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Research Article

Identification of Four Potential Epigenetic Modulators from the NCI Structural Diversity Library Using a Cell-Based Assay

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Epigenetic pathways help control the expression of genes. In cancer and other diseases, aberrant silencing or overexpression of genes, such as those that control cell growth, can greatly contribute to pathogenesis. Access to these genes by the transcriptional machinery is largely mediated by chemical modifications of DNA or histones, which are controlled by epigenetic enzymes, making these enzymes attractive targets for drug discovery. Here we describe the characterization of a locus derepression assay, a fluorescence-based mammalian cellular system which was used to screen the NCI structural diversity library for novel epigenetic modulators using an automated imaging platform. Four structurally unique compounds were uncovered that, when further investigated, showed distinct activities. These compounds block the viability of lung cancer and melanoma cells, prevent cell cycle progression, and/or inhibit histone deacetylase activity, altering levels of cellular histone acetylation.

1. Introduction

Pathologies such as human cancer result largely from the inappropriate silencing or activation of genes. It is well established that gene expression can be partly controlled by modulating the access of the transcriptional machinery to target genes through chemical modifications of DNA sequences or histones, the proteins that package DNA. These modifications are mediated by cellular enzymes, including DNA methyltransferases, histone acetyl transferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases, and histone kinases. Epigenetic enzymes function during development, helping orchestrate complex transcriptional programs that control differentiation pathways. During adult life, these enzymes continue to influence transcription by maintaining tissue-specific epigenetic and transcriptional patterns as well as by acting as coregulators for transcription factors. In many cancers, the regulation of transcriptional processes is altered partly because of the abnormal expression and/or function of epigenetic enzymes resulting in the silencing of tumor suppressor genes or in equivalent events that lead to unchecked cellular growth [1]. Indeed, it has been estimated that epigenetic changes are at least ten to forty times more frequent in cancers than genetic mutations [1–3]. Furthermore, specific mutations in HATs and HMTs have been found in relation to the cancer phenotype, suggesting them as potential targets for therapy [4]. Thus, effective treatment of cancer will, at least in part, necessitate the chemical targeting of the cancer epigenome.

Over the last two decades, drugs have been identified that modulate the pathways mediated by a subset of epigenetic enzymes. Because cancer cells often have abnormally silenced tumor suppressor genes or overexpressed oncogenes, mediated by epigenetic pathways, these drugs have been studied in preclinical contexts. Of the known compounds, however, only a few have shown success in clinical settings, with toxicities observed for most other compounds due to their global, unspecific effects on cell function [5–9]. This has led to new drug discovery and drug development efforts at industrial and academic laboratories over the last few years. These programs have used cell-based, *in vitro*, *in silico,*

or yeast systems to identify novel drugs or have developed second-generation compounds structurally related to already known inhibitors [10–18].

Altogether, more recent studies have yielded several new compounds that target epigenetic enzymes, primarily histone deacetylase family members and enzymes that modulate methylation [15, 19–22]. Some of these compounds offer limited benefit over existing drugs, since they are structurally closely related to known inhibitors of epigenetic enzymes, are unspecific, or lack substantial *in vivo* activity, due at least in part to limitations in drug screen design. To date, there are only a few epigenetic drugs approved by the FDA, including: 5-azacytidine and its deoxy derivative decitabine, both DNA methyltransferase inhibitors used for the treatment of myelodysplastic syndromes, vorinostat, and recently romidepsin, HDAC inhibitors used for the treatment of cutaneous T cell lymphoma [23–26]. There is, therefore, a persistent need to increase the number and diversity of available anticancer epigenetic modulators and to develop innovative, improved approaches for drug discovery.

Because screens that use *in vitro* or *in silico* approaches may lead to hits that prove to be toxic, insoluble, or inefficient when taken to the *in vivo* setting, using systems in which drugs are directly tested in cells, as was done for some of the original HDAC inhibitors [10, 11, 18], can save time and effort on followup studies of drugs that are only effective *in vitro* or would require substantial chemical optimization. Here, we report the characterization and use of a cell-based assay in which a locus containing an easily quantifiable marker, green fluorescent protein (GFP), is epigenetically silenced, and derepressed chemically by known epigenetic modulators targeting both histone acetylation and DNA methylation. We have now successfully used this system, the Locus Derepression assay (LDR) [27, 28], to screen the NCI's structural diversity library to identify novel compounds with epigenetic activity. Four confirmed hits from the screen were further investigated for their anticancer properties and their ability to inhibit histone deactylases. We found that two of our hits potently blocked the viability of both lung cancer and melanoma cells and that one of them caused cancer cells to accumulate in the G2/M phase, preventing cell cycle progression. A third hit inhibited deacetylase activity *in vitro* and in cells, but on its own had little toxicity, while a fourth compound selectively inhibited the viability of melanoma cells compared to lung cancer cells.

