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Research Article

Molecular Cancer Therapeutics

Selective Inhibition of Histone Deacetylases Sensitizes Malignant Cells to Death Receptor Ligands

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Abstract

Evasion of death receptor ligand–induced apoptosis represents an important contributor to cancer development and progression. Therefore, molecules that restore sensitivity to death receptor stimuli would be important tools to better understand this biological pathway and potential leads for therapeutic adjuncts. Previously, the small-molecule 4-(4-chloro-2-methylphenoxy)-*N*-hydroxybutanamide (that we propose be named droxinostat) was identified as a chemical sensitizer to death receptor stimuli, decreasing the expression of the caspase-8 inhibitor FLIP. However, the direct targets of droxinostat were unknown. To better understand the mechanism of action of droxinostat and highlight new strategies to restore sensitivity to death receptor ligands, we analyzed changes in gene expression using the Connectivity Map after treating cells with droxinostat. Changes in gene expression after droxinostat treatment resembled changes observed after treatment with histone deacetylase (HDAC) inhibitors. Therefore, we examined the effects of droxinostat on HDAC activity and showed that it selectively inhibited HDAC3, HDAC6, and HDAC8 and that inhibition of these HDACs was functionally important for its ability to sensitize cells to death ligands. Thus, we have identified a selective HDAC inhibitor and showed that selective HDAC inhibition sensitizes cells to death ligands, thereby highlighting a new mechanism to overcome resistance to death receptor ligands. *Mol Cancer Ther; 9(1); 246–56. ©2010 AACR.*

Introduction

Blocks in the death receptor pathway of caspase activation can render malignant cells resistant to death receptor ligands such as Fas ligand and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; ref. 1). In addition, such defects can confer resistance to anoikis, cell death that occurs on detachment from the extracellular matrix, a process often dependent on the death receptor pathway (2). To better understand mechanisms conferring resistance to death receptor stimuli, we screened a chemical compound library to identify small molecules that sensitize resistant cells to CH-11 Fas activating antibody (FAS; ref. 3). From this screen, we identified several molecules, including *N*-[4-chloro-3-(trifluoromethyl)phenyl]-3-oxobutanamide (fasentin), which sensitized cells to death ligands by blocking glucose transporters and partially inhibiting glucose uptake (4).

This screen also identified 4-(4-chloro-2-methylphenoxy)-*N*-hydroxybutanamide (that we proposed be named droxinostat based on the data in this article) that sensitized cells to FAS and TRAIL by decreasing the mRNA and protein expression of the caspase-8 inhibitor c-Fas–associated death domain–like interleukin-1–converting enzyme-like inhibitory protein (FLIP; ref. 3). Through its ability to decrease FLIP, droxinostat also sensitized cells to anoikis and thereby prevented distant tumor formation in a mouse model of metastatic prostate cancer (2).

Although decreases in FLIP were functionally important for the ability of droxinostat to sensitize cells to FAS, TRAIL, and anoikis, its direct molecular targets were unknown (3). Through chemical and genetic approaches, this report shows that droxinostat is a selective inhibitor of histone deacetylase (HDAC) 3, 6, and 8. HDACs are a class of at least 18 enzymes in humans (5) that remove acetyl groups from lysine residues of histones. HDACs have multiple biological functions, including tightening the chromatin structure in the nucleosome and inhibiting gene transcription (6). More recently, additional functions for HDACs have been identified. For example, in certain circumstances, HDACs can promote gene transcription (7), regulate mitosis (8), and promote DNA repair (9).

HDAC inhibitors (HDACi) have proven clinical efficacy in the treatment of solid tumors and hematologic

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malignancies (10). The initial HDACis developed were pan-inhibitors, blocking all HDAC isoforms (11). Currently, efforts are under way to develop isoform-selective HDACis that will possess anticancer activity with fewer side effects. A challenge in this field is to identify HDACis with different patterns of inhibition and to understand the biological consequences of inhibiting selected HDACs. Here, we report that droxinostat is a selective inhibitor of HDAC3, HDAC6, and HDAC8.

Materials and Methods

Reagents

MS-275, trichostatin A (TSA), and sodium valproate were purchased from Sigma-Aldrich. CH-11 anti-Fas monoclonal antibody (FAS), to activate the Fas receptor, was obtained from Medical Biological Laboratories. TRAIL was obtained from Alexis Biochemicals.

Stock solutions (1 mmol/L) of droxinostat (**1**) and compounds **2** and **3** were prepared in water with 0.1% DMSO and stored at 4°C in polystyrene tubes. Stock solutions of MS-275 and TSA (100 mmol/L) were prepared in DMSO and stored at −20°C in polystyrene tubes. Stock solutions (1 mol/L) of sodium valproate were prepared in water and stored at 4°C.

Synthesis of Droxinostat and Analogues

All solvents and reagents for the syntheses of compounds **1** to **3** were purchased from suppliers and used without further purification. Characterization data can be found in the Supplementary Data.

4-(4-Chloro-2-Methylphenoxy)-*N*-Hydroxybutanamide (1, Droxinostat)

Ethyl 4-(4-Chloro-2-Methylphenoxy)Butanoate. Ethyl 4-bromobutyrate (1.27 mL, 8.8 mmol) was added to 4-chloro-2-methylphenol (1.13 g, 7.9 mmol) and anhydrous K_2CO_3 (1.22 g, 8.8 mmol) in *N*,*N*-dimethylformamide (7 mL). The mixture was stirred for 48 h at room temperature and then diluted in Et_2O (20 mL) and washed with 10% (v/v) aqueous HCl (3 × 20 mL) and distilled water (10 mL). The organic phase was separated, dried with anhydrous $MgSO_4$, and filtered, and solvent was removed under vacuum. Purification by silica flash chromatography (gradient 0–40% ethyl acetate in hexanes) provided the product as a colorless oil (1.87 g, 92% yield).

