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A Quantitative High-Throughput Screen Identifies Potential Epigenetic Modulators of Gene Expression

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Abstract

Epigenetic regulation of gene expression is essential in embryonic development and contributes to cancer pathology. We used a cell-based imaging assay that measures derepression of a silenced GFP reporter to identify novel classes of compounds involved in epigenetic regulation. This Locus Derepression (LDR) assay was screened against a 69,137-member chemical library using quantitative high-throughput screening (qHTS), a titration-response method that assays compounds at multiple concentrations. From structure-activity relationships of the 411 actives recovered from the qHTS, six distinct chemical series were chosen for further study. Forty-eight qHTS actives and analogs were counter screened using the parental line of the LDR cells, which lack the GFP reporter. Three series, 8-hydroxy quinoline, quinoline-8-thiol and 1,3,5-thiadiazinane-2-thione, were not fluorescent and re-confirmed activity in the LDR cells. The three active series did not inhibit histone deacetylase activity in nuclear extracts or reactivate the expression of the densely methylated p16 gene in cancer cells. However, one series induced expression of the methylated CDH13 gene and inhibited the viability of several lung cancer lines at submicromolar concentrations. These results suggest that the identified small molecules act on epigenetic or transcriptional components and validate our approach of using a cell-based imaging assay in conjunction with qHTS.

Keywords

epigenetic; small molecule; GFP; HTS; HDAC; cell assay; cancer

Introduction

Epigenetic control of gene expression is important in normal and disease processes including differentiation and cancer (reviewed in [1]) and is mediated in part by the methylation status of DNA at CpG sites as well as by chemical modifications of histones including acetylation, methylation, phosphorylation and ubiquitination (reviewed in [2]). In general, unmethylated DNA and acetylated histones at promoter sites are permissive to transcriptional activity

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whereas methylated DNA and hypoacetylated histones restrict transcription. Cancer cells are characterized by global DNA hypomethylation, the hypermethylation of specific genomic regions including the promoters of tumor suppressor genes such as p16, and by changes in histone modifications [1;3;4;5]. Many of the enzymes that mediate these modifications, including DNA methyltransferases (DNMT), and histone acetyltransferases, deacetylases (HDAC), methylases, and demethylases have been identified and are being intensively studied because of their potential as therapeutic targets. Chemical tools to modulate the functions of these proteins have been identified only for DNMTs and HDACs and they are relatively limited in number, structural diversity, and enzyme class specificity.

Several known compounds inhibit HDAC activity, such as trichostatin A (TSA), butyrate, apicidin and the cyclic peptide depsipeptide, with nM to mM potencies (reviewed in [6]. These agents inhibit Class 1 and 2 HDACs and some are being evaluated in clinical trials for the treatment of leukemias and solid tumors (reviewed in [7]. Indeed, the FDA recently approved the first epigenetic drug targeting HDACs, vorinostat (SAHA), for certain cases of T-cell lymphoma [8]. Small molecule inhibitors of Sir2, a class 3 HDAC, have also been identified using yeast assays [9;10] but have not yet been actively developed for therapeutic purposes. Cytidine analogs, such as 5-aza-deoxycytidine, inhibit DNA methyltransferases (DNMT) and are antineoplastic agents used to treat myelodysplastic syndrome and certain hematopoietic malignancies though they exhibit high toxicity (reviewed in [11].

Our aim is to develop techniques to find new small molecules that directly or indirectly modulate the epigenetic control of gene transcription. Such compounds could identify novel molecular targets, define new mechanisms of epigenetic or transcriptional regulation and potentially display antitumor or other therapeutic activity. To screen for epigenetic modulators, we combined two innovative approaches: a cell-based imaging assay that monitors activation of a silenced gene reporter [12;13] and a quantitative high-throughput screening (qHTS) method that tests compounds at multiple concentrations [14]. We identified three structural classes of compounds that derepress or induce transcription of a silenced gene. The compounds do not appear to inhibit HDAC or DNMT activities, and one series exhibits selective anti-tumor activity. These small molecules may target specific transcriptional pathways or regulate new targets involved in the epigenetic control of gene expression.

