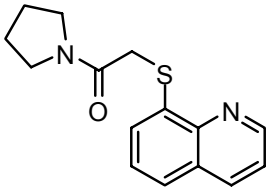
Project: High Throughput Screens for Epigenetic Modulators: Chemotype 2

Probe 2: 1-(pyrrolidin-1-yl)-2-(quinolin-8-ylthio)ethanone



SID: 26752291
NCGC00059731

PubChem Primary Bioassay Identifier (AID): 597

SID	IC50 (μM)	Antitarget	Selectivity*
26755514	5	AP1 signaling assay	≥7 (Inactive @38 μM)
26752291	8.9	AP1 signaling assay	≥4 (Inactive @38 μM)
17389072	7.9	AP1 signaling assay	≥5 (Inactive @38 μM)

*Selectivity = antitarget IC50/target IC50

Assigned Assay Grant #: X01-MH079860-01

Screening Center Name: NIH Chemical Genomics Center
Principal Investigator of Screening Center: Christopher Austin

Assay Submitter & Institution: Elisabeth D. Martinez, University of Texas Southwestern Medical Center

Assay or Pathway Target: Unidentified cellular targets

Assay provider information

Specific Aim: Identify small-molecule modulators of cellular epigenetic pathways.

Significance: Over the last several years, a few compounds have been identified that inhibit the methylation or de-acetylation pathways mediated by DNA methyltransferases and histone deacetylases. These compounds have immediate application in the treatment of cancers. as they reactivate aberrantly silenced tumor suppressor genes. There is clear need to identify additional small molecules that interact with these enzyme families. as well as new targets involved in the epigenetic control of gene expression. Our overall goal is to expand the available repertoire of small molecules that modulate gene expression and to evaluate their basic mechanism of action.

Rationale: A mammalian cell-based assay used to screen a chemical library in 1536-well plate format. Unlike methods used to date, this image-based assay has the advantage of

measuring the exact biological event it seeks to target: the reversal of transcriptional repression in mammalian cells.

Screening center information

Assay Implementation and Screening

PubChem Bioassay Name: qHTS Assay for Epigenetic Modulators

List of PubChem bioassay identifiers generated for this screening project (AIDs):

AID	Target	Concentration	Bioassay type
597	Unidentified	46μM – 0.59 nM	Primary qHTS
890	Unidentified	46μM – 5.5 pM	Confirmation
990	Unidentified	46μM – 5.5 pM	Counter screen
1035	HDAC	48 uM	Secondary Screen
1036	Cell viability	20μM – 1 nM	Secondary Screen
1037	Cell viability	20μM – 1 nM	Secondary Screen
1038	Cell viability	20μM – 1 nM	Secondary Screen
1039	Cell viability	20μM – 1 nM	Secondary Screen
1043	Cell viability	40μM – 1 nM	Secondary Screen
1041	P16 transcription	10μM	Secondary Screen
1042	CDH13 transcription	10μM	Secondary Screen

Primary Assay Description as defined in PubChem:

Overview:

The Locus Derepression (LDR) assay detects the derepression of a GFP reporter that is stably integrated in a region of the genome of murine c127i mammary cells, which is presumably silenced. GFP transcription in this construct is controlled by a CMV promoter, which is normally strong and constitutively active. However, this line was selected for lack of constitutive expression of the GFP protein. GFP production can be induced by incubating the cells with histone deacetylase or DNA methyltransferase inhibitors. Compounds that cause derepression of the locus to express GFP are identified by enumerating GFP positive cells using a laser-scanning microplate cytometer.

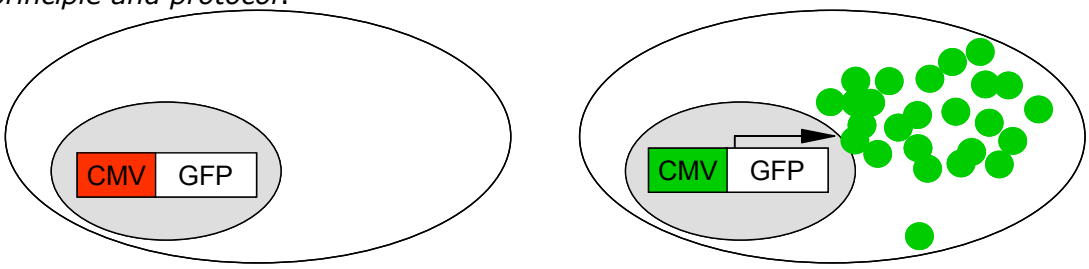
Protocol:

Two hundred and fifty cells in 5 uL per well were dispensed into black, clear-bottom 1536-well plates. Twenty-three nL compound was transferred to the assay plate and cells were incubated 30 hours at 37 C. Medium was removed, cells were washed two times with PBS, and GFP-positive cells were enumerated as fluorescent objects between the sizes of 20 to 120 um width and depth using an Acumen Explorer.

Center Summary of the Primary Screen:

Note: the below screen and probe characterization incorporates text that has been previously published (1).

Assay principle and protocol:



HDAC Inhibitor - +

LDR 1536-well qHTS Protocol			
Step	**Parameter**	**Value**	**Description**
1	Reagent	5 uL	250 cells/well
2	Compound	23 nL	46 uM – 0.6 nM
3	Time	30 hr	37°C incubation, 5 % CO_2
4	Aspirate	-4 uL	Remove medium
5	Reagent	6 uL	PBS
6	Aspirate	-4 uL	Remove PBS
7	Reagent	6 uL	PBS
8	Detector	EX 488	Acumen reader

Protocol Notes	
Step	**Notes**
1	1536-well TC treated black, clear-bottom plate. Media contained DMEM,10% FBS, Nonessential Amino Acids, 1 mM Sodium Pyruvate, Penicillin & Streptomycin
2	Libraries tested: Molecular Libraries SMR & NCGC Exploratory Collection- LOPAC 1280, Tocris, Prestwick, Pharmacopeia, TimTec, BUCMLD
3	
4	
5	
6	
7	
8	Acumen enumerated # cells/well- objects sized 20-120 um in X and Y dimension

Figure 1: LDR assay and protocol. *Top*, In the LDR assay, a GFP reporter, controlled by the normally constitutively active CMV reporter, is integrated in a transcriptionally silent genomic region in mouse C127 cells. Addition of a histone deacetylase (HDAC) inhibitor relieves transcriptional repression and permits induction of GFP. The LDR assay was used to screen for small molecules that derepress transcription of this reporter. *Bottom*, the protocol used to screen the LDR assay is shown.