2. Materials and Methods

2.1. Chemicals and Reagents. Dulbecco's modification of eagle's medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), and G418 were purchased from Mediatech (Manassas, VA). The penicillin/streptomycin solution, trypsin-EDTA, and 37% formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Hoechst 33342 was obtained from Invitrogen (Carlsbad, California). Trichostatin A and apicidin were obtained from Alexis Biochemicals, depsipeptide was a generous gift from Dr. David Schrump and 5-aza-2′deoxycytidine and sodium butyrate were purchased from Sigma/Aldrich. Hit compounds for followup were provided by the Drug Synthesis and Chemistry Branch, DTP, NCI. All drugs were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C.

2.2. Cell Culture and Materials. LDR cells were grown in DMEM media with 10% heat inactivated FBS and supplements, as previously described [29]. The C127 cells were grown in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% charcoal/dextran-treated FBS. Cells were cultured in a humidified incubator at a 37°C, 5% CO_2, and 95% air environment.

2.3. Drug Screen. LDR cells were plated into 96-well Nunc glass-bottom black plates at 8,000 cells/well and cultured in complete media at 37°C, 5% CO_2, and 95% air overnight. DMSO (0.5%) was used for the negative control and 25 mM sodium butyrate was used as the positive control. Twenty-four hours after seeding the cells into assay plates, cells were treated with the structural diversity chemical library (4 μM final). After a 24 hour treatment with the chemical library, the assay plates were subjected to fixation with 4% formaldehyde for 45 minutes. After fixation, the assay plates were washed 5 times with 100 μL of DPBS. The plates were stained with 0.3 μg/mL Hoechst 33342 in DPBS overnight at 4°C. The next day, assay plates were washed twice with 100 μL of DPBS, and the plates were sealed with aluminum sealing tape and were barcoded with a Velocity-11 VCode Bar Code Label Print and Apply Station (Menlo Park, CA). Images were acquired using the Discovery-1 imaging system, at 20X, 4 sites per well, 30 ms exposure for Hoechst 33342, and 600 ms exposure for GFP, and they were processed with Metamorph software. Hits were identified by manual examination of images for GFP expression. Hit compounds were cherry picked and subjected to quadruplicate testing in the LDR cell line and in the C127 parental cell line to confirm activity and eliminate false positive results due to fluorescent compounds.

2.4. Fluorescence Microscopy. For followup experiments, LDR cells were plated on four-well chambered glass slides (Lab-TekII, Nunc) at a density of 15,000 cells per well, treated with drug and incubated for 24 hours in 5% CO_2 at 37°C, and imaged either on a Nikon Eclipse TE2000-U fluorescence microscope equipped with a CCD Roper camera, an Olympus 1 × 70 system equipped with a Photometrics camera or on a CARV Metamorph system with an Orca II CCD camera. Metamorph software was used to process the images.

2.5. GFP Quantification. Fluorescent Activated Cell Sorting (FACS) was used to quantify GFP levels in LDR cells after various drug treatments in dose response studies. Briefly, cells were collected and pelleted, washed in PBS, and then resuspended in PBS at 100,000 cells/mL and subjected to analysis in BD FACSCalibur sorter. Data was analyzed using FlowJo software. Uninduced and vehicle treated cells were used as a negative control for gating.

2.6. Cell Viability Assays. Human cancer cell lines were plated in RPMI media with 5% heat-inactivated FBS, at 2000–4000 cells per well of 96-well flat-bottom plates. The next day, cells were treated with compounds with maximum concentrations ranging between 15 μM and 5 μM and allowed to incubate for 4 days in 5% CO_2 at 37°C. Cells were then treated with MTS reagent and absorbance measured according to the company's protocol (CellTiter 96 AQ_{ueous} Nonradioactive Cell Proliferation Assay, Promega).