Materials and Methods

Cell culture and general reagents

C127 (a kind gift of Dr. Gordon Hager) and LDR 6103 cells were maintained in DMEM with L-glutamine and high glucose, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, 1% v/v penicillin-streptomycin and 10% heat inactivated FBS. All lung cancer cells (provided by Dr. John D. Minna) were maintained in RPMI 1640 media supplemented with 10% FBS. Immortalized normal human bronchial epithelial cells (also provided by Dr. John D. Minna) were cultured in KSFM supplemented with pituitary extract and EGF, as described [15]. H358 cells have been previously described (Phelps, 1996) and are commercially available. Matched cell line pairs were established, with signed consent, from primary tumors or corresponding normal bronchial epithelial cells of two independent non-small cell lung cancer patients at the Hamon Center for Therapeutic Oncology Research (Sheridan and Minna, unpublished work). The human bronchial epithelial primary lines were then immortalized as described (Ramirez, 2004). TSA was acquired from Tocris, 5-azacytidine was purchased from SigmaAldrich, depsipeptide was the kind gift of Dr. David Schrump, and follow-up compounds were purchased from ChemDiv, Chembridge, Sigma-Aldrich, Asinex and Acros.

Preparation of compound libraries

The assay was screened against 69,137 compounds collected from the following sources with compound number in each collection indicated in parentheses; Sigma-Aldrich LOPAC collection (1280), Prestwick Chemical (1117), TimTec (280), Pharmacopeia (3000), Tocris (980), Boston University Center for Chemical Methodology and Library Development (718), the NIH Molecular Libraries Small Molecule Repository (59,783), the National Cancer Institute (1979). Compounds were prepared as previously described [14].

LDR assay and qHTS

Cells were assayed as described [13], Table 1). In brief, cells were harvested, passed through a 40 μ m filter, and suspended at 50,000 cells/mL in growth medium. Cells were seeded at 250 cells/6 μ L/well into black clear-bottom 1536-well plates using solenoid valve dispensers and compounds added by pin tool [16]. Following a 30 hr incubation at 37° C and 5% CO₂, the medium was aspirated, 6 μ L/well of PBS was added and then replaced by a second dispense of 6 μ L/well of PBS. The plates were read in the Acumen Explorer [17] using the following settings: 6 mW 488 nm laser, 660 V channel 1 PMT (500-530 nm), 1 $\times$ 8 μ m x and y scan resolution, 2.5 SD above background trigger threshold, and 10 μ m minimum and 100 μ m maximum feature size. The qHTS comprised 561 plates screened over eight days. Controls were added to each assay plate by pin tool in the following manner; sixteen 2-fold titrations in duplicate of aqueous sodium butyrate beginning at 4.25 M (columns 1 and 2), aqueous (column 3), and 4.25 M sodium butyrate (column 4).

HDAC assay

Compounds were assayed in 384-well black plates using a HDAC fluorometric kit (AK-500, Biomol International LP) according to the manufacturer's instructions except using half the volume of reagents.

qHTS Data Analysis

Screening data was corrected and normalized using Assay Analyzer (GeneData) as described [14]. Percent activity was calculated by normalization to the median values of the butyrate and aqueous controls present on each plate. For each compound, a titration response series was generated, curve-fit, and classified as described [14]. Data were deposited in Pubchem (AID 597).

Fluorescence microscopy

LDR cells were plated at 50,000 cells/well in two-well chamber slides (LabTek II) and incubated overnight at 37° C and 5% CO₂. Compounds were added from a 1000x DMSO stock for a final concentration of 20 μ M unless indicated otherwise. Following a second overnight incubation, cells were washed in PBS and imaged at 400x magnification using a Nikon Eclipse TE2000-U fluorescence microscope equipped with a CCD Roper camera. Cells were then treated for 6-12 hours with 2 μ M estradiol to induce nuclear translocation of the GFP-GER chimera. Cells were fixed in 4% paraformaldehyde, stained with 2 μ g/ml Hoescht 33342 and imaged as above. Images were processed with Metamorph software.