Identification of epigenetic modulators: The LDR assay was screened in 1536 well plate format against 69,128 compounds using a qHTS method that assays compounds at seven or more concentrations. Four hundred and eleven compounds (0.6% of the library) were found as activators that induced GFP expression in LDR cells. Of these, 14 and 51 compounds were associated with complete (Class 1) and partial (Class 2) titration-response curves, respectively, while the remaining 346 showed activity only at the highest tested concentration of 46 uM. Nine actives showed half maximal activity concentrations (AC50) of 1 uM or lower (Table 2).

qHTS summary of assay results:

	Assay Optimization	***Validation***	***qHTS***
Materials Received 6/27/05	11/2/05	11/9/05	12/27,29/05 1/10-14/06
Screening System	Kalypsys offline DMSO	Kalypsys offline LOPAC	Kalypsys robot
Plates Screened	2	10	422
Average Z'	0.52	0.69	0.23
Average S:B Ratio	40 +/- 3	143 +/- 51	48 +/- 18
# Wells	3,072	15,360	646,656
# Compounds	N/A	1,280	69,128
# Actives (% Library)	N/A	6 (0.5%)	411 (0.6%)
# Quality Actives (% library)	N/A	1 (0.08%)	49 (0.07%)
Quality Actives <1 uM EC50	N/A	1	4
Quality Actives 1-10 uM EC50	N/A	2	28

Table 1: Performance of LDR assay for assay optimization, validation, and qHTS steps. N/A, not applicable.

	Curve Class					
AC50 (uM)	1.1	1.2	2.1	2.2	3	Total
<1	3	1	0	1	4	9
>1 to 10	7	1	20	9	124	161
>10	1	1	15	6	218	241
Total	11	3	35	16	346	411
% library	0.016%	0.004%	0.051%	0.023%	0.503%	0.598%

Table 2: Curve class and potency distribution of LDR actives. Table adapted from (ref 1).

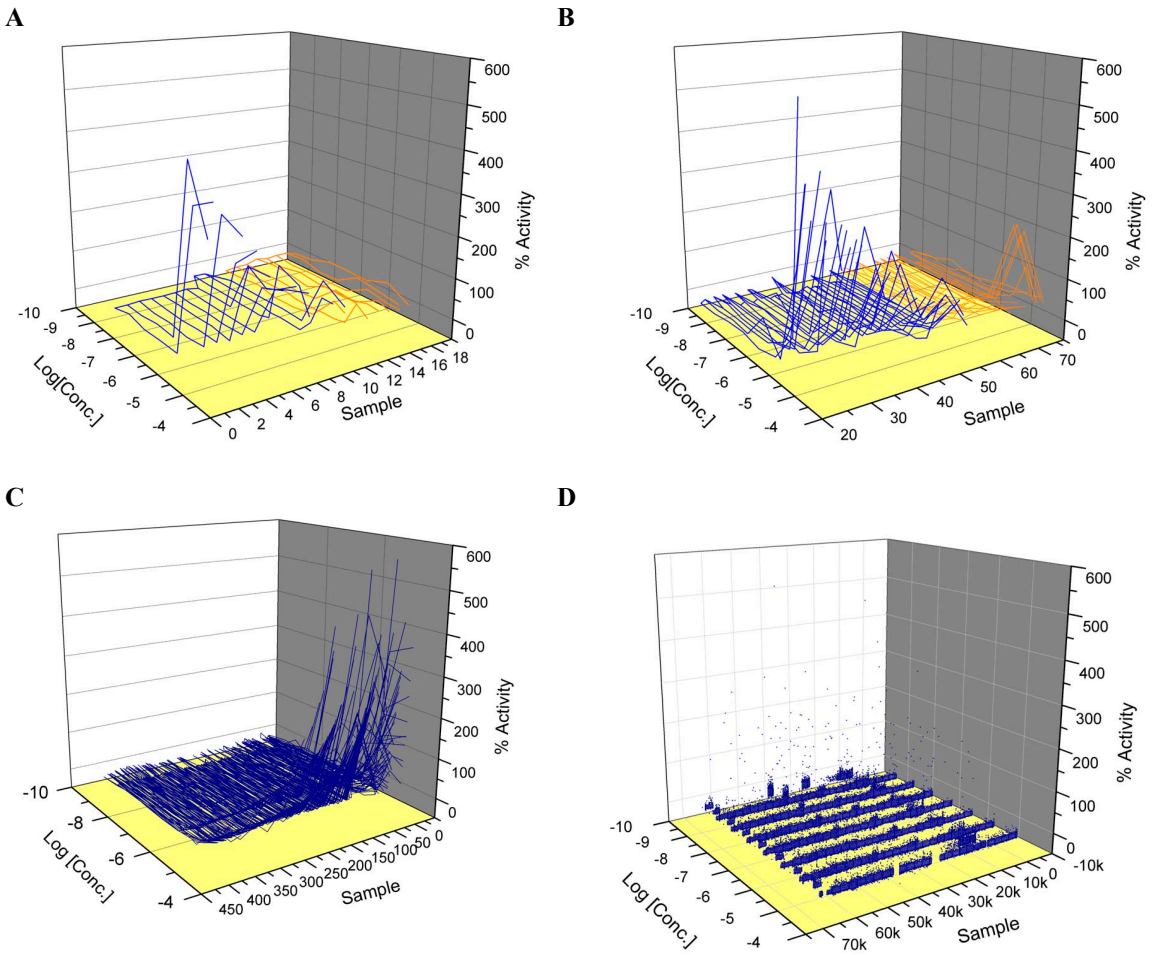


Figure 2: Activators from qHTS data (AID 597) binned into curve classifications 1-4 based on curve-fitting classification criteria. A) Class 1a (navy) and class 1b (orange) curves. B) Class 2a (navy) and class 2b (orange) curves. C) Class 3 curves (navy). D) Data with no apparent dose-response, curve class 4 (navy points).

SAR of series:
Class 1 and 2.1 actives were clustered and maximal common substructures (MCS) were extracted from clusters containing ≥ 3 actives. Each MCS was used to search the entire library to recover all analogs, including inactives. In addition, the core structure for each Class 1.1 compound was searched against the collection to find all related structures. The combined approaches yielded 6 series. 13 qHTS actives, representing five of the six series and two singletons, a combinatorial library containing the sixth series, and 35 commercially available analogs were chosen. All were counter screened against the parental C127 cells (which do not contain the GFP transgene) to identify fluorescent compounds, as well as retested on LDR cells. These studies indicated that two series were fluorescent false positives, and of the four nonfluorescent series, three confirmed activity in LDR cells and one did not show repeatable activity and was deemed inconclusive (Table 3).