4-(4-Chloro-2-Methylphenoxy)Butanoic Acid. A suspension of LiOH-H_2O (0.44 g, 10 mmol) in water (8 mL) was added to ethyl 4-(4-chloro-2-methylphenoxy)butanoate (1.77 g, 6.9 mmol) in 1,4-dioxane (17 mL). The mixture was stirred for 6 h at room temperature. The solvent was removed under vacuum, and the residual was dissolved in Et_2O (30 mL) and extracted with a mixture of saturated aqueous $NaHCO_3$ (15 mL) and distilled water (15 mL). The aqueous phase was separated and acidified with concentrated HCl. Suction filtration provided the product as a colorless powder (1.44 g, 91% yield).

4-(4-Chloro-2-Methylphenoxy)-N-Hydroxybutanamide (Droxinostat). A suspension of 4-(4-chloro-2-methylphenoxy)butanoic acid (1.44 g, 6.3 mmol) in $SOCl_2$ (8 mL) was heated to reflux under a N_2 atmosphere for 1 h. The excess thionyl chloride was removed under vacuum, and the residual was dissolved in tetrahydrofuran (150 mL). A solution of hydroxylamine hydrochloride (1.10 g, 16 mmol) and 4-methylmorpholine (2.7 mL, 25 mmol) in water (14 mL) was added to the solution of acyl chloride. The mixture was stirred for 16 h at room temperature and diluted with dichloromethane (250 mL) and saturated aqueous NH_4Cl (250 mL). The organic phase was separated, dried with anhydrous $MgSO_4$, and filtered, and the solvent was removed under vacuum. Precipitation from dichloromethane and hexanes provided the product as a beige powder (0.95 g, 62% yield).

4-(4-Chloro-2-Methylphenoxy)-*N*-Methoxybutanamide (2)

A suspension of 4-(4-chloro-2-methylphenoxy)butanoic acid (0.121 g, 0.53 mmol) in $SOCl_2$ (3 mL) was heated to reflux under a N_2 atmosphere for 1 h. The excess $SOCl_2$ was removed under vacuum, and the residual was dissolved in dichloromethane (5 mL). A suspension of methoxyamine hydrochloride (0.0460 g, 0.55 mmol) and 4-methylmorpholine (0.12 mL, 1.1 mmol) in tetrahydrofuran (5 mL) was added to the acyl chloride. The mixture was stirred for 6 h at room temperature and diluted with dichloromethane (10 mL) and saturated aqueous NH_4Cl (15 mL). The organic phase was separated, dried with anhydrous $MgSO_4$, and filtered, and the solvent was removed under vacuum. The product was obtained as a beige solid (0.12 g, 85% yield).

4-(4-Chloro-2-Methylphenoxy)-*N*-Hydroxy-*N*-Methylbutanamide (3)

A suspension of 4-(4-chloro-2-methylphenoxy)butanoic acid (0.121 g, 0.53 mmol) in $SOCl_2$ (3 mL) was heated to reflux under a N_2 atmosphere for 1 h. The excess $SOCl_2$ was removed under vacuum, and the residual was dissolved in dichloromethane (5 mL). A suspension of *N*-methylhydroxylamine hydrochloride (0.0460 g, 0.55 mmol) and 4-methylmorpholine (0.12 mL, 1.1 mmol) in tetrahydrofuran (5 mL) was added to the acyl chloride. The mixture was stirred for 6 h at room temperature and diluted with dichloromethane (10 mL) and saturated aqueous NH_4Cl (15 mL). The organic phase was separated, dried with anhydrous $MgSO_4$, and filtered, and the solvent was removed under vacuum. The product was obtained as a colorless solid (0.12 g, 91% yield).

Cell Culture

Cells were cultured in RMPI 1640 supplemented with 10% fetal bovine serum (Hyclone), penicillin (500 IU/mL), and streptomycin (50 μg/mL). All cells were cultured at 37°C in a humid atmosphere with 5% CO_2.

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Gene Expression Analysis

To assess the molecular response to droxinostat, Affymetrix HG U133 Plus 2.0 oligonucleotide array analysis was done on droxinostat-treated PPC-1 cells and compared with buffer control as previously described (4). Microarray data were visualized and fold differences (treated versus untreated) were calculated using GeneSpring GX v7.3 (Agilent Technologies). Gene Ontology analysis was done using the online tool GeneCODIS (Gene Annotation Co-occurrence Discovery) software (http://genecodis.dacya.ucm.es). Identification of drugs with similar gene expression signatures to droxinostat was achieved using the Connectivity Map (http://www.broad.mit.edu/cmap/) tool (12).

Cell Viability

Cell viability was assessed using the MTS reduction assay (Promega) according to the manufacturer's protocols as previously described (2). The percent relative cell viability was expressed as (absorbance of treated cells/absorbance of controls) × 100%.

HDAC Inhibition Assay

HDAC inhibition was assessed using the CycLex HDACs fluorometric assay (MBL International) according to the manufacturer's protocol and using crude nuclear extract from HeLa cells (principally HDAC1 and HDAC2). The relative activity was expressed as (fluorescence intensity of treated samples/fluorescence intensity of controls) × 100%. Assays to establish HDAC isoform inhibition selectivity were done by Reaction Biology Corp.

Molecular Docking

A model of droxinostat was built manually and optimized. A virtual model of HDAC8 was constructed from its crystal structure (Protein Data Bank ID code 1W22.pdb; ref. 13). Docking of droxinostat into the binding pocket of HDAC8 was explored using the FlexX program (14). Default, water-mediated binding parameters of the program as implemented in SYBYL7.1 were used during docking. The most promising of the top 30 (i.e., lowest docking score) were considered.

