of hematologic and solid tumors. HDAC inhibitors arrest the growth of primary cells, but they induce apoptosis or differentiation of tumor cells. Although the precise mechanism is unknown, differences in cell cycle checkpoints and chromatin structure may be responsible. Cellular polyamines regulate both cell cycle progression and chromatin structure. In tumors, polyamines are abundantly produced because of increased activity of the rate-limiting enzyme in polyamine synthesis, ornithine decarboxylase (ODC). To determine if polyamines contribute to the cellular response to HDAC inhibitors, we inhibited ODC activity with a-difluoromethylornithine. Polyamine depletion increased resistance to apoptosis induced by HDAC inhibitors. In addition, we found that ODC activity levels correlated with sensitivity to HDAC inhibitors in a panel of tumor cell lines. We conclude that polyamines participate in the cellular response to HDAC inhibitors and that ODC activity correlates with sensitivity to HDAC inhibitor-induced apoptosis. Thus, elevated polyamine levels might be a biomarker for tumor sensitivity to HDAC inhibitor-induced apoptosis. These findings warrant clinical evaluation of tumor samples to determine if high ODC activity levels predict sensitivity to HDAC inhibitors. [Mol Cancer Ther 2006;5(11):2777–85]

Introduction
As a new class of targeted chemotherapeutic agents, histone deacetylase (HDAC) inhibitors show significant promise against a variety of cancers in clinical trials (1, 2).

Most available HDAC inhibitors block the activity of all class I and II HDACs, thereby increasing acetylation of histone and nonhistone protein targets (3). Although HDAC inhibitors induce apoptosis or differentiation of transformed cells, they induce a reversible cell cycle arrest in primary cells (4).

HDAC inhibitors target cell cycle checkpoints and chromatin structure, which can be altered during tumorigenesis. In primary cells, HDAC inhibitors activate a G2 checkpoint that leads to cell cycle arrest (4). In most tumor cells, this G2 checkpoint is defective and the arrest cannot be maintained (5). Tumor cells then enter mitosis with hyperacetylated chromosomes that fail to properly align on the metaphase plate (4, 6). High doses of HDAC inhibitors prevent activation of the mitotic spindle checkpoint, and premature exit from this aberrant mitosis is followed by apoptosis (4, 6). Histone hyperacylation induced by HDAC inhibitors decondenses chromatin and induces global changes to chromatin structure (7–9). In many tumor cells, the nuclear architecture and chromatin structure are altered (7, 10). These alterations in global chromatin structure might mediate the response of tumor cells to HDAC inhibitors.

Primary and transformed cells differ in their levels of cellular polyamines and this difference might mediate their responses to HDAC inhibitors. Polyamines (putrescine, spermidine, and spermine) are abundant cations that bind and alter the conformation and action of nucleic acids and proteins (11). Because polyamines regulate both cell cycle progression and chromatin structure, they might play a role in the cellular response to HDAC inhibitors (12). Ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis, catalyzes the decarboxylation of ornithine into the higher-order polyamine, putrescine (Fig. 1A). Polyamines are also obtained from the diet, mainly from cheese, red meat, and intestinal bacteria (13, 14).

ODC is an early marker of tumorigenesis. It is often overexpressed or shows increased enzymatic activity in epithelial tumors, including colon, skin, prostate, and gastric cancers (15–19). Although ODC overexpression alone does not transform normal fibroblasts, it does lead to transormation of NIH 3T3 cells, and it cooperates with activated H-ras to promote tumorigenesis (20–22). The irreversible ODC inhibitor difluoromethylornithine (DFMO) can both prevent tumor formation and promote tumor regression in ODC/ras and ODC/K14—mitogen-activated protein/extracellular signal-regulated kinase double-transgenic mice (22, 23). ODC is a transcriptional target of myc and is up-regulated in tumors that overexpress myc (24–26). ODC is the critical downstream regulator of myc that leads to uncontrolled proliferation, and impairing ODC function...
with DFMO or lowering ODC levels by targeted knockout in mice prevents myc-induced lymphomagenesis (26).