2.7. Cell Cycle Analysis. H358 cells were treated with 1 μM 5-aza, 200 nM TSA, 5 μM NSC-159631, or DMSO and incubated for 24 hours in 5% CO_2 at 37°C. Cells were trypsinized and stored in 75% ethanol at −20°C and later washed in PBS and suspended in propidium iodide staining buffer (1x PBS, 0.1% Triton X-100, 0.5 mM EDTA, 50 μg/mL DNAse-free RNase, and 0.05 mg/mL propidium iodide). Samples were then analyzed in the UT Southwestern FACs core using a BD FACSCalibur sorter. Data was analyzed with FlowJo software.

2.8. HDAC Assays. HDAC activity was analyzed using the HDAC Assay Kit from Millipore/Upstate (17-356). Briefly, nuclear extract from Baf3 cells or purified, recombinant HDAC1 (Millipore/Upstate) was allowed to incubate with the fluorometric HDAC substrate according to the manufacturer's protocol. In a secondary activator reaction, the fluorophore is only cleaved from the deacetylated substrate, allowing for quantification. Fluorescence was quantified on a FLUOstar-Optima or a FLUOstar Omega plate reader (BMG Biosciences).

2.9. Western Blot Analysis. H358 cells were treated with 10 μM NSC-22206, 200 nM TSA, 0.5 μM NSC-159631, or DMSO for 24 hours, and protein was extracted using a lysis buffer (50 mM NaCl, 1 mM EDTA, 2.5 mM Tris pH 7.4, 0.1% SDS, and 1% NP-40). Protein was quantified and equal amounts of samples were run on 4%–12% SDS acrylamide gradient gels and transferred to nitrocellulose membranes. Membranes were incubated overnight with 0.05 μg/mL polyclonal rabbit anti-acetyl-Histone 3 antibody (Millipore/Upstate 06-599). Blots were washed and reincubated with anti-actin primary antibody (Santa Cruz no. 1616). Bands were imaged using enhanced chemiluminescence reagents from Thermo Scientific. For Supplementary Figure 2B available at doi: 10.1155/2011/868095 , the additional antibodies used were as follows: HDAC1 (Affinity Bioreagents PA1-860), acetylated tubulin (Sigma T6793) and H3K9me3 (Millipore 07-523).

3. Results and Discussion

3.1. Development of Cell-Based GFP Assay. To screen for epigenetic modulators, we developed a cell-based assay, consisting of C127 mouse mammary adenocarcinoma cells stably expressing a GFP construct linked to a portion of the estrogen receptor ligand binding domain, driven by the CMV promoter, which is susceptible to epigenetic repression. On a separate plasmid, the gene for neomycin resistance was also introduced. We selected cells that were neomycin resistant but whose GFP expression was constitutively silenced. After clonal expansion, GFP expression remained repressed. Upon treatment of cells with epigenetic modulators, we expected GFP production (Figure 1). The use of GFP allows for easy and automatic detection, applicable to both low and high throughput screening applications. The detailed design of this cell-based assay, named the locus derepression or LDR assay, has been described elsewhere [27, 28].