Quantitative PCR

Exponentially growing H358 cells were treated daily with fresh media containing the indicated concentrations of compounds for 72 hr. Cells were processed for RNA extraction using RNeasy (Qiagen). The extracted RNA was quantified, DNase treated and reverse transcribed as described [18]. The resulting cDNA was amplified in TaqMan real time quantitative PCR assays (Applied Biosystems) containing validated primers specific for CDH13

(Hs00169908.m1) and 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. Data was analyzed following the ddCt method as described [18]. 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.

Viability assays

H358 cells or matched lung cancer cell line pairs 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.

Results

The Locus Derepression (LDR) assay detects the derepression of a GFP reporter that is stably integrated in the mouse mammary carcinoma line, C127. In the vector, GFP transcription is controlled by a CMV promoter, which normally is strong and constitutively active. However, this cell line (referred to as LDR cells) was selected for lack of constitutive expression of the GFP reporter [12] presumably due to epigenetic silencing of the integration locus and/or methylation of the CMV promoter. GFP production can be induced by incubating the cells with HDAC inhibitors such as TSA or butyrate [13] or with DNMT inhibitors such as 5-aza-deoxycytidine [12] but not by general activators of transcriptional pathways such as serum, insulin or steroids (data not shown). Derepression of this reporter locus was measured by enumerating GFP-positive cells using a laser scanning microplate cytometer, the Acumen Explorer [13;17].

To find new small molecules that modulate epigenetic control of transcription, the LDR cells were screened as outlined in Table 1, against 69,137 compounds using qHTS, a method that assays compounds at multiple concentrations [14]. Following curve fit and classification of the titration-response data, 411 compounds (0.6% of the library) were found as activators that induced GFP expression (Table 2). Fourteen actives showed complete titration curves, of which 11 displayed full efficacy relative to control (response >80 %; Class 1.1 in the qHTS classification) (Inglese et al., 2006) and three showed partial efficacy (response <80 %, Class 1.2). An additional 51 compounds produced incomplete titration-response curves containing only the lower asymptote; of these, 35 displayed full efficacy (Class 2.1) and 16 showed partial efficacy (Class 2.2). The remaining 346 active compounds showed activity only at the highest concentration tested, or were associated with poorly fit curves (Class 3). Nine actives had half-maximal activity concentrations (EC₅₀) of ≤ 1 uM and 161 compounds had an EC₅₀ between 1 and 10 uM (Table 2).

To determine structure-activity relationships among the actives, compounds associated with Class 1 and 2.1 curves were clustered and maximal common substructures (MCS) were extracted from clusters containing three or more actives. Each MCS was then 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 six series (Table 3) for further investigation.

For follow-up studies, 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. These compounds were first counter screened against the parental C127 cells (which do not contain the GFP transgene) to identify fluorescent compounds. Each compound was titrated in 24 two-fold dilutions in duplicate beginning at 46 μ M while the combinatorial library was screened at the three highest qHTS concentrations (9, 2, and 0.4 μ M). The compound containing the 7-aminochromen-2-one core (series 4) and the pteridin-7-one-containing compounds from the combinatorial library (series 6) showed activity in the control cells (Figure 2A and data not shown), indicating these were fluorescent molecules that bound or permeated the cells. These series were therefore classified as false positives and eliminated from further consideration. All the other tested compounds showed no activity in the parental cells (Figure 2A and data not shown).

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 [19]. 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 3). 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 GFP-GER expression by these compounds.

Compounds that were negative in the fluorescence assays were next tested on the LDR cells. One of the two singletons showed activity upon retest of the library samples. This compound, 2-butyl-N-(3,4-dimethoxyphenyl) cyclopropanecarboxamide, displayed a poorly-fit Class 3 curve of 4 nM EC₅₀ in the qHTS that upon retest, displayed a Class 2.1 curve with a 14 μ M EC₅₀ (Table 4). Of the three compounds retested from the 1,3,5-triazine series identified in the qHTS (series 5), one compound showed no activity, and two did not show consistent activity at different times or with freshly prepared independent samples. In addition, five analogs showed no activity (Table 4). As the activity of series 5 could not be consistently reproduced, these compounds were not investigated further.