ODC activity regulates polyamine synthesis and has profound effects on cell growth, differentiation, and death (12). In tumors, increased polyamine levels are associated with increased cell proliferation and decreased apoptosis (12). Polyamine depletion leads to cell cycle arrest and a senescence-like phenotype, but does not induce apoptosis (27). However, the apoptotic response in polyamine-depleted cells is altered in a cell type– and stimulus-dependent manner (28). Polyamines affect gene expression by binding DNA and modulating chromatin structure (29). Polyamines modulate the response to HDAC inhibitors. To test this hypothesis, we investigated the role of polyamines in the cellular response to HDAC inhibitors. We also determined if ODC activity correlated with sensitivity to apoptosis induced by HDAC inhibitors in a panel of tumor cell lines.

**Materials and Methods**

**Cell Lines, Plasmids, and Reagents**

HCT116 human colon carcinoma cells were cultured in DMEM (Mediatech, Herndon, VA) or McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, Woodland, CA) or dialyzed FBS (Life Technologies Invitrogen Corporation, Carlsbad, CA), 1% penicillin-streptomycin, and 2 mmol/L L-glutamine (Life Technologies Invitrogen Corporation). Serum starvation was done in 0.1% FBS in DMEM for 4 days. H1299 human lung carcinoma cells, A431 human epithelial carcinoma cells, A549 human lung carcinoma cells, U2OS human osteosarcoma cells, and HeLa human cervical carcinoma cells (American Type Culture Collection, Manassas, VA) were grown in DMEM. Normal neonatal human foreskin fibroblasts (a gift from Thea Tlsty, University of California San Francisco) were grown in RPMI (Life Technologies Invitrogen Corporation) supplemented with 10% FBS.

Trichostatin A was dissolved in DMSO to 33.1 mmol/L (Wako Chemicals USA, Richmond, VA). Sodium butyrate (Sigma, St. Louis, MO) was dissolved in water at a stock concentration of 1 mol/L. Trapoxin A was dissolved in DMSO (a gift from M. Yoshida, University of Tokyo, Tokyo, Japan). DL-α-Difluoromethylornithine hydrochloride (Sigma) was suspended in water to 0.1 mol/L, and putrescine (P5780; Sigma) was dissolved in water to 1 mol/L.

**ODC Activity Assay**

ODC activity was measured as release of CO₂ from L-[1-C¹⁴]ornithine. Cells (1 × 10⁶) were trypsinized, washed with PBS, and lysed in 100 µL of ODC lysis buffer [25 mmol/L Tris-HCl (pH 7.4), 0.1 mmol/L EDTA, 0.1% Triton X-100, 0.1 mmol/L pyridoxyl-5-phosphate, 1 mmol/L DTT, 1× protease inhibitors)]. Lysates were filtered at ~80°C, thawed on ice, and centrifuged at 12,000 × g for 20 minutes at 4°C to remove cell debris. Supernatants were transferred to new Eppendorf tubes, and 25 µL aliquots were transferred to glass vials in triplicate. Released CO₂ was captured with a cotton swab, which was soaked with 80 µL hyamine hydroxide (Fisher Scientific, Fairlawn, NJ) through the hole in a stopper top (Kontes Glass, Vineland, and increase proliferation rates that may alter their response to HDAC inhibitors.

Differences in cell cycle checkpoints and chromatin structure between normal and transformed cells underlie their differential response to HDAC inhibitors. Tumor cells often have elevated levels or activity of ODC, leading to elevated polyamine levels. The resulting effect on cell cycle checkpoints and chromatin structure might play a role in their apoptotic response to HDAC inhibitors. We hypothesized that polyamines modulate the response to HDAC inhibitors. To test this hypothesis, we investigated the role of polyamines in the cellular response to HDAC inhibitors. We also determined if ODC activity correlated with sensitivity to apoptosis induced by HDAC inhibitors in a panel of tumor cell lines.