Immunoblot Analysis

Protein extracts were obtained by washing cells with PBS (pH 7.4) and suspending in Laemmli buffer [60 mmol/L Tris (pH 6.8), 10% glycerol, 20% SDS] containing protease inhibitors (Complete tablets; Roche). Immunoblot assays were done as previously described (4). Briefly, equal amounts of protein, as determined by Bio-Rad detergent-compatible assay, were subjected to SDS-PAGE (4–20% gradient gels; ISC BioExpress) followed by transfer to nitrocellulose membranes. Acetylated lysine antibody (Cell Signaling Technology) was incubated at a dilution of 1:1,000 (v/v), HDAC2 and HDAC3 (Bio Vision) at 1:200 (v/v), HDAC6 and HDAC8 (Santa Cruz Biotechnology, Inc.) at 1:1,000 (v/v), mouse monoclonal anti–β-actin (Sigma) at 1:10,000 (v/v), and mouse monoclonal anti–α-tubulin (Sigma) at 1:10,000 (v/v). Secondary antibodies consisted of horseradish peroxidase–conjugated goat anti-rabbit IgG or goat anti-mouse IgG (GE Healthcare). Detection was done by the enhanced chemiluminescence method (Immobilon, Millipore).

HDAC Silencing by Lentiviral-Delivered RNA Interference

Construction of hairpin-pLKO.1 vectors (carrying a puromycin antibiotic resistance gene) containing short hairpin RNA (shRNA) sequences and production of shRNA viruses have been described in detail (15). The shRNAs targeting the HDAC coding sequences are as follows: HDAC2 (accession no. NM_001527), 5′-GCAAATACTATGCTTGTCAATT-3′; HDAC3 (accession no. NM_003883), 5′-GCACCTAGTGTCCAGATTCAT-3′; HDAC6 (accession no. NM_006044), 5′-CGGTAATGGAACTCAGCACAT-3′; and HDAC8 (accession no. NM_018486), 5′-CCAATGCCTGATTGACGGAAT-3′. Lentiviral infections were done essentially as described (15). Briefly, adherent cells were treated with 0.5 mL of the virus (high titer) followed by overnight incubation (37°C, 5% CO_2) without removing the virus. The next day, viral medium was replaced with fresh medium containing puromycin (2 μg/mL) to select a population of resistant cells.

Quantitative Reverse Transcription-PCR

First-strand cDNA was synthesized from 1 μg of DNase-treated total cellular RNA using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocols. Real-time PCR assays were done in triplicate with 5 ng of RNA equivalent cDNA, SYBR Green PCR Master mix (Applied Biosystems), and 400 nmol/L of gene-specific primers. Reactions were processed and analyzed on an ABI 7900 Sequence Detection System (Applied Biosystems). Forward/reverse PCR primer pairs for human cDNAs were as follows: FLIP, 5′-CCTAGGAATCTGCCTGATAATCGA-3′/5′-TGGGATATACCATGCATACTGAGATG-3′; 18S, 5′-AGGAATTGACGGAAGGGCAC-3′/5′-GGACATCTAAGGGCATCACA-3′; DR4, 5′-AGAGAGAAGTCCCTGCACCA-3′/5′-GTCACTCCAGGGCGTACAAT-3′; and DR5, 5′-CACCAGGTGTGATTCAGGTG-3′/5′-CCCCACTGTGCTTTGTACCT-3′. Relative mRNA expression was determined using the $\Delta\Delta C_T$ method as described (3).

Results

Droxinostat Inhibits HDAC Activity

Recently, we identified 4-(4-chloro-2-methylphenoxy)-*N*-hydroxybutanamide (that we proposed be named droxinostat based on the data below) that sensitized selected resistant cells to stimuli of the death receptor pathway of caspase activation, including FAS and TRAIL, and anoikis (Supplementary Fig. S1; refs. 2, 3). However, the direct molecular targets and effectors of droxinostat were unknown.

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To identify its targets, we analyzed changes in gene expression after treatment of PPC-1 prostate cancer cells with droxinostat for 24 hours. After this incubation, cells were harvested and gene expression was measured by microarray analysis and compared with cells treated with buffer alone. We identified 414 probe sets differentially expressed 4-fold or greater; 135 of these were differentially expressed 8-fold or greater. We subjected our 8-fold differentially expressed data set to Connectivity Map (http://www.broad.mit.edu/cmap/) analysis (12) to identify other drugs that induced changes in gene expression similar to droxinostat. Strikingly, this analysis identified that droxinostat induced a gene expression signature most closely resembling those of TSA and vorinostat, two known HDACis. In addition, genes involved in transcription comprised the most significantly overrepresented group in the data set based on Gene Ontology analysis. Thus, taken together, these data suggest that droxinostat is a novel HDACi.

As changes in gene expression suggest that droxinostat may inhibit the activity of HDACs, we evaluated the effects of droxinostat on HDAC activity in cultured cells. PPC-1 prostate, OVCAR-3 ovarian, U937 leukemia, HT29 colon, and T47D breast cancer cells were treated with droxinostat or the known HDACi TSA, and levels of acetylated histones H3 and H4 were measured by immunoblotting. Consistent with its effects as a HDACi, droxinostat, like TSA, increased levels of acetyl histones H3 and H4 (Fig. 1A). Increased levels of acetyl histones H3 and H4 were observed within 4 hours of treatment with droxinostat and preceded its ability to sensitize to FAS that was only observed after 16 hours of treatment (Supplementary Fig. S2).

To determine whether droxinostat was a direct inhibitor of HDAC enzymatic activity, HeLa cell nuclear extracts were treated with increasing concentrations of droxinostat in a fluorometric assay of HDAC activity. Droxinostat inhibited HDAC activity at concentrations