Compounds containing 8-hydroxy quinoline, quinoline-8-thiol and 1,3,5-thiadiazinane-2-thione cores (series 1-3), confirmed activity on LDR cells (Figure 2B-D, Table 3). For the 8-hydroxy quinolines (series 1), 28 unique compounds with substitutions at the R1, R2, R4, and R5 positions were tested (Table 3). Three of four qHTS actives reproduced activity with similar or lower potencies. Of the 24 analogs containing the 8-hydroxy quinoline core, seven showed Class 1.1 curves with EC₅₀ between 3 and 16 μ M, five displayed Class 2 curves of 38 μ M EC₅₀ or greater, and twelve were inactive (Table 4). Analog **1c** (Figure 1) was initially scored as inactive on LDR cells as detected by a laser-scanning imager, yet by fluorescence microscopy was active, indicating this molecule to be a weak positive (data not shown). No discernible SAR from series 1 could be ascertained.

For series 2, which contained a quinoline-8-thiol core, both qHTS actives (**2a** and **2b**, Figure 1) confirmed as independent samples having potencies of 7 and 9 μ M (Figure 2C and Table

4). The testing of three analogs indicated that a 1-(pyrrolidin-1-yl)ethanone substitution at R1 did not alter potency (Table 4) while phenyl- or benzyl-acetamide substitutions were inactive (data not shown).

Series 3 comprised two actives containing the 1,3,5-thiadiazinane-2-thione core. The 3,5-dimethyl form (**3a**, Figure 1), identified from the qHTS, and a 3,5 diethyl analog (**3b**, Figure 1) showed potencies of about 7 μ M. A second analog (**3c**, Figure 1), with phenyl substitutions at R1 and R2, was inactive (Figure 2D, Table 4).

In summary, the follow-up investigation showed that of the initial six series, three were confirmed active by testing the original and/or independent samples of library compounds, as well as their analogs. Of the remaining three series, one showed inconclusive activity, and two were false positive due to fluorescence. Eight of thirteen (62%) library compounds identified by the qHTS confirmed activity upon retest including one fluorescent compound, while three were inactive, and two were inconclusive due to inconsistent activity (Table 4). Thirty-five analogs from four series were tested and 14 (40%) were active with their potencies ranging from 3 μ M to above 46 μ M, the highest tested concentration.

Since known HDAC inhibitors such as butyrate and TSA induce GFP-GER in LDR cells [12], we examined whether the LDR actives inhibit HDAC activity *in vitro*. While 5 μ M TSA completely inhibited HDAC enzymatic activity in HeLa nuclear extracts, none of the tested actives blocked activity when assayed at 48 μ M (Figure 4A). In addition, these compounds did not block HDAC activity in whole cell extracts of LDR cells (data not shown). NSC3852 (5-nitroso-8-quinolinol) is a reported HDAC inhibitor [20] and shares the 8-hydroxy quinoline core of series 1. While this molecule induced GFP-GER with a 2.8 μ M EC₅₀ (Table 4), it did not inhibit HDAC activity in HeLa extracts at 48 μ M (4A). Though NSC3852 could inhibit HDAC activity by 80 % at 190 μ M, 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 (NSCLC) H358 cells harbor methylated CpG islands at the CDH13 and p16 promoters, with the latter being densely methylated and fully silenced by this modification [21;22]. H358 cells were incubated with compounds for 3 days and CDH13 and p16 transcript levels 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 4B and C). Nicotinamide, an inhibitor of sirtuins [23], 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.

As known epigenetic drugs inhibit cancer cell growth *in vitro* and in some cases *in vivo* [24; 25;26;27], we evaluated LDR actives for potential anti-tumor activity. H358 cells were treated with compounds for four days and viability was assessed by MTS reduction. TSA terminated cells with an IC₅₀ of 40 nM while 5-azadeoxycytidine showed incomplete killing at 10 μ M, the highest tested concentration. Series 1 compounds killed cells with IC₅₀ values ranging from 0.1 to 2.4 μ M while series 2 and 3 showed little or no effect (Figure 4D, Table 6).