**Figure 1.** Pretreatment of human HCT116 colon carcinoma cells with DFMO increases resistance to TSA-induced cell death. A, polyamine synthesis pathway. ODC enzymatic activity is required for the first step in polyamine synthesis, in which ornithine is decarboxylated to produce the higher-order polyamine putrescine. DFMO inhibits the activity of ODC, and the activity of ODC in cellular lysates can be measured as release of CO₂ from L-[1-C¹⁴]ornithine. B, ODC activity assays were done on cell lysates from HCT116 cells grown in dialyzed FBS (dFBS) to ensure no exogenous source of polyamines was available. After 4 d of treatment with 1 mmol/L DFMO or DFMO and 100 µmol/L putrescine, ODC activity was blocked. Columns, means for two experiments done in triplicate; bars, SD. C, cells were pretreated with increasing amounts of DFMO for 4 d and then treated with 0.5 µmol/L TSA for 72 h. Cell viability was measured with an MTT assay. Values are medians, upper and low quartiles, and upper and low extremes for at least three experiments done in quadruplicate. ***, P < 0.0001, unpaired t test. D, cells were pretreated with 1 mmol/L DFMO for up to 4 d and then treated with 0.5 µmol/L TSA for 72 h. Cell viability was measured with an MTT assay. Values are medians, upper and low quartiles, and upper and low extremes for at least three experiments done in quadruplicate. ***, P < 0.0001 unpaired t test.
NH). ODC assay buffer (20 μL) containing 2 μL l-[1-\(^{14}\)C]ornithine hydrochloride (Amersham, Piscataway, NJ) was added to each vial and incubated for 2 hours at 37°C. Reactions were terminated with an equal volume of 10% TCA injected with a syringe through the rubber stopper top and left overnight at room temperature. The cotton swab was transferred to a scintillation vial, and 5 mL scintillation fluid were added and left overnight at room temperature. Liquid scintillation counting was done for 1 minute. Protein concentration was measured on 5 μL aliquots of supernatants in duplicate with the DC Protein Assay (Bio-Rad, Hercules, CA). ODC activity was determined as picoatoms of CO\(_2\) released (cpm) per milligram of protein per hour.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Assay

Cell viability was measured with an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells (10,000 per well) were plated in 96-well plates in 100 μL DMEM. HDAC inhibitors were added 24 hours after plating at various concentrations in 100 μL DMEM. After 72 hours of drug exposure, the medium was replaced with 5 μL MTT (Sigma; 5 mg/mL in PBS) in 195 μL DMEM for 4 to 6 hours. The medium was removed, and 0.04 N HCl in isopropanol was added to dissolve the formazan. Absorbance at 570 nm was read with a microplate reader.

Cell Cycle Analysis

Floating and attached cells were collected, washed with PBS, and resuspended in PBS in 12 × 75 mm polystyrene tubes (Fisher, Pittsburgh, PA). An equal volume of propidium iodide staining solution (PIPERES buffer: 10 mmol/L PIPES, 100 mmol/L NaCl, 2 mmol/L MgCl\(_2\), 0.1% Triton X-100) containing 20 μg/mL propidium iodide and 200 μg/mL RNase A was added, and the cells were incubated for 20 minutes at room temperature. The nuclei were analyzed by flow cytometry on a FACSCalibur system (BD Biosciences, San Jose, CA) with Cell Quest software (BD Biosciences). Cell cycle analysis was done with FlowJo software (TreeStar, Ashland, OR).