3.2. Characterization of the LDR Assay. To determine if the GFP construct in LDR cells was under epigenetic regulation and if so, what classes of epigenetic modulators would derepress the locus, LDR cells were treated with different structural classes of HDAC inhibitors and with DNA methyltransferase inhibitors. The cells were then analyzed for GFP expression by visualization with a fluorescent microscope (Figure 2(a)), evaluated in dose-response experiments (Figure 2(b)) and/or quantified by fluorescence-activated cell sorting (FACS) (Figure 2(c)). Class I/II HDAC inhibitor trichostatin A (TSA), a hydroxamic acid, was added to the cells at a concentration of 165 nM, and GFP expression was measured. Treating cells with increasing concentrations of TSA ranging from 16 nM to 827 nM demonstrated that the induction of GFP in LDR cells was dose dependent (Figure 2(b)). The short chain fatty acid butyrate, also a Class I/II HDAC inhibitor, was able to induce expression of GFP as measured by both microscopy (Figure 2(a), middle left panel) and FACS (Figure 2(c)). Butyrate induction of GFP expression was also seen to be dose dependent when tested with concentrations ranging from 1 mM to 100 mM (Figure 2(b)). Similar results were observed with apicidin, a cyclic tetrapeptide HDAC inhibitor. Cells treated with 5 μM apicidin had a significantly increased green fluorescent population, as shown by FACS (Figure 2(c)). Apicidin also derepressed the GFP construct in a dose-dependent manner, at concentrations between 0.1 μM and 10 μM (Figure 2(b)). We also evaluated a Class I-specific HDAC inhibitor, the cyclic depsipeptide FR901228 also known as simply depsipeptide or romidepsin, which potently induced GFP production at 25 ng/mL (Figure 2(a), bottom left panel). Taken together, these results demonstrate that the GFP construct in LDR cells is under epigenetic regulation which can be overcome by any structural type of HDAC inhibitor. As expected from their functionally distinct roles, inhibition of class 3 HDACs by nicotinamide had no effect on GFP production although Sirt 1 enzymatic activity was markedly diminished in vitro (data not shown). Interestingly, BIX-01294, an inhibitor of G9a histone methyltransferase [15], did not induce GFP expression at up to 10 μM doses over a two day exposure, suggesting that histone methylation by G9a is not required for CMV silencing in LDR cells.

To test whether the silenced locus was also under the control of DNA methylation (which was possible due to the presence of a large CpG island on the CMV promoter), we measured the effects of a DNA methyltransferase inhibitor, 5-aza-2′deoxycytidine (5-aza) and found that it too was able to induce GFP expression (Figure 2(a), bottom right panel). Similar results were obtained with 5-aza-cytidine.

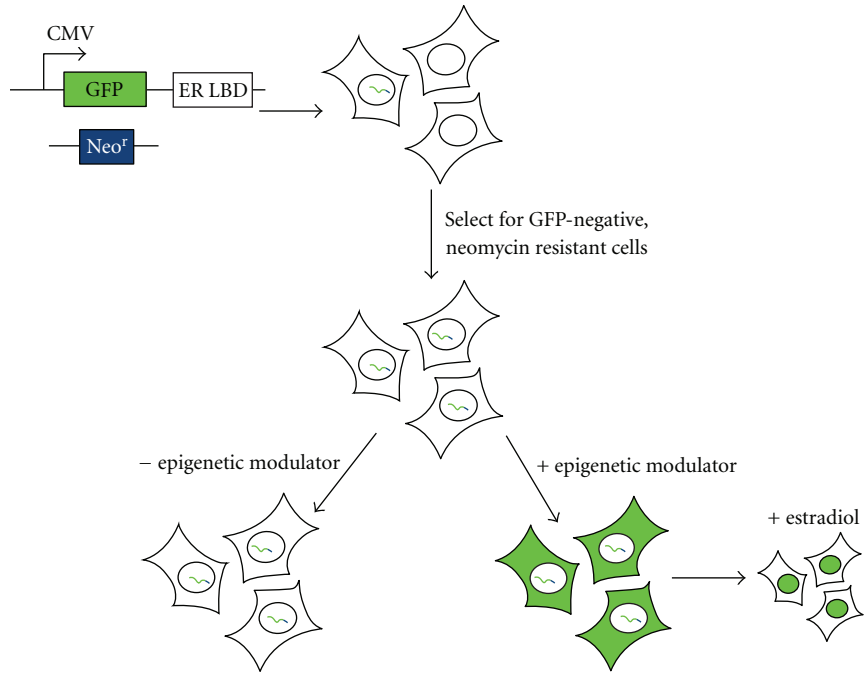


Figure 1: Schematic representation of the process of creating transgenic mouse mammary adenocarcinoma cells (locus derepression assay cells or LDR) [29]. A construct containing the GFP gene linked to the estrogen receptor ligand-binding domain, driven by the CMV promoter and a neomycin resistance marker, was stably transfected into cells. GFP negative cells, stably expressing the neomycin resistant gene, were selected. With the addition of an epigenetic modulator, such as an HDAC inhibitor or a DNA methyltransferase inhibitor, the cells express GFP and can be visualized by fluorescence microscopy. When cells are then treated with estradiol, the GFP fusion protein translocates to the nucleus.