To test whether the LDR actives were selective against tumor cells, we tested two NSCLC and their matched normal bronchial epithelial cells, both derived from patient samples (see

Materials and 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 μM IC_{50} but did not reduce the viability of normal line 2 at concentrations up to 0.4 μM (Figure 5). One active and one inactive compound were tested from series 2 and 3, all of which showed little to no activity (Table 6). For one matched set, **2a** decreased viability in both tumor and normal lines by about 50% at 20 μM . Of the three series 1 compounds tested, **1a** and **1b** were potent and selective for both tumor lines (Figure 5, Table 6). **1a** was 10- to 30-fold selective for the tumor lines with an IC_{50} between 0.2-0.3 μM while **1b** was 7- to 12-fold selective with an IC_{50} of about 1 μM . **1c** displayed 3- to 10-fold selectivity for the normal lines with a potency of 1-2 μM .

Discussion

In this study, we have used a cell-based imaging assay to screen for small molecules that activate expression of a silenced locus. Using qHTS, we identified six chemically distinct series of compounds that derepress or induce the GFP-GER reporter. Follow-up experiments revealed that compounds within three series were true reproducible actives, one series was inconclusive, and two were false-positive fluorescent. Compounds from each of the three active series did not inhibit HDAC enzymatic activity *in vitro* nor reactivate expression from the densely methylated p16 promoter, suggesting that these small molecules modulate other targets involved in transcriptional derepression or induction.

The percentage of qHTS actives re-testing positive was lower than expected for titration-response screening. Of the 13 qHTS actives retested, eight (62%) confirmed activity in all experiments performed, including one fluorescent compound (Table 4). This percentage was lowered because two triazine compounds (series 5) showed inconsistent activity and one showed no activity. The two triazine compounds that did not show repeatable activity in LDR cells were considered inconclusive and scored negative for retest. The inconsistent or lack of activity of series 5 and of the two other compounds that failed retests (a singleton and a series 1 compound that both showed Class 1 curves in the qHTS) may relate to the assay biology, cell state, or variability in sample impurities below the detection level of analytical compound QC but sufficient to affect the assay signal output. If the two inconsistent series 5 compounds are included as positive, the retest percentage becomes 77%, with 10 of 13 qHTS actives confirming activity in the follow up LDR assay.

To date, small molecule screens for modulators of epigenetic activity have utilized either *in vitro* enzyme assays [28;29], low-throughput yeast [9;30] or mammalian cell-based assays [31], or have measured upregulation of an active transcriptional reporter [32]. Our assay and screening approach is innovative and offers several advantages to finding new compounds and targets. As the LDR assay detects the transcriptional derepression of a silenced reporter in cells, this assay is not biased toward a particular enzyme or activity, therefore permitting the detection of new and diverse targets. Furthermore, since the LDR cells measure the derepression or induction of a silenced gene, it can potentially detect distinct regulatory activities, such as chromatin remodeling events, which would not necessarily be identified in assays measuring upregulation of an activated locus.

The qHTS method provided benefits that would not have been realized by screening this assay at a single concentration. Because the compound collection was screened at multiple concentrations, we identified 100 compounds as active that were inactive at the highest tested concentration, presumably because of cytotoxicity or compound precipitation. Screening at only one concentration entails the risk of missing bioactive molecules that are cytotoxic or insufficiently efficacious at the chosen concentration.

The LDR assay is unique in several respects. It is highly selective: only 411 actives (0.6% of the library) were identified from the qHTS, of which 14 (0.02%) displayed Class 1 curves. This selectively arose from several characteristics of the assay. First, active compounds were identified by *inducing* a signal in live cells, so potent (submicromolar IC₅₀) cytotoxic compounds, which are a frequent source of false positives in assays based on signal inhibition, were not recovered. Second, since the silencing of the GFP-GER reporter in the LDR cells is stable, the background fluorescence (i.e., “leakiness”) is very low, giving the assay a high signal to background ratio (48-fold in the qHTS). Third, fluorescent artifacts were minimized by washing the cells before imaging and by using a laser-scanning cytometer to enumerate individual cells or cell groups within each well rather than using a fluorescence plate reader, which detects a population-averaged signal of the entire well.