Annexin V Immunostaining

To quantitate apoptosis, cells were immunostained with Annexin V–FITC, which detects phosphatidylserine that is translocated to the outer plasma membrane early during apoptosis. HCT116 cells were cultured for various times with 1 μmol/L trichostatin A (TSA) and stained with Annexin V–FITC and propidium iodide in 10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L CaCl\(_2\) for 15 minutes at room temperature in the dark. Cells were subsequently analyzed by flow cytometry and FlowJo software. Cells were identified as live (Annexin V–FITC negative, propidium iodide negative), early apoptotic (Annexin V–FITC positive, propidium iodide negative), or late apoptotic (Annexin V–FITC positive, propidium iodide positive).

Acid Extraction of Histones

Cells (≈ 0.5 × 10^7) were harvested, washed in cold PBS, and lysed in 400 μL Triton extraction buffer (TBE: PBS containing 0.5% Triton-X 100, 2 mmol/L phenylmethylsulfonyl fluoride, 0.02% sodium azide, and 5 mmol/L sodium butyrate) for 10 minutes on ice with gentle agitation. Lysates were centrifuged at 2,000 rpm for 10 minutes at 4°C, and the cell pellet was washed in 200 μL TBE, resuspended in 100 μL of 0.2 N HCl, and shaken overnight at 4°C. The samples were centrifuged at 2,000 rpm for 10 minutes at 4°C, and the supernatants were transferred to a new Eppendorf tube. Protein concentrations were measured with the Bradford protein assay (Bio-Rad).

Western Blot Analysis

Cells were lysed in 50 mmol/L Tris-HCl (pH 7.5), 0.5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP40, and 1× complete protease inhibitors (Roche, Penzberg, Germany), and protein concentration was determined with the DC Protein Assay (Bio-Rad). Protein samples were separated by electrophoresis on 15% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in TBS-Tween [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.1% Tween 20] and probed with antiacetylated α-tubulin (Sigma) or anti-α-tubulin (Sigma) at a 1:2,000 dilution, antiacetylated histone H3 (Upstate Biotechnology, Lake Placid, NY), anti-histone H3 (Upstate), or antiacetylated histone H4 (Upstate) at a 1:1,000 dilution.

Results

Inhibition of ODC by DFMO Increases Resistance to TSA-Induced Cell Death in HCT116 Cells

To determine if cellular polyamines are involved in HDAC inhibitor–induced cell death, we used human HCT116 colon carcinoma cells, which are sensitive to TSA-induced cell death (IC\(_{50}\) 0.34 μmol/L). In lyses of HCT116 cells, ODC activity was assayed by measuring CO\(_2\) released from \(^{14}\)C]ornithine (Fig. 1A). HCT116 cells had high ODC activity levels (≈ 1,200 cpm/mg protein/h; Fig. 1B). Pretreatment with 1 mmol/L DFMO for 4 days completely inhibited ODC activity (Fig. 1B). Treatment of HCT116 cells with 0.5 μmol/L TSA for 72 hours reduced viability to ~20% in an MTT assay (Fig. 1C). Pretreatment with increasing amounts of DFMO for 4 days before TSA treatment increased viability to ~75% (Fig. 1C). When the cells were pretreated for 0 to 4 days with 1 mmol/L DFMO and then treated for 72 hours with 0.5 μmol/L TSA, viability was increased with the duration of DFMO treatment (Fig. 1D). Therefore, cells were pretreated with 1 mmol/L DFMO for 4 days in all subsequent experiments. Thus, ODC activity or the level of cellular polyamines is involved in the cellular response to HDAC inhibitor–induced cell death in HCT116 cells.