However, other molecules, such as general transcriptional activators including hormones, steroids, and amino acids, were incapable of inducing GFP production, as were proteasome inhibitors or general stress conditions such as hypoxia and starvation (data not shown). These experiments show that the GFP construct is, indeed, silenced epigenetically and can be derepressed by inhibiting HDAC as well as DNA methyltransferase pathways (and potentially other epigenetic cascades), emphasizing the assay's utility in finding novel epigenetic modulators.

To confirm that GFP expression induced by drug treatment was dependent upon *de novo* transcription of the locus and did not, for example, involve increased stability or translation of an already transcribed message, we measured the effects of actinomycin-D on GFP production. We cotreated LDR cells with 200 nM TSA or 25 nM depsipeptide, in the presence or absence of 0.5 μg/mL actinomycin-D, a known inhibitor of transcription. Actinomycin-D prevented the production of GFP in response to both TSA (Figure 3(a) and 3(b)) and depsipeptide (Figures 3(c) and 3(d)). This confirms that the expression of the silenced locus requires new transcription, engaging the pathways the assay was designed to exploit.

3.3. Drug Screens Using the LDR Assay and Characterization of Hit Molecules. To adapt LDR cells for use in screening applications, we first isolated a subset of the most responsive transgenic cells, which showed a 70%–90% response to 25 mM butyrate (Figure 4(a)) and expanded them clonally. These cells grew well in 96-well plate format and produced robust GFP signal (Figure 4(b)). We used these LDR cells for drug screens to identify potential novel epigenetic modulators that would turn on the expression of GFP in cells. Cells were plated in 96-well plates, grown overnight, and treated for 24 hours with 4 μM concentrations of compounds from the NCI structural diversity library, which was chosen in order to maximally query chemical space for new active structures. After fixation, cells were visualized using a Discovery-1 automated fluorescent microscope which captured two-by- two frames in each well (Figure 4(b)). Hits were manually scored for GFP expression, and of the 2,080 compounds screened, 71 of them were primary hits. However, after eliminating the autofluorescent compounds by counter screening on parental C127 cells, and retesting the remaining hits through cherry picking, there were 4 confirmed hits which were followed up (Table 1). These confirmed hits were validated at the protein level by measurements of GFP protein translocation in response to estradiol, making use of the estrogen receptor ligand-binding domain which is expressed in LDR cells downstream of GFP on the same construct (see Supplementary Figure 1) [28].

The hits from this library are not only structurally distinct from each other, but also from other known HDAC and DNA methyltransferase inhibitors, providing structural diversity to this chemical class. To analyze if these compounds were active at a single dose or showed