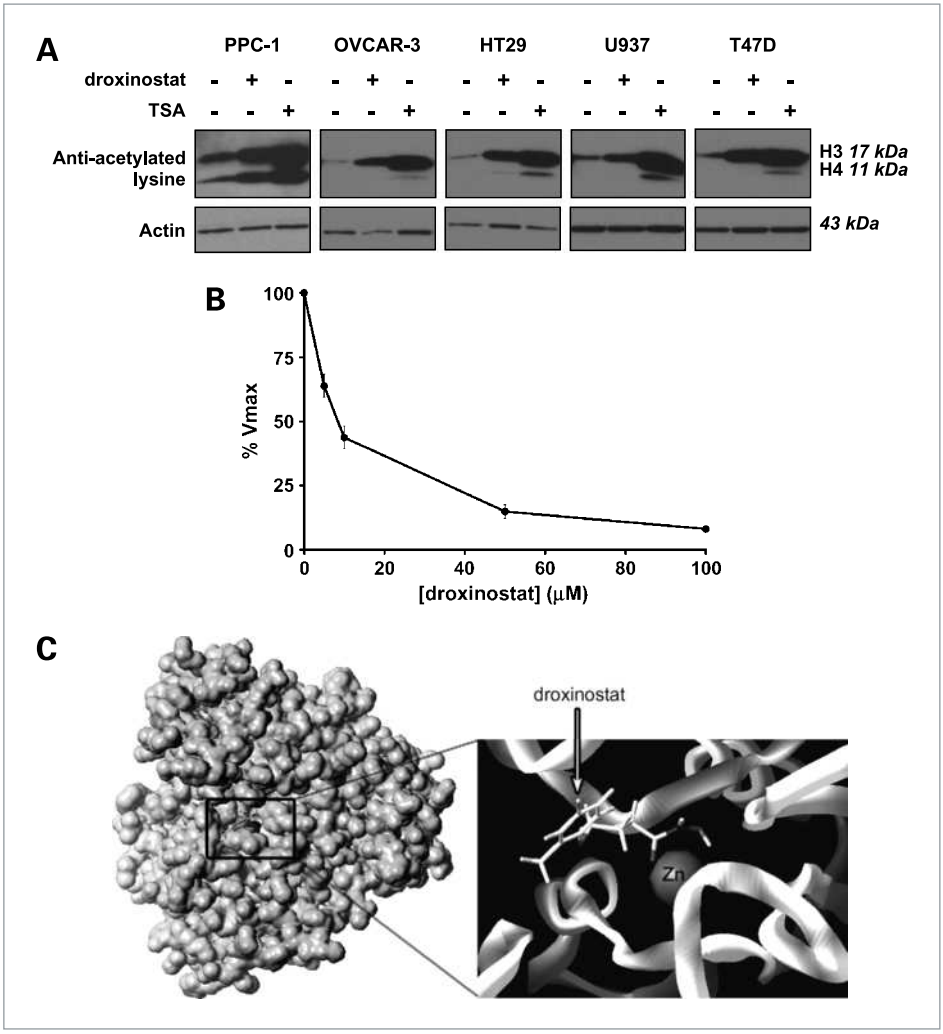

Figure 1. Droxinostat inhibits HDAC activity. **A,** PPC-1, OVCAR-3, HT29, U937, and T47D cancer cells were treated for 16 h with droxinostat (50 μmol/L), TSA (500 nmol/L), or buffer control. After incubation, total cellular proteins were isolated and analyzed by SDS-PAGE immunoblotting using antibodies against acetylated lysine and actin. **B,** extracts from HeLa cells were treated with increasing concentrations of droxinostat and deacetylase activity was measured using a fluorescent assay as described in Materials and Methods. *Points,* mean percent HDAC activity compared with extracts treated with buffer control from independent experiments done in triplicate; *bars,* SD. **C,** a virtual model of HDAC8 was constructed based on its known crystal structure. Docking of droxinostat into the binding pocket of HDAC8 was explored using the FlexX program with default, water-mediated binding parameters.

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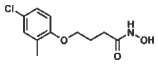
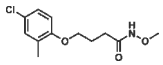
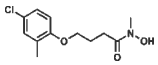
Table 1. The hydroxamic acid moiety is important for the effects of droxinostat on HDAC activity and FAS sensitization

Compound	Structure	HDAC IC_{50} (μmol/L)	LD_{50} –FAS (μmol/L)	LD_{50} +FAS (μmol/L)
Droxinostat (**1**)		10 ± 1	>80	20 ± 2
2		>80	>80	>80
3		>80	>80	>80

NOTE: Extracts from HeLa cells were treated with increasing concentrations of droxinostat and analogues **2** and **3** with substituted hydroxamic acid moieties, and deacetylase activity was measured as described in Materials and Methods. The IC_{50} represents the concentration of the compound to inhibit 50% of the deacetylase activity compared with control-treated extracts. PPC-1 cells were treated with increasing concentrations of droxinostat and analogues **2** and **3** with substituted hydroxamic acid moieties with and without FAS (100 ng/mL). Sixteen hours after incubation, cell growth and viability were determined by the MTS assay. Data represent the mean ± SD of independent experiments done in triplicate.

similar to those required to sensitize cells to the death ligand FAS and TRAIL (Fig. 1B).

The Hydroxamic Acid Moiety in Droxinostat Is Important for Its Effects as a FAS Sensitizer and a HDACi

Droxinostat contains a hydroxamic acid moiety, which is frequently found in HDAC-inhibiting molecules. To investigate its relevance to the effects of droxinostat as a FAS sensitizer and inhibitor of HDAC activity, we synthesized analogues of droxinostat in which the hydroxamic acid residue was methylated either at the oxygen or at the nitrogen atom, compounds **2** and **3**, respectively. PPC-1 cells were treated with increasing concentrations of these analogues with and without FAS or TRAIL. Analogues possessing a methylated hydroxamic acid residue were inactive and neither sensitized cells to FAS or TRAIL nor inhibited HDAC enzymatic activity. Thus, the hydroxamic acid moiety of droxinostat is functionally important for its ability to sensitize cells to FAS and TRAIL and inhibit HDAC activity (Table 1; data not shown).

To investigate the mechanism by which droxinostat inhibits HDACs, we constructed a virtual model of HDAC8 based on its known crystal structure (13). Docking of droxinostat into the binding pocket of HDAC8 was done using the FlexX program (14). The resulting structures possessing the most favorable docking scores consistently showed a chelating interaction between the droxinostat hydroxamic acid moiety and the zinc ion in the active site of HDAC8 (Fig. 1C). The aromatic ring of droxinostat is oriented toward the cytosol in a similar manner to the observed orientation of TSA bound to HDAC8 in the solid state structure of that complex (13).