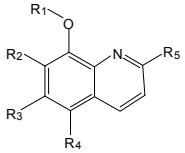
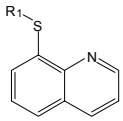
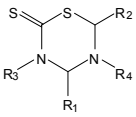
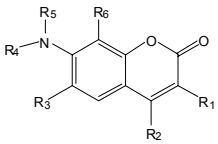
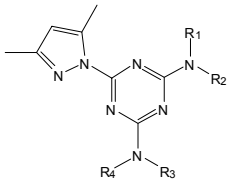
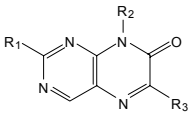
	Series	Structural Class	% Active Samples (# Tested)[1]		Potency Range, uM[1]	
Active	1		15% (143) 80% (5) 50% (24)	qHTS Retest Analogs	3-32 5-36 3- >46	qHTS Retest Analogs
Active	2		57% (7) 100% (2) 33% (3)		0.6-32 7-9 7	
Active	3		40% (10) 100% (1) 33% (3)		5-32 8 7	
Fluorescent	4		21% (33) 0% (1)[2] -		0.2-32 - -	
Inconclusive Activity	5		43% (7) 0% (6)[3] 0% (6)		5-20 - -	
Fluorescent	6		2% (1000)[2] - -		0.8-6 - -	

Table 3: Activity and structure of initial LDR series. [1]For each series, percentage and number (in parentheses) of qHTS actives (top row), retest compounds (middle row), and analogs (bottom row). [2]Fluorescent. [3]Compound activity was not repeatable. Table adapted from (ref 1).

Probe Characterization

Representatives from series 1-4 were characterized further in parental C127 and LDR cells. When tested on C127 cells, only compound 4 was active, indicating this compound was a fluorescent false positive (Figure 3). As a further counter screen to identify potential fluorescent false positives, compound-treated LDR cells were assayed for nuclear translocation of the GFP reporter. In LDR cells, GFP is fused to a glucocorticoid estrogen receptor chimeric protein (GFP-GER) that is retained predominantly in the cytosol by the glucocorticoid receptor portion. However, upon estradiol binding to the ligand binding domain of the estrogen receptor portion, GFP-GER undergoes nuclear translocation (2). The cytosol-to-nuclear translocation of GFP-GER provided an easy means to confirm that the fluorescence induced by active compounds in the LDR cells arose from the expression of the GFP-GER reporter. LDR cells were treated with compounds or vehicle, incubated overnight at 37 °C, and the following day imaged by fluorescent microscopy, before and after estradiol stimulation. Cells treated with 200nM TSA to induce GFP-GER showed cytosolic fluorescence that became nuclear after estradiol addition (Figure 4). In contrast, LDR cells treated with the fluorescent but inactive compound 4 showed cytosolic fluorescence in either the presence or absence of estradiol stimulation. Treatment of LDR cells with actives from Series 1, 2 or 3 resulted in a predominantly cytosolic signal that became clearly nuclear in the presence of estradiol (Figure 3), supporting the hypothesized induction of induce GER-GFP expression by these compounds. Compounds 1a-e, 2a-b, and 3a-b retested in LDR cells (Figure 3), indicating these were confirmed actives.

The confirmed actives were tested in several secondary assays: inhibition of HDAC activity, derepression of methylated gene expression, and killing of lung cancer lines. None of the compounds tested inhibited HDAC activity in HeLa cell extracts at 48 uM, suggesting these series are not HDAC inhibitors. NSC3852 (5-nitroso-8-quinolinol) is a reported HDAC inhibitor (3) and shares the 8-hydroxy quinoline core of Series 1. While this molecule induced GFP-GER with a 2.8 uM EC50, it did not inhibit HDAC activity in HeLa extracts at 48 uM (Figure 5A). Though NSC3852 could inhibit HeLa HDAC activity by 80 % at 190 uM, it also inhibited an unrelated protease enzyme assay by 40 % (data not shown), indicating some nonspecific activity at this concentration. These results suggest that Series 1-3 are not general HDAC inhibitors, but rather may target different epigenetic enzymes or specific HDACs of low abundance in HeLa or LDR extracts.

We next ascertained whether the LDR actives could reactivate the expression of endogenous genes silenced by promoter methylation. Human non-small cell lung cancer H358 cells harbor methylated CpG islands at the CDH13 and p16 promoters, with the latter being densely methylated and fully silenced by this modification (4,5). H358 cells were incubated with compounds for 3 days, and CDH13 and p16 transcript levels were measured by real time quantitative RT PCR. The HDAC inhibitors, depsipeptide and TSA, and the DNMT inhibitor, 5-azadeoxycytidine, reversed CDH13 silencing by 10 to 1000 fold, while only 5-azadeoxycytidine reactivated p16 expression (Figure 5B and C). Nicotinamide, an inhibitor of sirtuins (6), and Series 2 and 3 compounds did not derepress either gene. Like depsipeptide and TSA, Series 1 compounds induced CDH13 but not p16 gene expression. Of this series, NSC3852 was the most potent, inducing expression 85 fold over basal levels while the others induced expression by 4 to 12 fold. These results indicate the LDR actives do not behave as DNMT inhibitors to derepress transcription of both p16 and CDH13 genes. To test whether the LDR actives were selective against tumor cells, we tested two non-small cell lung cancer lines and their matched normal bronchial epithelial cells, both derived from

patient samples (see Methods for cell line development details). After four days of treatment, TSA killed both NSCLC lines and one normal line with 0.1-0.4 uM IC50, but did not reduce the viability of normal line 2 at concentrations up to 0.4 uM (Figure 5D). One active and one inactive compound were tested from Series 2 and 3, all of which showed little to no activity (Table 4). For one matched set, 2a decreased viability in both tumor and normal lines by about 50% at 20 uM. Of the three series 1 compounds tested, 1a and 1b were potent and selective for both tumor lines (Figure 6, Table 4). 1a was 10- to 20-fold selective for the tumor lines with an IC50 between 0.2-0.3 uM, while 1b was 7- to 12-fold selective with an IC50 of about 1 uM. 1c displayed 3- to 10-fold selectivity for the normal lines with a potency of 1-2 uM.