The steep Hill slope (≥ 3) of the titration-response curves of almost all actives is intriguing. The steep Hill slopes do not appear to arise from the design of the qHTS, as this effect was seen with known HDAC inhibitors as well as active compounds, and with testing at 24 two-fold dilutions as well as the 7 five-fold dilutions used in the qHTS (Figure 2), [13]. Hill slopes greater than 1 can indicate positive cooperativity. In the LDR assay, the high Hill values may indicate that derepression of a silenced locus may be a cooperative threshold event analogous to an all-or-none switch, in contrast to a more graded response seen with upregulation of transcriptionally active genes [33;34].

The targets of the three active LDR series are not known. The tested compounds do not appear to be HDAC inhibitors as they did not block *in vitro* HDAC activity at 48 μ M concentrations or higher. Indeed, only NSC3852, a proposed HDAC inhibitor [20] that contains the 8-hydroxy quinoline core of series 1, inhibited *in vitro* HDAC activity at 190 μ M but much of this activity appeared to be nonspecific (data not shown). NSC3852 kills MCF7 breast carcinoma cells at 2-10 μ M IC₅₀ [20] and as we show here, killed NSCLC tumor lines at 0.3-4 μ M IC₅₀ (Table 6). However, NSC3852 was a less potent and selective inhibitor of tumor cell viability than **1a** or **1d** (Figure 5, Table 6). These new actives may have greater anti-tumor potential and preferentially target cancer cells over normal cells. As several series 1 actives did not inhibit HDAC activity *in vitro* yet showed relatively potent and selective inhibition of cancer cells, we speculate that their primary targets are not HDACs.

The actives do not appear to be DNMT inhibitors either as they failed to reactivate the expression of the methylated p16 gene under conditions in which 5-azadeoxycytidine robustly induced it. Series 1 compounds induced CDH13 but not p16, similar to Class 1 and 2 HDAC inhibitors, TSA and depsipeptide. This result may indicate that series 1 actives can act as weak HDAC inhibitors in some contexts but may be stronger modulators of other epigenetic components or gene expression regulatory networks. The present data do not exclude either possibility. The reactivation of CDH13 but not p16 expression by series 1 molecules suggest however, that these agents may alter reversible epigenetic marks but not dense DNA methylation similar to the effects of known HDAC inhibitors on the T-cadherin promoter [35]. The pathways affected by series 2 and series 3 compounds remain unknown.

Compounds in the three identified series may act on other modulators of chromatin structure or transcriptional regulation. For instance, a number of enzymes have been identified that modify histones by pathways other than acetylation to regulate transcriptional activity. These modifications include histone methylation, phosphorylation, ubiquitination, sumoylation, and deimination among others (reviewed in [2]). The enzymes that add or remove such modifications could be potential targets of the small molecule actives identified by this assay. Future work will involve identifying the targets of the LDR actives, delineating their mode of action, and screening additional libraries of diverse small molecules to identify further active

series. These compounds will be useful chemical tools to understand the molecular nature of epigenetic and/or transcriptional regulatory pathways.

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Abbreviations

DMSO, dimethyl sulfoxide; GER, glucocorticoid-estrogen receptor chimera; GFP, green fluorescent protein; LDR, Locus Derepression; HDAC, histone deacetylase; TSA, Trichostatin A..

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Figure 1. Structures of selected compounds from the qHTS and follow up testing
Compounds were from three active series, 8-hydroxy quinoline (**1a-e**), quinoline-8-thiol (**2a-c**) and 1,3,5-thiadiazinane-2-thione (**3a-c**), as well as a fluorescent 7-aminochromen-2-one series (**4**). Compounds **2c**, **3c** and **4** were inactive when tested on LDR cells (grey shading).

Figure 2. 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 1-4 at the indicated concentrations for 30 hrs, washed with PBS, and cell objects enumerated by a laser-scanning microplate cytometer. Only compound 4 was active in parental C127 cells, indicating this was a fluorescent molecule. Grayed symbols are masked data points.