Droxinostat Selectively Inhibits HDAC3, HDAC6, and HDAC8

Multiple HDAC enzymes have been identified, of which HDAC1 to HDAC10 are expressed in malignant cells (16–27). To understand the spectrum of HDACs inhibited by droxinostat and determine whether selective HDAC inhibition is sufficient to sensitize cells to FAS and TRAIL, we examined the spectrum of HDAC enzymes inhibited by droxinostat. The deacetylation activity of recombinant HDAC1 to HDAC10 was measured after incubation with increasing concentrations of droxinostat (Table 2). Droxinostat selectively inhibited HDAC3, HDAC6, and HDAC8 with an IC_{50} of 16.9 ± 5.0, 2.47 ± 1.09, and 1.46 ± 0.11 μmol/L, respectively, but did not inhibit HDAC1, HDAC2, HDAC4, HDAC5, HDAC7, HDAC9, and HDAC10, with an IC_{50} of >20 μmol/L. In contrast, the known pan-HDACi TSA inhibited all tested HDACs with an IC_{50} in the low nanomolar range (data not shown; ref. 28). Thus, droxinostat exhibits differential selectivity among the HDAC enzymes. Although droxinostat was a more potent inhibitor of HDAC6 and HDAC8 compared with HDAC3, its inhibition of HDAC3 may be functionally important for its ability to sensitize to FAS and TRAIL, as FAS and TRAIL sensitization occurred at an IC_{50} of 20 ± 2 and 39 ± 2 μmol/L, respectively.

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Given the ability of droxinostat to selectively inhibit HDAC3, HDAC6, and HDAC8, we evaluated the ability of other HDACis to sensitize cells to FAS and TRAIL. PPC-1 cells were treated with increasing concentrations of droxinostat, the pan-HDACis TSA and valproic acid, or the selective HDAC1 inhibitor MS-275 (11, 29) with and without FAS. Similar to the effects of droxinostat, TSA, valproic acid, and MS-275 sensitized PPC-1 to FAS (Fig. 2). A similar effect was observed on these HDACis to sensitize cells to TRAIL (data not shown).

To determine whether basal levels of HDAC3, HDAC6, and HDAC8 could explain sensitivity to droxinostat, we measured the expression of these HDACs in a panel of five droxinostat-sensitive and three droxinostat-resistant cell lines. By immunoblotting, basal levels of the HDACs did not predict the ability of this compound to sensitize cells to FAS or TRAIL (data not shown). We explored whether droxinostat inhibited HDAC activity in cells where droxinostat did not sensitize to FAS. In HT29 cell lines, droxinostat did not sensitize to FAS but inhibited HDAC activity as measured by increases in acetyl H3 and H4 levels (Fig. 1A). Likewise, droxinostat also increased acetyl histones H3 and H4 and decreased FLIP in MDA468 breast cancer cells but did not sensitize them to FAS (Supplementary Fig. S3). Of note, MDA468 and HT29 cells have detectable levels of CD95 and these levels did not change after droxinostat treatment. Thus, the failure of droxinostat to sensitize some cells to FAS is not due to failure to inhibit the selected HDACs and decrease FLIP but rather the downstream response to HDAC inhibition.

Genetic Inhibition of Individual HDACs and Sensitization to Death Receptor Ligands

To further explore the effects of selective HDAC inhibition on FAS and TRAIL sensitization, we selectively knocked down HDAC3, HDAC6, and HDAC8 with shRNA in PPC-1 cells. Target knockdown was confirmed by immunoblotting (Fig. 3A). We then evaluated the effects of selective HDAC inhibition on FAS and TRAIL sensitization. Individual knockdown of HDAC3, HDAC6, and HDAC8 was sufficient to sensitize PPC-1 cells to TRAIL, and individual knockdown of HDAC8 was sufficient to sensitize cells to FAS (Fig. 3B). Of note, however, knockdown of HDAC3 and HDAC6 did not sensitize cells to FAS. To determine whether knockdown of individual shRNA was sufficient to explain the activity of droxinostat, we treated PPC-1 cells with knockdown of HDAC3, HDAC6, or HDAC8 with droxinostat and FAS or TRAIL. In cells with knockdown of these individual HDACs, droxinostat continued to sensitize to FAS and TRAIL (Fig. 3C). Thus, knockdown of all three isoforms may be necessary to completely recapitulate the effects of droxinostat.

Previously, we showed that droxinostat sensitized cells to FAS and TRAIL by decreasing FLIP mRNA and protein (3). We also showed that inhibition of FLIP expression by droxinostat seemed functionally important as overexpression of FLIP or the caspase-8 inhibitor cytokine response modifier A blocked the ability of droxinostat to sensitize cells to FAS (Supplementary Fig. S4). To determine whether selective knockdown of HDAC3, HDAC6, and HDAC8 could decrease FLIP expression, we measured changes in FLIP after infection of cells with HDAC3, HDAC6, or HDAC8 shRNA in a lentiviral vector. By quantitative reverse transcription-PCR, knockdown of these HDACs led to small decreases in FLIP mRNA but, at most, only minor decreases in protein expression (Fig. 3D). In contrast, knockdown of HDAC2, which was not inhibited by droxinostat, had no effect on FLIP mRNA expression. Prior studies have shown that HDACis can sensitize to FAS and TRAIL by increasing death receptor expression. Therefore, we examined changes in cell surface CD95 expression by flow cytometry and DR4 and DR5 expression by PCR. Knockdown of individual HDACs had no effect on the surface expression of CD95 and DR4/DR5 mRNA expression. Thus, knockdown of all three HDAC isoforms is likely necessary to see the full effects of the compound.

Table 2. Droxinostat selectively inhibits HDAC3, HDAC6, and HDAC8

HDAC isoform	Droxinostat IC_{50} (μmol/L)
1	>20
2	>20
3	16.9 ± 5.0
4	>20
5	>20
6	2.47 ± 1.09
7	>20
8	1.46 ± 0.11
9	>20
10	>20

NOTE: Recombinant HDAC1 to HDAC10 were treated with increasing concentrations of droxinostat, and deacetylase activity was measured using a fluorescent assay as described in Materials and Methods. Data represent the mean and SD from two independent experiments (n = 4).