Polyamine Depletion Increases Resistance to TSA-Induced Cell Death

To show that DFMO increased resistance to TSA-induced cell death by depleting cellular polyamines, we supplemented the medium with 100 μmol/L putrescine during the pretreatment with 1 mmol/L DFMO. Putrescine is normally produced by ODC, but when ODC is inhibited,
addition of putrescine to the culture medium restores normal polyamine levels (31). HCT116 cells pretreated with both DFMO and putrescine were as sensitive as control cells to increasing amounts of TSA, whereas cells pretreated with DFMO alone were more resistant to TSA-induced cell death (Fig. 2A). Furthermore, cells cotreated with both DFMO and putrescine, which reverses the TSA-resistant phenotype, had no ODC activity, indicating that endogenous polyamine production was blocked (Fig. 1A). Thus, depletion of cellular polyamines leads to resistance to the cytotoxic effects of TSA. Polyamine depletion also led to resistance to TSA-induced cell death in several other human cell lines, including H1299 lung carcinoma cells (Fig. 2B), A431 epithelial carcinoma cells, and A549 lung carcinoma cells (data not shown). However, addition of exogenous polyamines (putrescine or spermidine) or over-expression of ODC did not increase the sensitivity to TSA in any cell line examined (data not shown). Although we did not measure polyamine levels, their levels are tightly regulated and addition of exogenous polyamines might not have resulted in significant increases in intracellular polyamine levels (28).

Polyamine Depletion Leads to Resistance to Different Structural Classes of HDAC Inhibitors

To determine if depletion of cellular polyamines leads to resistance to all HDAC inhibitors or is specific to TSA, we depleted polyamines from HCT116 cells with DFMO and tested their sensitivity to other HDAC inhibitors. Polyamine depletion led to resistance to cell death induced by the cyclic tetrapeptide trapoxin A (Fig. 2C) and the short-chain fatty acid sodium butyrate (Fig. 2D). Furthermore, cotreatment with both DFMO and putrescine restored the sensitive phenotype as in control cells.

**G1 Cell Cycle Arrest Induced by Polyamine Depletion Is Not Sufficient to Increase Resistance to HDAC Inhibitor–Induced Cell Death**

To determine the effect of polyamine depletion on the cell cycle, HCT116 cells were left untreated or were pretreated with DFMO or with DFMO and putrescine, and were treated with TSA for various times. Propidium iodide staining of nuclei from cells treated with DFMO showed a dramatic G1 arrest (Fig. 3A, middle left). Polyamine depletion was responsible for this arrest as cells cotreated with DFMO and putrescine did not arrest (Fig. 3A, bottom left). Treatment of control cells with TSA doubled the number of cells in G2-M at 12 hours and increased the number of dead cells (sub-G1 DNA content) to ~50% at 24 hours and ~70% at 48 hours (Fig. 3A, top row). Treatment with TSA for 12 hours did not cause an accumulation of cells in G2-M in cells treated with DFMO, and ~30% of these cells maintained a G1 arrest at 48 hours (Fig. 3A, middle row). The TSA response of cells cotreated with DFMO and putrescine was similar to that of control cells (Fig. 3A, bottom row). These findings suggest that G1 arrest induced by polyamine depletion could protect cells from HDAC inhibitor–induced cell death by preventing them from reaching G2-M and undergoing aberrant mitosis and apoptosis.

Next, we determined if induction of G1 arrest in HCT116 cells by a different method also led to resistance to TSA-induced cell death. After serum starvation of cells with 0.1% FBS/DMEM for 4 days, propidium iodide staining of nuclei revealed a G1 arrest (Fig. 3B, bottom left). Serum-starved cells were as sensitive to TSA-induced cell death as cells cultured in 10% FBS/DMEM or 10% dialyzed FBS (Fig. 3C). These findings suggest that the influence of polyamines on the response to HDAC inhibitors is independent of G1 arrest.

Because up-regulation of p21 by HDAC inhibitors reduces their cytotoxicity (32), we wished to determine if the potential up-regulation of p21 during DFMO-induced G1 cell cycle arrest played a role in increasing resistance to HDAC inhibitor–induced cell death. Wild-type HCT116 cells and a cell line in which p21 was disrupted by homologous recombination (33) were treated with DFMO and then with TSA for various times. We found that both cell lines became resistant to TSA treatment to the same extent, indicating that p21 induction is not involved in the mechanism by which DFMO leads to resistance to TSA-induced cell death (data not shown).