Figure 3. Nuclear localization of GFP-GER in compound-treated LDR cells upon addition of estradiol, an ER ligand

LDR cells were incubated for 24 hr at 37 C after treatment with vehicle, 200 nM TSA, or 10 uM compound representative of four LDR series. Following the addition of 2 uM estradiol, cells were incubated for 6-12 hr 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 upon addition of estradiol. The cytosolic fluorescence in cells incubated with DMSO or an inactive (**4**), did not translocate to the nucleus upon estradiol stimulation, indicating the signal did not derive from GFP-GER.

Figure 4. 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 μ M TSA, or 48 μ M indicated LDR compound for 20 min at ambient temperature and fluorescence was measured. Background-subtracted activity is expressed as a percent of uninhibited extract and the results of two independent experiments are shown. N, NSC3852.
 (B,C) H358 cells were treated with 10 μ M compound for three days and RNA extracted, reverse transcribed and subject to qPCR 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 four 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 5. 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 four days and viability was measured using MTS. Vehicle controls showed 100% viability. Error bars represent the standard deviation from four wells.

Table 1**qHTS protocol for LDR assay**

Step	Parameter	Value	Description
1	Reagent	5 uL	250 cells/well
2	Library Compounds	23 nL	46 uM to 0.5 nM titration series
3	Controls	23 nL	Butyrate intra-plate titration
4	Incubation Time	30 hr	Compound interaction with targets
5	Aspirate	-4 uL	Remove medium
6	Reagent	6 uL	PBS
7	Aspirate	-4 uL	Remove medium
8	Reagent	6 uL	PBS
9	Assay Readout	488 nm	Acumen Explorer
Notes			
1	Black clear bottom plates, 8-tip dispense of cells into all wells		
2	Pintool transfer of library to columns 5-48		
3	Pintool transfer of butyrate titration into columns 1 and 2, water into column 3, and butyrate into column 4		
4	37 C, 5% CO ₂ incubator		
5&7	32-tip aspiration leaving ~1-2 uL residual volume		
6&8	PBS addition via 8-tip dispense		
9	500-530 nm emission, fluorescent objects 20 to 120 um in size enumerated for eachwell		

Table 2

Potency and curve class of qHTS actives

EC ₅₀ (uM)	Curve Class				Total
	1.1	1.2	2.1	2.2	
<1	3	1	0	1	9
1 to 10	7	1	20	9	161
>10	1	1	15	6	241
Total	11	3	35	16	411
% Library	0.016%	0.004%	0.051%	0.023%	0.598%

Table 3
Activities and potencies of selected classes of LDR actives

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

¹ Percent active samples of total samples tested (in parentheses) and potency range from qHTS (top row), follow-up library retests (middle row) and follow-up analogs (bottom row).

² Fluorescent compounds.

³ The activity of two compounds was not consistent and therefore inconclusive.