Discussion

HDACs inhibit gene transcription, and their aberrant activity in malignancy is associated with tumor progression and chemoresistance (reviewed in ref. 30). To date, four classes of HDACs have been identified (5): class I (zinc-dependent enzymes showing homology to the yeast protein RPD3; HDAC1, HDAC2, HDAC3, and HDAC8), class II (zinc-dependent enzymes showing homology to the yeast protein HDA1; HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10), class III (the structurally distinct NAD-dependent sirtuins: Sirt1–Sirt7), and class IV (HDAC11, which has characteristics of both class I and class II). The initial HDACis identified,

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such as TSA and vorinostat, are pan-HDACis and block the enzymatic activity of all HDAC classes with similar potency (11).

Side effects of the pan-HDACi vorinostat, which is approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma, include grade 3 thrombocytopenia, fatigue, dehydration, and hypernatremia (31). Potentially, HDACis that block selective isoforms can maximize antitumor effects and minimize side effects compared with pan-inhibitors. Although this hypothesis has not been tested in clinical trials, data from mice with knockouts of individual HDACs suggest that isoform-specific inhibitors may be better tolerated than pan-inhibitors. For example, cardiotoxicity including cardiac hypertrophy and fibrosis have been observed in HDAC9 and HDAC5 knockout mice but not in HDAC1 and HDAC2 knockout mice (32, 33). Furthermore, HDAC7 knockout mice are not viable (34). However, we have not conducted toxicology studies with droxinostat, so we cannot be certain how the toxicity of this compound compares to other pan or selective HDACis.

Previously, we identified droxinostat that sensitized resistant cells to stimuli of the death receptor pathway of caspase activation, but its direct molecular targets were unknown (3). Here, we showed that droxinostat is a selective inhibitor of HDAC3, HDAC6, and HDAC8. Other HDACis that contain a hydroxamic acid residue, such as TSA, vorinostat, Scriptaid, CBHA, and panobinostat, are broader acting among class I, II, and IV HDACs (11). In general, these compounds show stronger inhibition of HDAC1 to HDAC7 and weaker inhibition of HDAC8. For example, vorinostat inhibits HDAC3 and HDAC6 more actively than HDAC8 (11). Linkerless analogues of vorinostat possessing the hydroxamic acid pharmacophore without the long flexible spacer selectively inhibit

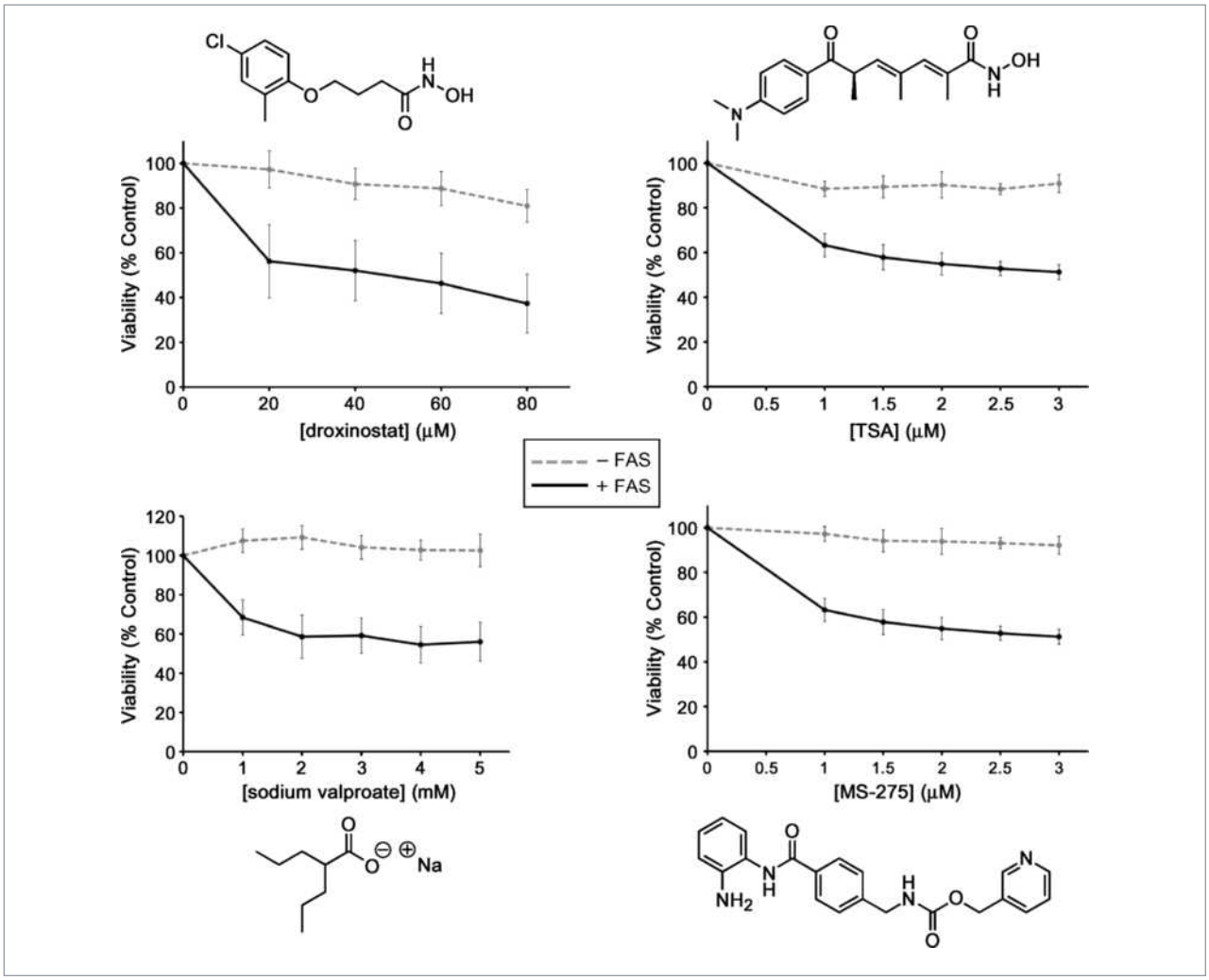

Figure 2. HDACis sensitize resistant cells to FAS. PPC-1 prostate cancer cells were treated for 16 h with increasing concentrations of droxinostat or the known HDACis TSA, valproic acid, or MS-275 with and without FAS (100 ng/mL). After incubation, cell growth and viability were assessed by the MTS assay. *Points,* mean percent viability compared with control-treated cells from independent experiments done in triplicate; *bars,* SD.