**Polyamine Depletion Blocks HDAC Inhibitor–Induced Apoptosis in HCT116 Cells**

We next asked if polyamine depletion blocked apoptosis in HCT116 cells. Control cells and cells pretreated with DFMO or with DFMO and putrescine were treated with...
A mol/L TSA for 0 to 3 days, and apoptosis was assessed by staining with Annexin V and propidium iodide. As shown by flow cytometric analysis, apoptosis levels at 18 hours were similar in polyamine-depleted and control cells (Fig. 4, first two columns). However, polyamine-depleted cells had lower levels of apoptosis than control cells at both 48 and 72 hours after TSA treatment (Fig. 4, third and fourth columns). After 72 hours of TSA treatment, twice as many polyamine-depleted cells were alive compared with the control cells (Fig. 4). HCT116 cells pretreated with both DFMO and putrescine underwent apoptosis to the same extent as control cells (Fig. 4, bottom row). Thus, polyamine depletion blocks apoptosis in response to HDAC inhibitors in a fraction of cells.

Global Chromatin Structure Is Altered in Polyamine-Depleted Cells

To determine if polyamine depletion affects the ability of TSA to enter the cell, inhibit HDAC activity, or induce histone hyperacetylation, histones were acid extracted from control, DFMO, and DFMO/putrescine–pretreated cells that were treated or not with TSA for up to 6 hours. Equal levels of histone proteins were extracted under each condition (Fig. 5A, top). Western blot analysis showed that TSA induced histone hyperacetylation to the same extent in control and in polyamine-depleted cells (Fig. 5A, middle). Furthermore, an antibody to unmodified histone H3 showed equal levels of H3 protein under all conditions (Fig. 5A, bottom). Control, DFMO, and DFMO/putrescine–treated cells were also lysed in a low-salt buffer that leaves most of the histone proteins associated with the DNA in the insoluble fraction. Even without TSA treatment, hyperacetylated histones were released from polyamine-depleted cells (Fig. 5B). After TSA treatment, hyperacetylated histones were released into the soluble fraction to a greater extent in the polyamine-depleted cells (Fig. 5B). TSA induced hyperacetylation of α-tubulin equally under all conditions, and blotting for α-tubulin showed equal loading of the gels (Fig. 5B). These results suggest that depletion of polyamines by DFMO alters chromatin structure, allowing easier extraction of acetylated histones under these lysis conditions.

ODC Activity Is Elevated in Tumor Cells and Correlates with Sensitivity to HDAC Inhibitor–Induced Apoptosis

ODC activity is tightly regulated in primary cells but often elevated in tumor cells (12), which may sensitize them to HDAC inhibitor–induced apoptosis. To test this hypothesis, we measured ODC activity in various cell lines.
ODC activity was lower in primary human neonatal foreskin fibroblasts than in all tumor cell lines examined (Fig. 6A). Among the tumor cell lines, HCT116 cells had the highest ODC activity levels and were the most sensitive to HDAC inhibitor–induced apoptosis. In addition, tumor cell lines with high ODC activity correlated with increased sensitivity to TSA-induced apoptosis (Fig. 6A).

**TSA-Induced Increases in ODC Activity Correlate with Sensitivity to HDAC Inhibitors**

Next, we determined if TSA induces ODC activity in our panel of cell lines after 16 hours of TSA treatment. ODC activity was increased in several tumor cell lines (HCT116, A431, and A549) but not in other cell lines (U20S, H1299, and human neonatal foreskin fibroblasts; Fig. 6B and data not shown). Increases in ODC protein levels were confirmed by Western blotting (data not shown). Moreover, the ability of TSA to induce ODC activity also correlated with increased sensitivity to TSA-induced apoptosis (Fig. 6C).