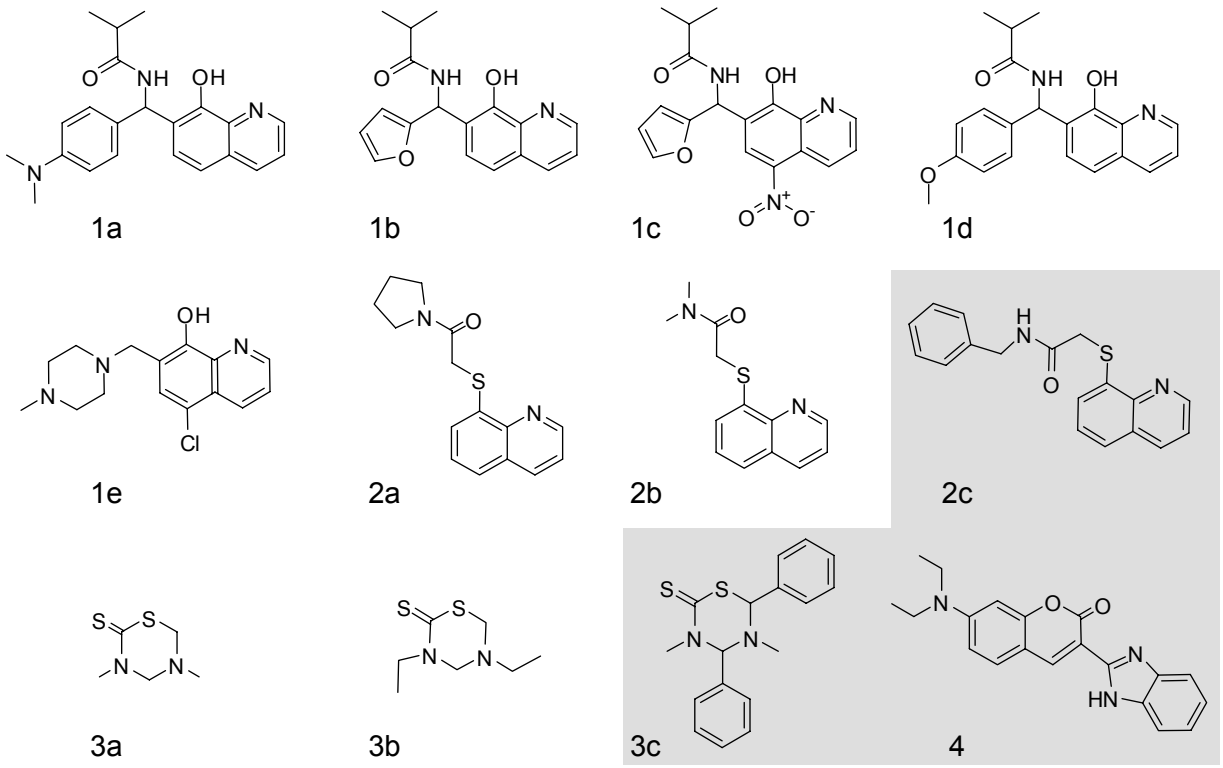


Figure 3: Selected compounds from each of the LDR probe series 1-3 and an inactive fluorescent compound (4). Compounds in grey shading are inactive. Figure from (ref 1).

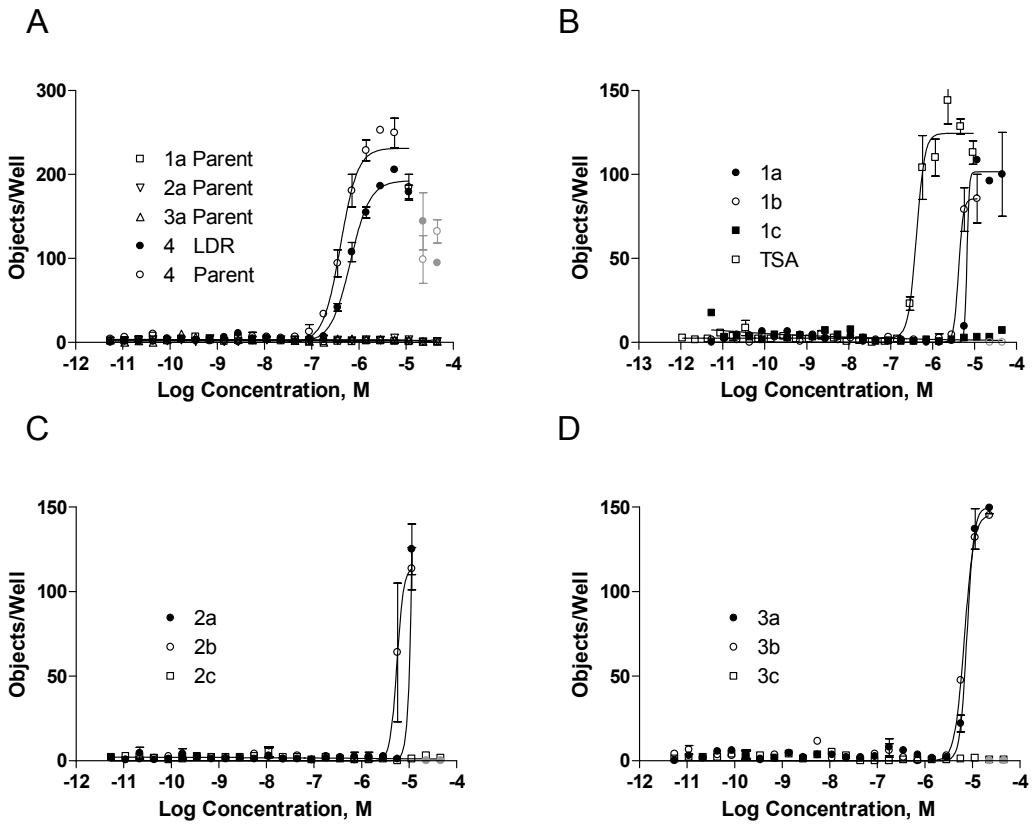


Figure 4: Activities of selected compounds on LDR and parental cells. Parental C127 (A) or LDR (A–D) cells were incubated with TSA or compounds from series 1 to 4 at the indicated concentrations for 30 h and washed with PBS before cell objects were enumerated by a laser-scanning microplate cytometer. Only compound 4 was active in parental C127 cells, indicating that this was a fluorescent molecule. Grayed symbols are masked data points. Compounds in grey shading are inactive. Figure and text from (ref 1).