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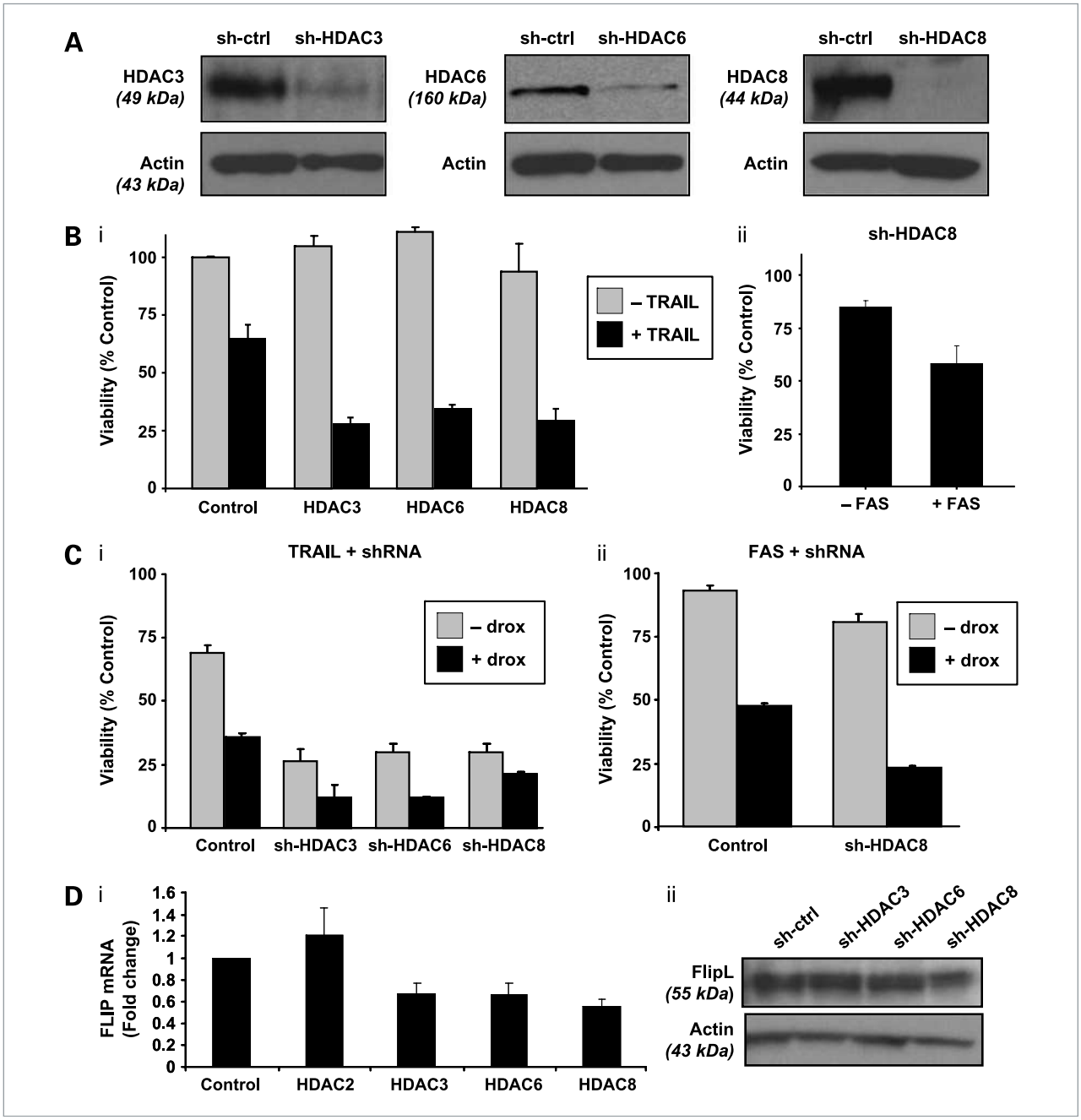

Figure 3. Genetic knockdown of HDAC3, HDAC6, and HDAC8 and sensitization to death receptor ligands. PPC-1 prostate cancer cells were infected with lentivirus containing shRNA control sequences or shRNA targeting HDAC3, HDAC6, or HDAC8, and populations of infected cells were selected. **A,** target knockdown was confirmed by SDS-PAGE immunoblotting using antibodies against HDAC3, HDAC6, and HDAC8 and actin. **B,** *i,* cells stably infected with shRNA targeting HDAC3, HDAC6, HDAC8, or control sequences were treated with TRAIL (50 ng/mL) or buffer control. After incubation for 16 h, cell growth and viability were measured by MTS assay. *ii,* cells stably infected with shRNA targeting HDAC8 or control sequences were treated with FAS (100 ng/mL) or buffer control. *Columns,* mean percent viability compared with cells infected with control shRNA and treated with buffer alone from independent experiments done in triplicate; *bars,* SD. **C,** *i,* cells stably infected with shRNA targeting HDAC3, HDAC6, HDAC8, or control sequences were treated with TRAIL (50 ng/mL) with or without droxinostat (40 μmol/L). After incubation for 16 h, cell growth and viability were measured by MTS assay. *ii,* cells stably infected with shRNA targeting HDAC8 or control sequences were treated with FAS (100 ng/mL) with our without droxinostat (40 μmol/L). *Columns,* mean percent viability compared with cells infected with control shRNA and treated with buffer alone; *bars,* SD. **D,** *i,* total cellular RNA was isolated from cells stably infected with shRNA targeting HDAC2, HDAC3, HDAC6, HDAC8, or control sequences. FLIP long mRNA expression was measured relative to 18S RNA by real-time reverse transcription-PCR. *Columns,* mean percent of FLIP/18S expression relative to controls ($\Delta\Delta C_T$ normalization) from independent experiments done in triplicate; *bars,* SE. *ii,* total protein was isolated from PPC-1 cells, stably infected with shRNA targeting HDAC3, HDAC6, HDAC8, or control sequences, and analyzed by SDS-PAGE immunoblotting using antibodies against FLIP and actin.