**Discussion**

This study shows that polyamines modulate the cellular response to HDAC inhibitors and that high ODC activity levels in tumor cell lines correlate with increased sensitivity to HDAC inhibitor–induced apoptosis. In several human cell lines, depletion of cellular polyamines with the ODC inhibitor DFMO increased resistance to various structural classes of HDAC inhibitors, including the hydroxamic acid TSA, the cyclic tetrapeptide trapoxin A, and the short-chain fatty acid sodium butyrate. The resistance to apoptosis was due to the depletion of cellular polyamines and not just the inhibition of ODC activity, as addition of exogenous putrescine during DFMO treatment did not alter the response to HDAC inhibitors. Polyamine depletion induced a G1 cell cycle arrest that was not sufficient to increase resistance to HDAC inhibitor–induced apoptosis. Polyamine depletion blocked apoptosis in a percentage of HCT116 cells. HDAC inhibitors induced histone hyperacetylation to an equal extent in control and polyamine-depleted cells, but global chromatin structure was altered only in polyamine-depleted cells. In tumor cell lines, high levels of ODC activity correlated with increased sensitivity to HDAC inhibitor–induced apoptosis, suggesting that...
polyamine levels might be a potential biomarker predictive of tumor sensitivity to HDAC inhibitors.

The tumor-selective induction of apoptosis by HDAC inhibitors is thought to act through altered G2 checkpoints and chromatin structure in tumor cells (4, 7). Because ODC activity increases with both the G1-S and S-G2 transitions, the associated changes in polyamine levels regulate important cell cycle checkpoints (34, 35). Inhibition of polyamine biosynthesis leads to G1 or S-G2 arrest that is reversed by the addition of exogenous polyamines (36–38). In HCT116 cells, polyamine depletion or serum starvation each led to a G1 cell cycle arrest, but serum starvation did not alter the sensitivity to HDAC inhibitor–induced apoptosis. Therefore, G1 arrest after polyamine depletion did not protect cells from HDAC inhibitor–induced apoptosis by preventing them from reaching G2-M, where HDAC inhibitors would induce hyperacetylated chromosomes to undergo an aberrant mitosis, leading to apoptosis.

The levels of apoptosis 18 hours after treatment with HDAC inhibitors were similar in polyamine-depleted and control HCT116 cells, but were lower in the polyamine-depleted cells at 48 and 72 hours. Therefore, polyamine depletion did not delay apoptosis at early time points after treatment with the HDAC inhibitor but blocked apoptosis in a percentage of cells; at 72 hours, ~20% of the polyamine-depleted cells were still alive. This suggests that polyamines affect the apoptotic response of tumor cells treated with HDAC inhibitors. Polyamine depletion in tumor cells also alters the response to other chemotherapeutic agents, depending on the apoptotic stimulus and cell type (39). For example, polyamine-depleted IEC-6 rat intestinal epithelial cells show increased susceptibility to apoptosis induced by staurosporin but are protected from apoptosis induced by tumor necrosis factor-α or camptothecin (40, 41). Many chemotherapeutic agents affected by polyamine levels target cell cycle checkpoints or chromatin, which are altered in tumor cells.

Using gentle low-salt lysis conditions under which most histones remain associated with the DNA in the insoluble fraction, we detected a greater release of hyperacetylated histones in polyamine-depleted cells both before and after HDAC inhibitor treatment. This likely reflects an alteration in chromatin structure after polyamine depletion. Polyamine-depleted cells were able to take up TSA, block HDAC activity, and induce histone hyperacetylation to the same extent as control cells. Polyamines play a role in higher-order chromatin structure, and polyamine depletion alters chromatin structure in mammalian cells (30, 42, 43). HDAC inhibitors also induce global reorganization of chromatin structure (44). Therefore, changes in chromatin structure after polyamine depletion might be responsible for the altered response to HDAC inhibitors in polyamine-depleted cells.