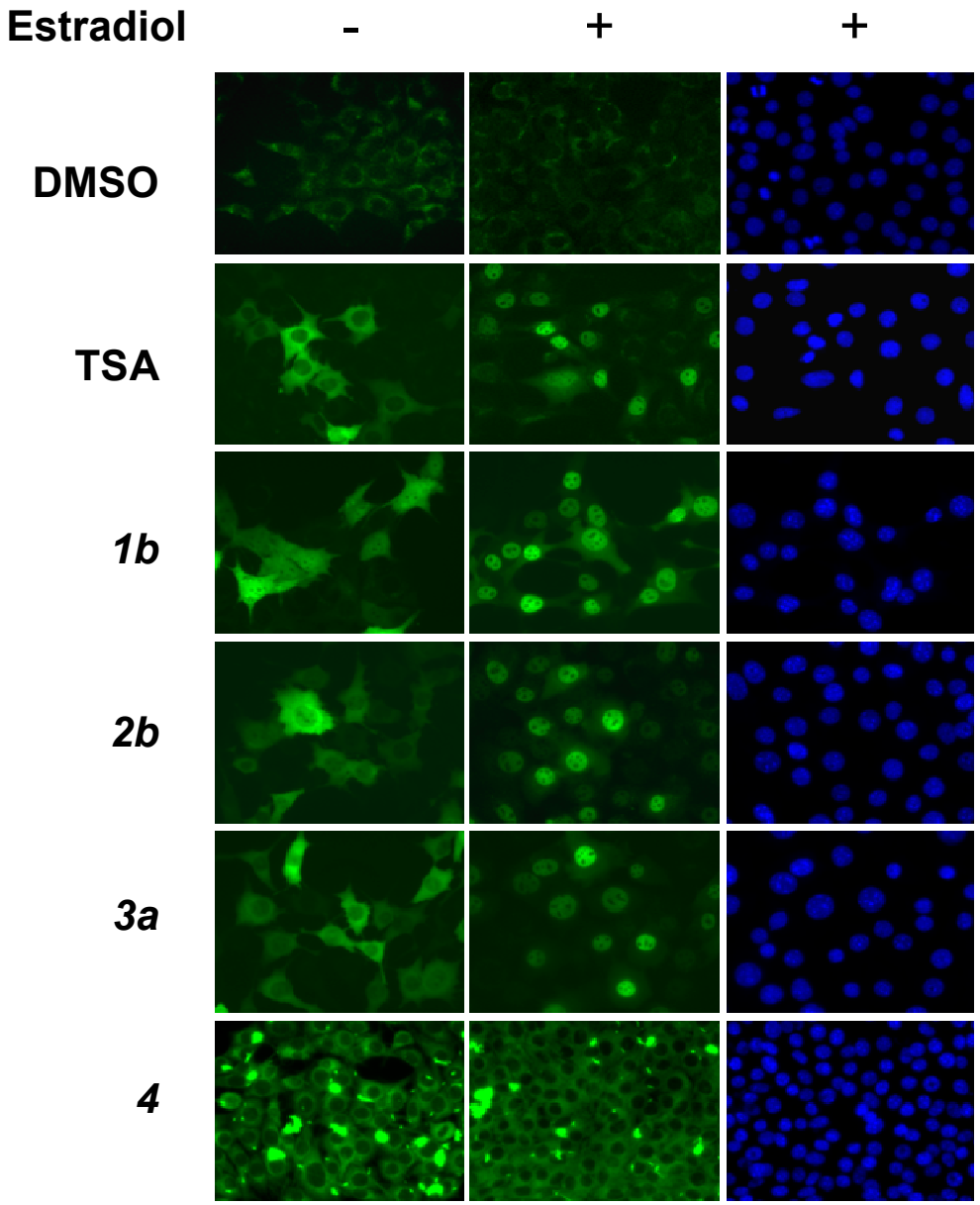


Figure 5: Nuclear localization of GFP–GER in compound-treated LDR cells on the addition of estradiol. LDR cells were incubated for 24 h at 37 C after treatment with vehicle, 200 nM TSA, or 10 uM compound representative of four LDR series. Following the addition of 2 lM estradiol, cells were incubated for 6 to 12 h at 37 C, fixed, stained with Hoescht to visualize nuclei, and imaged by fluorescent microscopy. In cells treated with active compounds, cytosolic GFP–GER underwent nuclear translocation on the addition of estradiol. The cytosolic fluorescence in cells incubated with DMSO or an inactive (4) did not translocate to the nucleus on estradiol stimulation, indicating that the signal did not derive from GFP–GER. Figure and text from (ref 1).