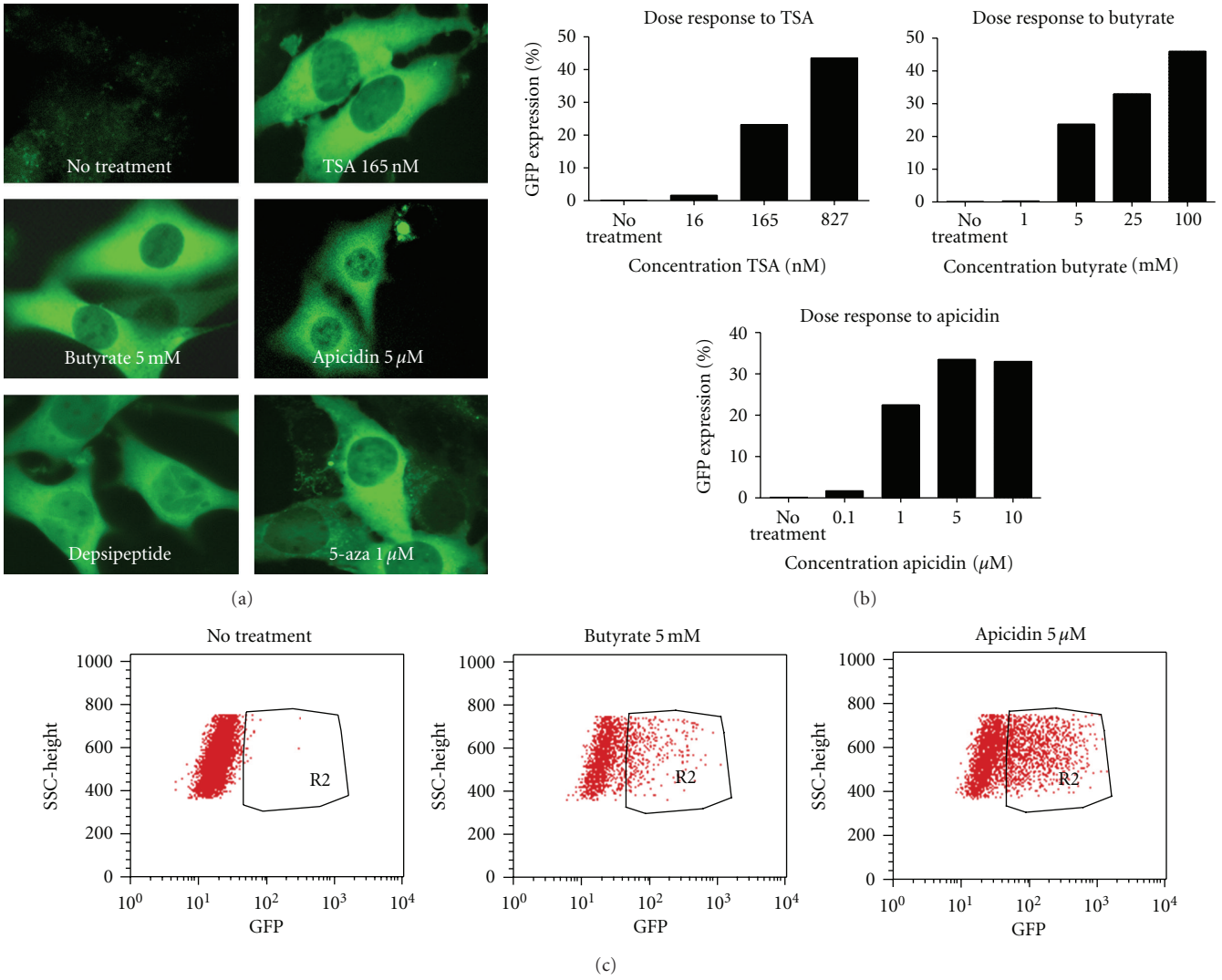
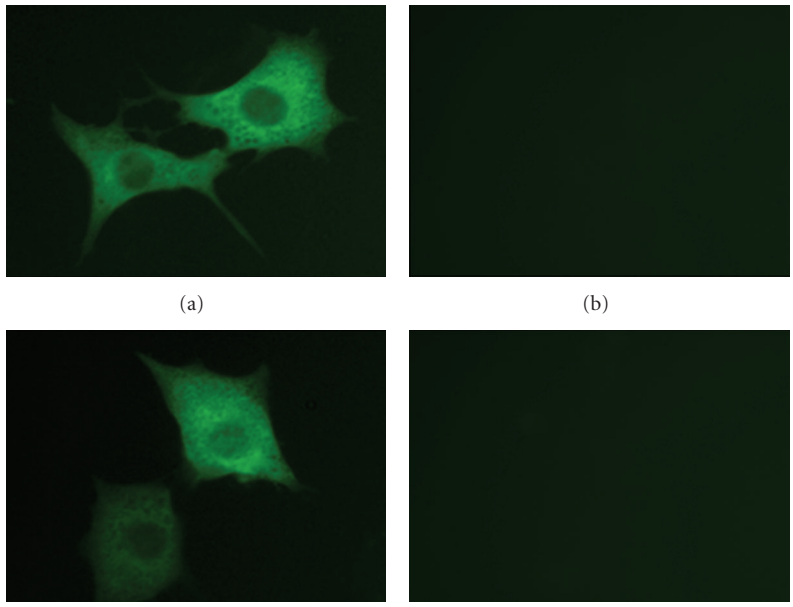


Figure 2: Characterization of cell-based LDR assay. (a) Visualization of GFP expression in cells treated with various known epigenetic modulators. TSA, butyrate, apicidin, and depsipeptide are HDAC inhibitors and 5-aza-deoxycytidine is a DNA methyltransferase inhibitor. (b) Cells exhibit dose-dependent GFP expression in response to HDAC inhibitors. (c) FACS analysis reveals an increase in number of cells expressing GFP when treated with butyrate or apicidin, compared to untreated cells.