Polyamines influence gene expression in part by regulating chromatin conformation, and polyamine depletion may affect the cellular response to HDAC inhibitors through changes in gene expression (11). Additionally, ODC is induced in response to some death signals, and polyamines regulate apoptosis by modulating the activation or expression of several apoptotic proteins (39). We found that TSA induced ODC expression and activity in cell lines that were sensitive to the apoptotic effects of HDAC inhibitors. Treatment of mouse MC-26 colon cancer cells with sodium butyrate increases ODC activity and polyamine levels due to an increased cellular requirement for polyamines (45). Therefore, in tumor cells, induction of ODC by TSA treatment might alter chromatin structure or the apoptotic response of cells.

Biomarkers that predict which tumors will respond to treatment with HDAC inhibitors are needed to direct future clinical studies. HCT116 colon carcinoma cells, which are extremely sensitive to the cytotoxic effects of TSA (IC50 0.34 μmol/L), had high ODC activity (~1,200 cpm/mg protein/h), whereas the more TSA-resistant lung carcinoma cell line H1299 (IC50 5.76 μmol/L) had low ODC activity (100 cpm/mg protein/h). Cell lines with high levels of ODC activity were more sensitive to HDAC inhibitor–induced apoptosis, and this correlation was

**Figure 6.** Elevated ODC activity levels and TSA-induced ODC activity in tumor cell lines correlate with increased sensitivity to TSA-induced apoptosis. A, ODC activity levels and IC50 values for each cell line plotted on a double log plot. The calculated IC50 values for each cell line by TSA were determined from 0.5 mol/L TSA, and ODC activity assays were done on cell lysates. Points, means of at least three experiments done in triplicate; bars, SE. C, change in ODC activity induced by TSA (slope of the response to TSA treatment for 16 h) and IC50 values for each cell line plotted on a double log plot. A Spearman rank correlation test was used to assess the relationship of the two variables.
ODC Activity Levels Predict HDAC Inhibitor Sensitivity

statistically significant in the panel of tumor cell lines we used. In addition, the ability of TSA to further induce ODC activity correlated with increased sensitivity to HDAC inhibitor–induced apoptosis. In microarray studies, HDAC inhibitor treatment of various cell lines induces ODC mRNA (46, 47). We found that TSA treatment increased ODC protein levels (data not shown). However, ODC protein and activity levels do not directly correlate, as ODC antizyme responds to increased levels of polyamines by targeting ODC for degradation by the proteosome (48). Tumors can overexpress antizyme inhibitor, which stabilizes ODC by trapping ODC antizyme and thereby promoting high levels of ODC activity (49). The complex regulation of polyamine synthesis, which can be altered in various ways in tumor cells, is an early event in tumorigenesis and seems to be a potential marker for the therapeutic value of treatment with HDAC inhibitors.

This study shows that polyamines are involved in the cellular response to HDAC inhibitors and that high levels of ODC activity and the ability of TSA to further increase ODC activity correlate with increased sensitivity to HDAC inhibitors. Therefore, polyamine levels are a potential biomarker in tumor cells that may predict their sensitivity to HDAC inhibitors. Polyamine biosynthesis is dysregulated in tumors with mutations in MYC, APC, KRAS, TP53, antizyme inhibitor, and eIF4E (12). Polyamines and histone acetylation may act together to modify global chromatin structure. Histone hyperacetylation induced by HDAC inhibitors seems to prevent proper activation of the spindle checkpoint (4, 6). Polyamine depletion may alter global chromatin structure in a manner that activates the spindle checkpoint to prevent tumor cells treated with HDAC inhibitors from entering an aberrant mitosis and undergoing apoptosis. This should be studied by examining polyamine-depleted cells for effects on chromosomal alignment and mitotic defects after treatment with HDAC inhibitors. Further studies will determine if polyamine depletion increases resistance to HDAC inhibitor–induced apoptosis through effects on gene expression or effects on chromatin structure. Examination of tumor samples for a correlation between polyamine levels and sensitivity to HDAC inhibitors is also warranted.

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