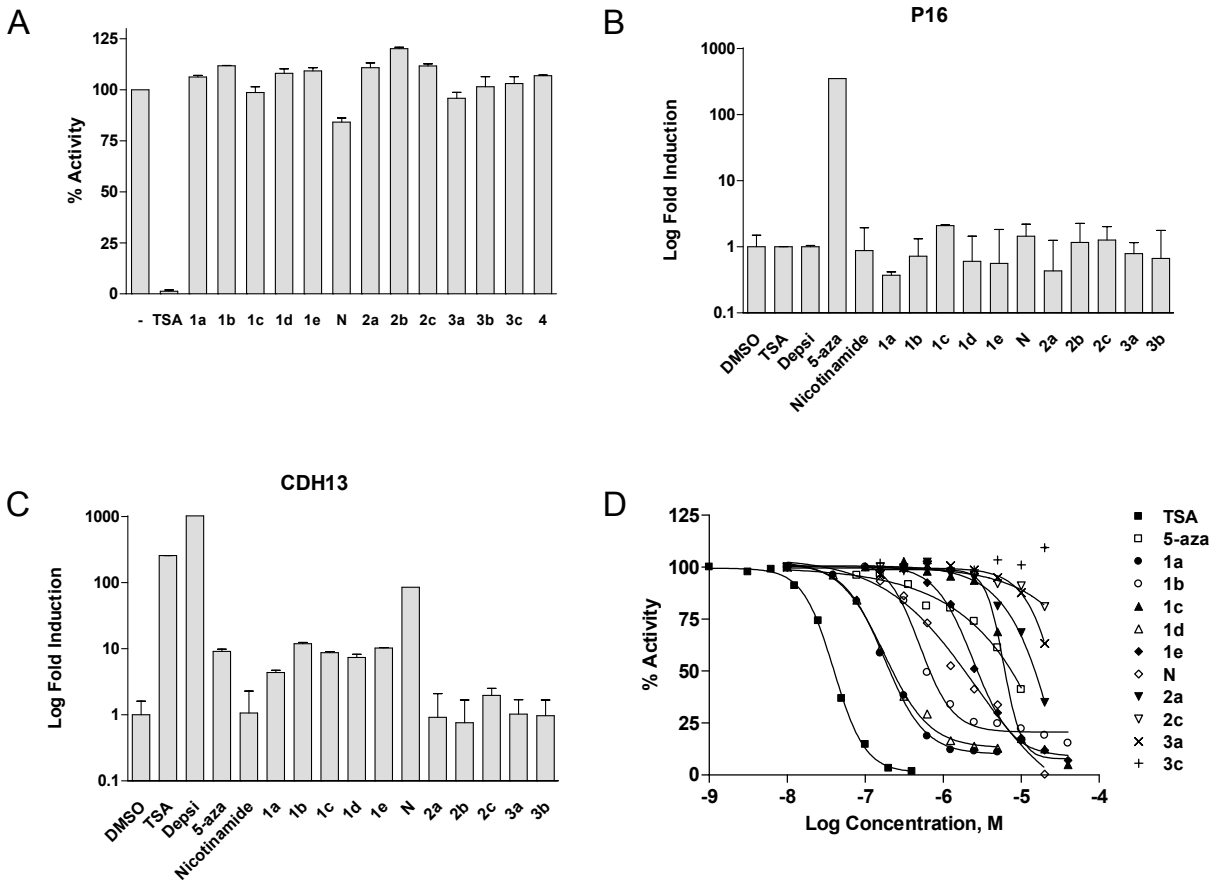


Figure 6: Effect of LDR compounds on HDAC activity, gene expression, and H358 viability. (A) HeLa nuclear extracts and acetylated profluorescent substrate were incubated with vehicle, 0.5 uM TSA, or 48 uM indicated LDR compound for 20 min at ambient temperature, and fluorescence was measured. Background-subtracted activity is expressed as a percentage of uninhibited extract, and the results of two independent experiments are shown. N, NSC3852. (B,C) H358 cells were treated with 10 uM compound for 3 days and RNA extracted, reverse transcribed, and subjected to quantitative PCR to detect levels of CDH13 (B) and p16 (C) expression. Error bars represent the standard deviation from triplicate wells. (D) H358 cells were incubated with the indicated concentrations of compounds for 4 days, and viability was measured using MTS. Data are normalized to the untreated control (100% viability). Vehicle controls showed 100% viability. Error bars represent the standard deviation from four to eight wells. Figure and text from (ref 1).

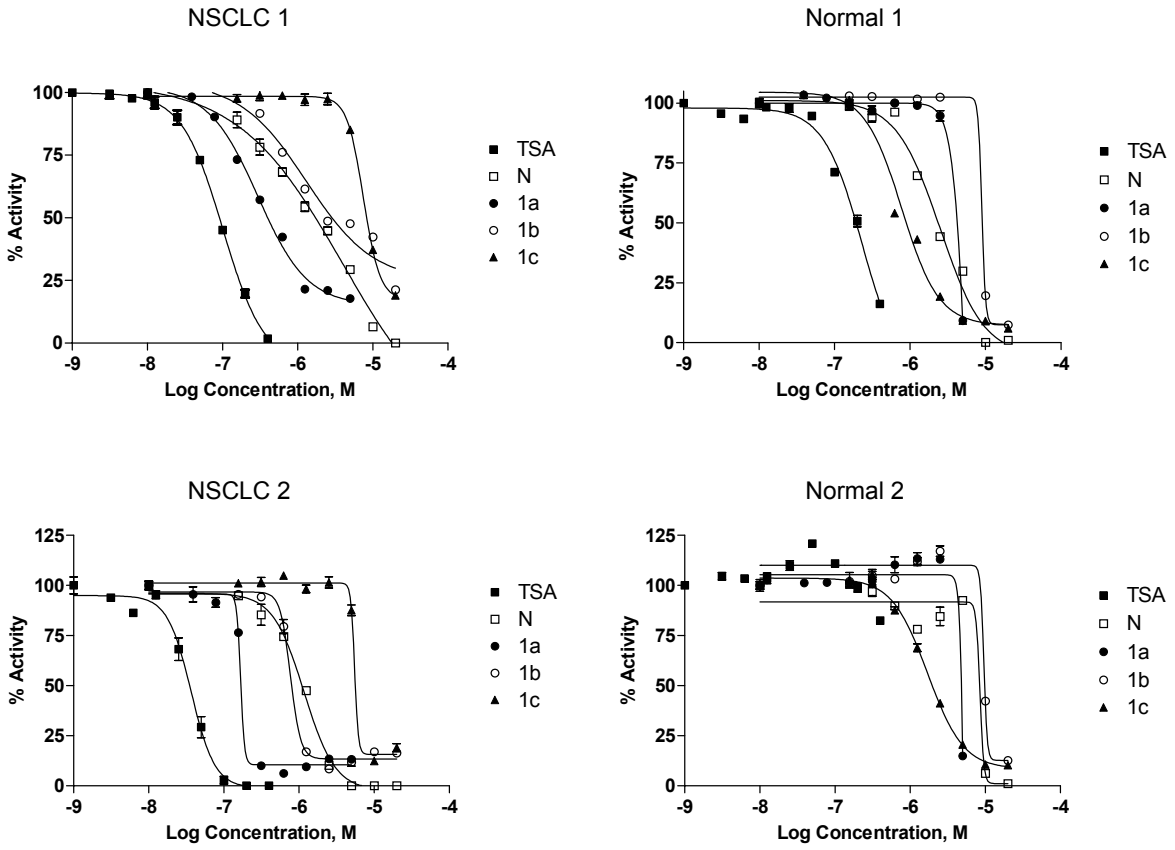


Figure 7: Effect of LDR compounds on matched patient-derived lung tumor and normal cells. Matched NSCLC and normal cells derived from two patients were treated with the indicated concentrations of compounds for 4 days, and viability was measured using MTS. Vehicle controls showed 100% viability. Error bars represent the standard deviation from four wells. N, NSC3852. Figure and text from (ref 1).

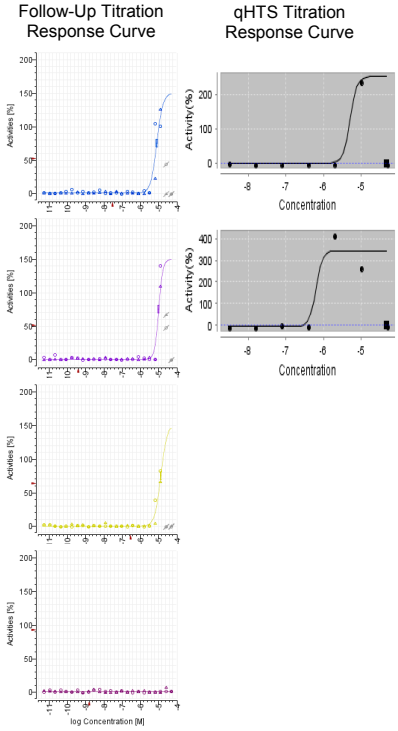
LDR	H358	NSCLC 1	Normal 1	NSCLC 2	Normal 2

	EC50 (uM)	CDH131	IC50 (uM)	IC50 (uM)	IC50 (uM)	IC50 (uM)	IC50 (uM)
TSA	0.41	256	0.04	0.1	0.23	0.37	-
1a	8.1	4	0.18	0.3	3	0.17	5.5
1b	4.6	12	0.5	1.3	9	0.77	9.6
1c	-2	9	0.6	7.5	0.78	5.5	1.7
1d	7.8	7	0.1	ND	ND	ND	ND
1e	6.1	10	2.4	ND	ND	ND	ND
N	2.8	85	0.26	4	2.5	1.2	8.5
2a	8.9	1	20	20	20	-	-
2b	7	1	ND	ND	ND	ND	ND
2c	-	2	-	-	-	-	-
3a	7.9	1	>20	-	-	-	-
3b	7	1	ND	ND	ND	ND	ND
3c	-	1	-	-	-	-	-

Table 4. Summary of biological activities of selected LDR compounds. [1]Fold induction at 10 uM. [2]LDR positive by fluorescent microscope. -, no activity; ND, not determined; N, NSC3852. Table and text from (ref 1).