Table 4

Potency and curve class of qHTS and follow-up actives

Sample #	Compound #	Series	Compound ID	qHTS EC ₅₀ (uM)	F/U EC ₅₀ (uM)	qHTS Class	F/U Class
1	1	1	NCGC00073186-01	3.2	not active	1.1	4
2	2	1 (1b) ¹	NCGC00161878-01	4.0	4.6	1.1	1.1
3	2	1 (1b)	NCGC00161878-02	not tested	7.6	not tested	1.1
4	3	1	NCGC00029936-01	5.0	15	1.1	1.1
5	4	1	NCGC00072825-01	5.0	36	1.1	2.1
6	5	1	NSC3852	not tested	2.8	not tested	1.1
7	6	1	NCGC00161906-01	not tested	5	not tested	1.1
8	7	1 (1e)	NCGC00161898-01	not tested	6.1	not tested	1.1
9	8	1 (1d)	NCGC00161899-01	not tested	7.8	not tested	1.1
10	9	1	NCGC00161897-01	not tested	8	not tested	1.1
11	10	1 (1a)	NCGC00161893-01	not tested	8.1	not tested	1.1
12	11	1	NCGC00161892-01	not tested	16	not tested	1.1
13	12	1	NCGC00161904-01	not tested	38	not tested	2.1
14	13	1	NCGC00161880-01	not tested	>46	not tested	2.2
15	14	1	NCGC00161877-01	not tested	>46	not tested	2.2
16	15	1	NCGC00161895-01	not tested	>46	not tested	2.2
17	16	1	NCGC00161881-01	not tested	>46	not tested	2.2
18	17	1 (1c)	NCGC00161884-01	not tested	not active ²	not tested	4 ²
19	18	1	NCGC00161879-01	not tested	not active	not tested	4
20	19	1	NCGC00161882-01	not tested	not active	not tested	4
21	20	1	NCGC00161883-01	not tested	not active	not tested	4
22	21	1	NCGC00161885-01	not tested	not active	not tested	4
23	22	1	NCGC00161887-01	not tested	not active	not tested	4
24	23	1	NCGC00161888-01	not tested	not active	not tested	4
25	24	1	NCGC00161889-01	not tested	not active	not tested	4
26	25	1	NCGC00161890-01	not tested	not active	not tested	4
27	26	1	NCGC00161891-01	not tested	not active	not tested	4
28	27	1	NCGC00161894-01	not tested	not active	not tested	4
29	28	1	NCGC00161896-01	not tested	not active	not tested	4
30	29	2 (2a)	NCGC00059731-01	0.6	8.9	1.1	1.1
31	30	2 (2b)	NCGC00054838-01	5.0	7	3	1.1
32	31	2	NCGC00161905-01	not tested	7.4	not tested	1.1
33	32	2	NCGC00161902-01	not tested	not active	not tested	4
34	33	2	NCGC00161903-01	not tested	not active	not tested	4
35	34	3 (3a)	NCGC00080412-01	6.3	7.9	1.1	1.1
36	35	3 (3b)	NCGC00161907-01	not tested	7	not tested	1.1
37	36	3	NCGC00161900-01	not tested	not active	not tested	4
38	37	4	NCGC00074931-02	0.2	0.6	1.1	1.1
39	38	5	NCGC00026384-01	6.3	not active ³	1.1	4
40	39	5	NCGC00026384-02	not tested	not active	not tested	4
41	40	5	NCGC00078675-01	13	not active	2.1	4
42	41	5	NCGC00078675-02	not tested	not active	not tested	4
43	42	5	NCGC00080570-01	20	not active ³	3	4
44	43	5	NCGC00080570-03	not tested	not active	not tested	4
45	44	5	NCGC00159314-02	not tested	not active	not tested	4
46	45	5	NCGC00079872-02	not tested	not active	not tested	4
47	46	5	NCGC00159311-02	not tested	not active	not tested	4
48	47	5	NCGC00159313-01	not tested	not active	not tested	4
49	48	5	NCGC00159313-01	not tested	not active	not tested	4
50	49	5	NCGC00159314-01	not tested	not active	not tested	4
51	50	5	NCGC00159315-01	not tested	not active	not tested	4
52	51	5	NCGC00159315-01	not tested	not active	not tested	4
53	52	singleton	NCGC00022125-01	0.004	14	3	2.1
	53	singleton	NCGC00021470-01	3.2	not active	1.2	4

¹ Parentheses indicate compounds in Figure 1.

² Weak active in LDR cells as detected by fluorescent microscopy but not by Acumen.

³ Activity was observed initially but not consistently in subsequent tests. F/U- follow-up.

Table 5

Summary of follow up testing of qHTS actives and analogs

Series	Total	Active	% Active	Inconclusive	Fluorescent	Inactive
qHTS candidates	6	3	50%	1	2	0
	13	7	54%	2	1	3
Analog	35	14	40%	0	0	22

Table 6

Summary of biological activities of selected LDR compounds

	LDR EC ₅₀ (uM)	CDH13 ¹	H358 IC ₅₀ (uM)	NSCLC 1 IC ₅₀ (uM)	Normal 1 IC ₅₀ (uM)	NSCLC 2 IC ₅₀ (uM)	Normal 2 IC ₅₀ (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	-	-	-	-	-

¹ Fold induction at 10 uM.

² LDR positive by fluorescent microscope. -, no activity; ND, not determined; N, NSC3852