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HDAC8 (24), showing that despite limited structural information, progress is being made in the design of isoform-selective HDACis (28). Further selectivity among the HDACs is possible, with, for example, MS-275, which belongs to the benzamide family of HDACis, selectively inhibiting HDAC1, HDAC2, and HDAC3 but not HDAC6 or HDAC8 (11, 29). In comparison, droxinostat shows comparable inhibition of HDAC6 and HDAC8 (IC_{50} = 2.47 and 1.46 μmol/L, respectively) but weaker inhibition of HDAC3 (IC_{50} = 16.9 μmol/L). Interestingly, this pattern of HDAC inhibition by droxinostat is unique among HDACis, even those based on the hydroxamic acid pharmacophore. Moreover, although droxinostat is isoform selective, it is not class selective, acting as an inhibitor of members of both class I and class II HDACs. The structure of droxinostat incorporates a hydroxamic acid Zn^{2+} binding group, a relatively short linker (compared with many HDACis), and an aromatic hydrophobic cap group. Unfortunately, despite extensive structure-activity studies of HDACis, the structural features necessary to promote isoform or class selectivity are relatively poorly understood (28), and the structural basis for the observed selectivity of droxinostat is not clear.

Although a useful chemical probe, the relatively high LD_{50} value for droxinostat *in vitro* suggests that it would not be optimal for use *in vivo* or as a therapeutic agent. However, its unique combination of HDAC3, HDAC6, and HDAC8 isoform inhibition may make it a lead for the design of more potent selective HDACi analogues. In the context of developing these analogues, detailed pharmacokinetic and toxicology testing would be required to fully evaluate the potential efficacy of these compounds *in vivo*.

Droxinostat sensitized some but not all of the tested cell lines to death ligands FAS and TRAIL. However, other cell lines, such as DU145 prostate cancer cells, were not sensitized to FAS by droxinostat. This result is consistent with a report by Inoue et al. (35), who showed that selective inhibition of HDAC1 and HDAC2, but not HDAC3 or HDAC8, sensitized K562 leukemia cell line and DU145 to TRAIL. Taken together, we hypothesize that the pattern of HDAC inhibition required to sensitize cells to death ligands varies with the cell type under investigation.

Currently, it is uncertain why different cell lines differ in their susceptibility to selective HDAC inhibition. Our study showed that differences in sensitivity were not due to differences in basal levels of HDAC expression nor the ability of droxinostat to increase acetylation of histones H3 and H4 or decrease FLIP. Potentially, differences in sensitivity between cell lines may reflect how individual HDACs modulate regulators of the death receptor pathway of caspase activation.

In this study, we also evaluated whether knockdown of individual HDACs with shRNA was sufficient to recapitulate the effects of FAS. Knockdown of HDAC3, HDAC6, or HDAC8, but not HDAC2, slightly reduced levels of FLIP mRNA. However, significantly more robust reductions in FLIP were seen after treatment with droxinostat. Knockdown of HDAC3, HDAC6, or HDAC8 sensitized cells to TRAIL, and knockdown of HDAC8 sensitized cells to FAS. The reason for the differential effect on individual HDAC knockdown on FAS and TRAIL sensitization is unknown. However, it may indicate that, in these cells, lesser amounts of FLIP knockdown are required to sensitize cells to TRAIL. Supporting this hypothesis, the PPC-1 cells are more resistant to FAS than TRAIL at baseline. However, we also concede that inhibition of individual and multiple HDACs may also sensitize to death ligands through mechanisms unrelated to decreasing FLIP.

Knockdown of individual HDACs was not able to recapitulate the effects of droxinostat and the molecule continued to sensitize cells to FAS and TRAIL despite single HDAC knockout. We speculate that to recapitulate the effects of droxinostat on both FLIP expression and death receptor sensitization, we would need to simultaneously knockdown HDAC3, HDAC6, and HDAC8. We attempted these experiments but were unsuccessful in selecting cells with the triple knockdown. Therefore, in the absence of the triple knockdown, we also need to acknowledge that droxinostat may also have other targets that enable it to sensitize cells to death ligands and inhibit FLIP expression.

We and others have reported that chemical and genetic inhibition of HDACs leads to decreased expression of FLIP mRNA (36–43). However, the mechanism by which inhibiting HDACs decreases FLIP expression is uncertain. Moreover, as discussed above, HDAC inhibition may sensitize to death ligands through additional mechanisms independent of FLIP. Generally, HDAC inhibition is regarded as a strategy to increase gene transcription (44). However, in examining the gene expression profile of cells treated with HDACis, there are an equal number of genes downregulated as upregulated. Although we cannot exclude a direct effect of HDAC inhibition on FLIP mRNA transcription, it is more likely that HDAC inhibition upregulates a transcriptional repressor of FLIP. Future work will decipher the link between HDAC inhibition and the downregulation of FLIP. Such studies would include examining changes in acetyl histones associated with the FLIP promoter. These studies would help determine whether the effects on FLIP mRNA by droxinostat were a direct or indirect effect of its ability to inhibit HDACs.

In summary, we identified a selective small-molecule inhibitor of HDAC3, HDAC6, and HDAC8 and this selectivity profile is unique among reported HDACis, including those in clinical use or undergoing clinical evaluation. Thus, we have identified a novel therapeutic strategy to overcome resistance to death ligands. In addition, droxinostat could be a tool for elucidating the isoform-specific cellular functions of HDAC3, HDAC6, and HDAC8 and a lead for a novel therapeutic agent to sensitize resistance tumors to recombinant TRAIL.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

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