Series 2

Structure	MW	PubChem CID	Followup AC50 (uM)	qHTS AC50 (uM)	qHTS Curve Class	Sample Supplier	QC Method	% Purity
NCGC00054838-01	246	851301	7.0	5.0	3	DPISMR	LC-MS-ELS	98
NCGC00059731-01	272	975656	8.9	0.6	1.1	DPISMR	LC-MS-ELS	98
NCGC00161905-01	245	2947668	7.4			Chembridge	UV & ELDS	>90
NCGC00161902-01	294	673932	inact			Chembridge	UV & ELDS	>90



Has this compound been provided to the MLSMR?

Yes, NCGC00059731 (Series 2) has been provided to the MLSMR.

Canonical SMILES: C1CCN(C1)C(=O)CSC2=CC=CC3=C2N=CC=C3 (NCGC00059731)

InChI: InChI=1/C15H16N2OS/c18-14(17-9-1-2-10-17)11-19-13-7-3-5-12-6-4-8-16-15(12)13/h3-8H,1-2,9-11H2 (NCGC00059731)

Description of secondary assays used to optimize/characterize probe structure:

AID 890 Assay Overview:

The Locus Derepression assay detects the derepression of a GFP reporter that is stably integrated in a region of the genome of murine c127i mammary cells that is presumably silenced. GFP transcription in this construct is controlled by a CMV promoter, which normally is strong and constitutively active. However, this line was selected for lack of constitutive expression of the GFP protein. GFP production can be induced by incubating the cells with histone deacetylase or DNA methyltransferase inhibitors. Compounds that cause derepression of the locus to express GFP are identified by enumerating GFP positive cells using a laser-scanning microplate cytometer.

AID 890 Assay Protocol Summary:

Two hundred and fifty cells in 5 uL per well were dispensed into black, clear-bottom 1536-well plates. Twenty-three nL compound was transferred to the assay plate and cells were incubated 30 hours at 37 C. Medium was removed, cells were washed two times with PBS, and GFP-positive cells were enumerated as fluorescent objects between the sizes of 20 to 120 um width and depth using a laser-scanning microplate imager (Acumen Explorer Ex3, TTP Labtech)

AID 990 Assay Overview:

This assay is a counter screen for the Locus Derepression (LDR) assay (PubChem AID 597). The LDR assay detects the derepression of a GFP reporter that is stably integrated in a region of the genome of murine c127i mammary cells that is presumably silenced. Compounds that cause derepression of the locus to express GFP are identified by enumerating GFP positive cells using a laser-scanning microplate cytometer. However, fluorescent inactive compounds that bind or enter LDR cells might be scored as active in this assay. To counter screen such fluorescent false positives, compounds were tested on parental c127i cells that do not contain the GFP reporter. and fluorescent cells were enumerated using a laser-scanning microplate cytometer. Compounds active in the parental c127i cells are likely fluorescent.

AID 990 Assay Protocol Summary:

Two hundred and fifty cells in 5 uL per well were dispensed into black, clear-bottom 1536-well plates. Twenty-three nL compound was transferred to the assay plate and cells were incubated 30 hours at 37 C. Medium was removed, cells were washed two times with PBS, and fluorescent cells were enumerated as objects between the sizes of 20 to 120 um width and depth using a laser-scanning microplate imager (Acumen Explorer Ex3, TTP Labtech).

AID 1035 Assay Overview:

This secondary assay characterizes selected actives identified in the qHTS of the Locus Derepression (LDR) assay (PubChem AID 597). The LDR assay detects the derepression of a

GFP reporter that is stably integrated in a region of the genome of murine c127i mammary cells that is presumably silenced. Compounds that cause derepression of the locus to express GFP are identified by enumerating GFP positive cells using a laser-scanning microplate cytometer. Selected LDR actives were assayed for inhibition of histone deacetylase (HDAC) activity in HeLa nuclear extracts using a HDAC fluorometric kit (AK-500, Biomol International LP). This assay uses a substrate containing an acetylated lysine side chain that is deacetylated by HDAC enzymatic activity to generate a fluorophore.

AID 1035 Assay Protocol Summary

HeLa nuclear extracts and acetylated profluorescent substrate were incubated with vehicle, 0.5 uM TSA, or 48 uM indicated LDR compound for 20 min at ambient temperature and fluorescence was measured. Background-subtracted activity was expressed as a percent of uninhibited extract. Tests were performed in 25 uL volume in 384-well black plates using the HDAC assay kit AK-500 (Biomol International LP).

AID 1036 and 1037 Assay Overview:

This secondary assay characterizes selected actives identified in the qHTS of the Locus Derepression (LDR) assay (PubChem AID 597). The LDR assay detects the derepression of a GFP reporter that is stably integrated in a region of the genome of murine c127i mammary cells that is presumably silenced. Compounds that cause derepression of the locus to express GFP are identified by enumerating GFP positive cells using a laser-scanning microplate cytometer. Selected LDR actives were assayed for killing of NSCLC 1 and NSCLC 2, patient-derived nonsmall cell lung carcinoma lines. Known epigenetic drugs, like histone deacetylase inhibitors, can block cancer cell growth. NSCLC 1 cell viability was measured using Cell Titer 96 AQueous One (Promega), an assay which detects the ability of metabolically active cells to reduce a tetrazolium salt substrate (MTS) into a soluble formazan product that can be measured by colorimetry.

AID 1036 and 1037 Assay Protocol Summary

NSCLC 1 cells were plated in 96-well flat bottom tissue culture dishes (Corning) and grown overnight at 37 C and 5% CO2, then treated with compounds, incubated 4 days, and assayed for viability using the Cell Titer 96 AQueous One kit (Promega) according to the manufacturer's protocol. Absorbance at 490 nm and 650 nm (reference) was measured by a Spectra Max (Molecular Devices). Data were normalized to the untreated control (100% viability). Each cell line was tested in one or two independent assays, each containing 4 or 8 replicates.