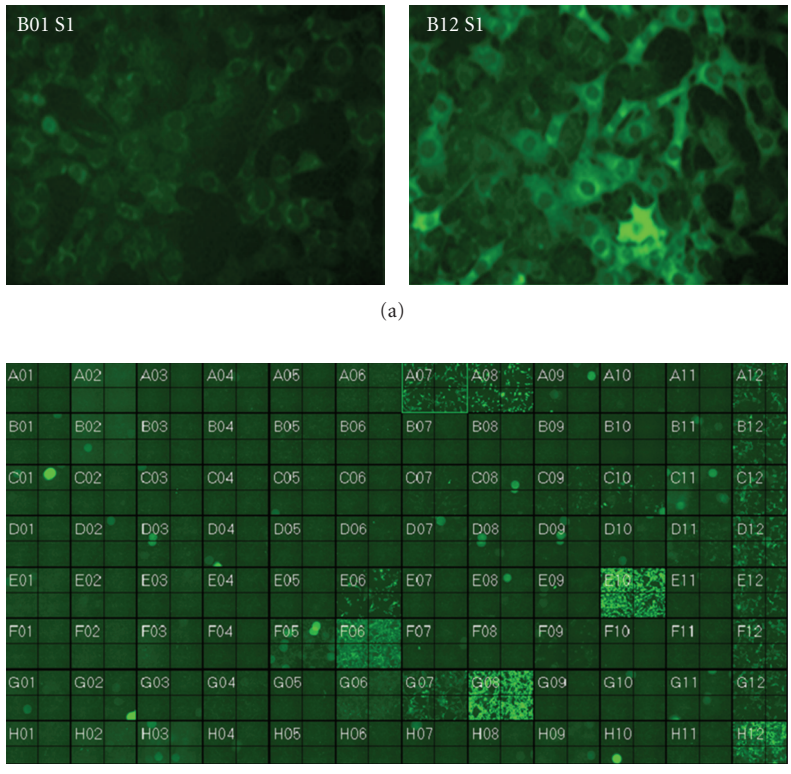
dose responsiveness, two of our hits were analyzed in a concentration series. Secalonic acid D (NSC-159631) [30], was added in concentrations ranging between 10 nM and 10 μM, and NSC-22225 was tested between 500 nM and 10 μM. GFP induction in response to secalonic acid D was strongly dose dependent showing a half-maximal activation between 1–5 μM (Figure 5(a)), while NSC-22225 showed a weaker but nonetheless clear dose dependence (Figure 5(b), note toxicity to mouse LDR cells at 10 μM). A search for compounds structurally related to our hits, yielded NSC-22206 and NSC-22214, which vary from NSC-22225 only in the nature of the metal salt. As expected, these related molecules also induced GFP expression in LDR cells but had a slightly lower potency than the primary hit (Figure 5(c) and data not shown).

Epigenetic modulators have been postulated to have anticancer properties because they can mediate the reexpression of silenced tumor suppressor genes and in a manner, that is not yet fully understood, can block cell cycle progression [1, 18, 22, 31, 32]. To evaluate the ability of LDR hits to inhibit the viability of human cancer cells, we performed standard MTS assays on nonsmall cell lung cancer and melanoma cells [33, 34]. Table 2 shows the average IC_{50} values obtained in 1–5 experiments each done with 8 replicates. While NSC-22225 and its related compound were incapable of blocking human cancer cell growth, other hits inhibited cancer cell viability with IC_{50}'s ranging from 90 nM to 9 μM. Interestingly, while NSC-150117 and secalonic acid D were effective against both lung cancer and melanoma cells of uveal origin, NSC-693322 preferentially targeted melanoma cells, suggesting that this compound may interfere with a signaling cascade essential for melanoma but not lung cancer cell survival. Secalonic acid D, known to have activity as a kinase inhibitor, was the hit with the broadest activity



(a) (b) (c) (d)