AID 1038 and 1039 Assay Overview:

This secondary assay characterizes selected actives identified in the qHTS of the Locus Derepression (LDR) assay (PubChem AID 597). The LDR assay detects the derepression of a GFP reporter that is stably integrated in a region of the genome of murine c127i mammary cells that is presumably silenced. Compounds that cause derepression of the locus to express GFP are identified by enumerating GFP positive cells using a laser-scanning microplate cytometer. Selected LDR actives were assayed for killing of Normal 1 and Normal 2, patient-derived normal bronchial epithelial lines matched to NSCLC 1 and NSCLC 2, respectively, nonsmall cell lung carcinoma lines from the same patient. The Normal 1 and 2

lines assay the cytoxicity of test compounds on normal lung cells. Normal 1 and 2 cell viability was measured using Cell Titer 96 AQueous One (Promega), an assay which detects the ability of metabolically active cells to reduce a tetrazolium salt substrate (MTS), into a soluble formazan product that can be measured by colorimetry.

AID 1038 and 1039 Assay Protocol Summary

Normal 1 cells were plated in 96-well flat bottom tissue culture dishes (Corning) and grown overnight at 37 C and 5% CO2, then treated with compounds, incubated 4 days, and assayed for viability using the Cell Titer 96 AQueous One kit (Promega) according to the manufacturer's protocol. Absorbance at 490 nm and 650 nm (reference) was measured by a Spectra Max (Molecular Devices). Data were normalized to the untreated control (100% viability). Each cell line was tested in one or two independent assays, each containing 4 or 8 replicates.

AID 1041 Assay Protocol Summary
This secondary assay characterizes selected actives identified in the qHTS of the Locus Derepression (LDR) assay (PubChem AID 597). The LDR assay detects the derepression of a GFP reporter that is stably integrated in a region of the genome of murine c127i mammary cells that is presumably silenced. Compounds that cause derepression of the locus to express GFP are identified by enumerating GFP positive cells using a laser-scanning microplate cytometer. Selected LDR actives were assayed for the induction of p16 gene expression, a gene silenced by dense methylation of its promoter. Treatment of cells with DNA methyltransferase inhibitors like 5-azadeoxycytidine induce p16 gene expression. Compounds active in this assay may affect components involved in DNA methylation or gene transcription.

AID 1041 Assay Protocol Summary

Exponentially growing H358 non-small cell lung cancer cells were treated daily with fresh media containing the indicated concentrations of compounds for 72 hr. Cellular RNA was extracted, quantified, DNAse treated and reverse transcribed. The resulting cDNA was amplified in TaqMan real time quantitative PCR assays (Applied Biosystems) containing validated primers specific for p16 (Hs00233365_m1). Reactions were performed on an ABI Prism 7900HT, with an initial 2 min preincubation at 50 C, followed by 10 min at 95 C and then 40 cycles of 95 C for 15 seconds and 60 C for 1 min. GAPDH and 18S ribosomal RNA were used as references. Gene expression was measured by fold-induction over control DMSO-treated samples (36-37 average cycle time). Increases corresponded to at least 2-3 cycle time differences over DMSO controls, which were in the measurable range. Decreases in expression were considered insignificant as measurement of the DMSO samples were at the low limit of detection. Reactions were run in triplicate and error bars represent standard deviations.

AID 1042 Assay Protocol Summary

This secondary assay characterizes selected actives identified in the qHTS of the Locus Derepression (LDR) assay (PubChem AID 597). The LDR assay detects the derepression of a GFP reporter that is stably integrated in a region of the genome of murine c127i mammary cells that is presumably silenced. Compounds that cause derepression of the locus to express GFP are identified by enumerating GFP positive cells using a laser-scanning

microplate cytometer. Selected LDR actives were assayed for the induction of CDH13 gene expression, a gene silenced by methylation of its promoter. Treatment of cells with DNA methyltransferase inhibitors, like 5-azadeoxycytidine, or with HDAC inhibitors, like trichostatin A, induce CDH13 gene expression. Compounds active in this assay may modulate components involved in DNA methylation, histone acetylation or gene transcription.

AID 1042 Assay Protocol Summary

Exponentially growing H358 non-small cell lung cancer cells were treated daily with fresh media containing the indicated concentrations of compounds for 72 hr. Cellular RNA was extracted, quantified, DNAse treated and reverse transcribed. The resulting cDNA was amplified in TaqMan real time quantitative PCR assays (Applied Biosystems) containing validated primers specific for CDH13 (Hs00169908.m1). Reactions were performed on an ABI Prism 7900HT, with an initial 2 min preincubation at 50 C, followed by 10 min at 95 C and then 40 cycles of 95 C for 15 seconds and 60 C for 1 min. GAPDH and 18S ribosomal RNA were used as references. Gene expression was measured by fold-induction over control DMSO-treated samples (36-37 average cycle time). Increases corresponded to at least 2-3 cycle time differences over DMSO controls, which were in the measurable range. Decreases in expression were considered insignificant as measurement of the DMSO samples were at the low limit of detection. Reactions were run in triplicate and error bars represent standard deviations.

AID 1043 Assay Overview:

This secondary assay characterizes selected actives identified in the qHTS of the Locus Derepression (LDR) assay (PubChem AID 597). The LDR assay detects the derepression of a GFP reporter that is stably integrated in a region of the genome of murine c127i mammary cells that is presumably silenced. Compounds that cause derepression of the locus to express GFP are identified by enumerating GFP positive cells using a laser-scanning microplate cytometer. Selected LDR actives were assayed for killing of H358 non-small cell lung carcinoma (NSCLC) cells. Certain epigenetic drugs, like histone deacetylase inhibitors, can block cancer cell growth. H358 cell viability was measured using Cell Titer 96 AQueous One (Promega), an assay which detects the ability of metabolically active cells to reduce a tetrazolium salt substrate (MTS), into a soluble formazan product that can be measured by colorimetry.

AID 1043 Assay Protocol Summary

Viability assays: H358 cells were plated in 96-well flat bottom tissue culture dishes (Corning) and grown overnight at 37 C and 5% CO2, then treated with increasing concentrations of compounds, incubated for four days, and assayed for viability using the Cell Titer 96 AQueous One kit (Promega) according to the manufacturer's protocol. Absorbance at 490 nm and 650 nm (reference) was measured by a Spectra Max (Molecular Devices). Data were normalized to the untreated control (100% viability). Each cell line was tested in one or two independent assays, each containing 4 or 8 replicates.

Known probe properties:

Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool:

NCGC00059731:
Molecular Weight 272.36534 [g/mol]
Molecular Formula C15H16N2OS
XLogP 2.4
H-Bond Donor0
H-Bond Acceptor 2
Rotatable Bond Count 3
Exact Mass 272.098334
MonoIsotopic Mass 272.098334
Topological Polar Surface Area 33.2
Heavy Atom Count 19

Compound is prepared in DMSO at 10 mM stock concentration. Assays described above have 0.6% DMSO final concentration in buffer.

Probe availability:

Aliquots of NCGC00059731 (Series 2) are available from the NCGC upon request.

Appendices

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