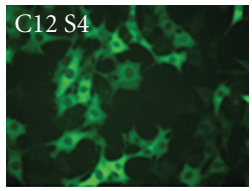
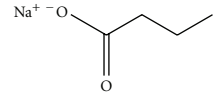
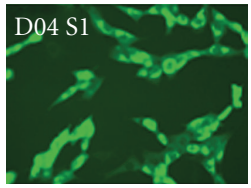
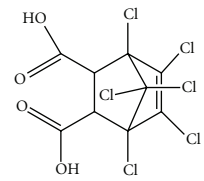
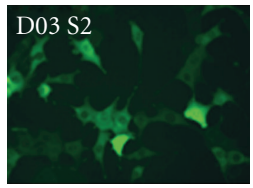
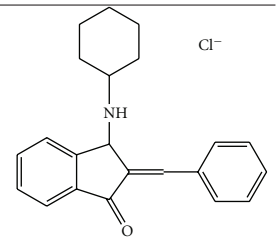
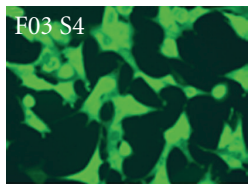
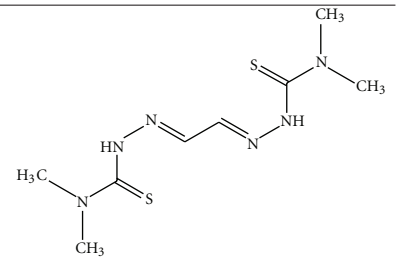
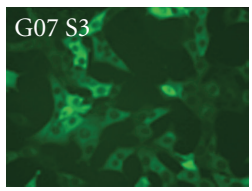
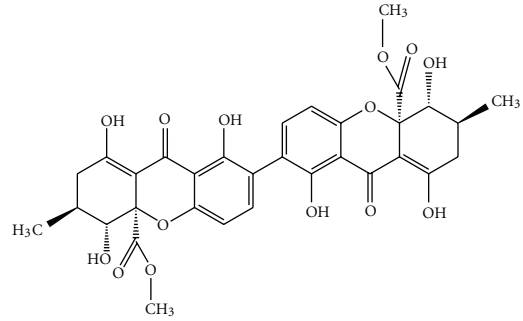
Figure 3: GFP expression requires *de novo* transcription. (a) Cells treated with 200 nM TSA express GFP. (b) When transcription inhibitor actinomycin-D (0.5 μg/mL) is added to LDR cells together with TSA, no GFP expression is observed. The same pattern is seen with 25 nM depsipeptide, with and without actinomycin-D in (c) and (d), respectively.



(a) (b)

Figure 4: Transgenic LDR cells used in a low-throughput drug screen. (a) Highly responsive cells chosen for the assay were expanded clonally, showing 70%–90% response to 25 mM butyrate (right), as compared to vehicle treated cells (left). (b) A 96-well plate used to screen compounds from the NCI structural diversity library was visualized after treatment with 4 μM compounds in DMSO, using a Discovery 1 fluorescent microscope platform and processed on the fly for 2×2 visualization per well. Hits are easily detectable by marked increase in fluorescence. Parental untransfected mouse mammary adenocarcinoma cells were used to counter screen.

Table 1: Hits from the screen are diverse in their structure. Butyrate was used as a positive control. The nonautofluorescent hits caused cells to express GFP, but have unique structures.

Name/ID	LDR Phenotype	Structure
Butyrate		
NSC-22225		
NSC-150117		
NSC-693322		
Secalonic Acid D; NSC-159631		

across all human cancer lines tested. To evaluate whether its ability to inhibit cell viability was specific for cancer versus normal cells, we measured its effects on a pair of matched lines derived from the lung cancer and the normal lung epithelium from the same patient. This revealed that secalonic acid D lacks specificity and inhibits a mechanism common to the survival of both normal and cancer cells (Figure 6(a)). This mechanism involves, at least in part, blocking of cell cycle progression in G2/M of the cell cycle, similar to the effects of TSA, as can be seen in Figure 6(b).

To directly test whether LDR hits had the ability to inhibit HDAC activity, we performed in vitro HDAC assays in nuclear extracts, using a commercially available substrate conjugated to a fluorophore, which is cleaved only from deacetylated substrate in an activation step. Figure 6(c) demonstrates that at 10 μM, only NSC-22206 inhibited HDAC activity. The lack of inhibition by NSC-22225 was surprising, leading us to confirm that the activity of NSC-22206 was specific and not the result of inhibition of the coupled activator reaction used in this assay. NSC-22206 did not affect the coupled reaction and was effective in inhibiting purified HDAC1 (Figure 6(c)) and in partly inhibiting HDAC8 (data not shown). In terms of the HDAC inhibition by NSC-22206, it must be concluded that its metal

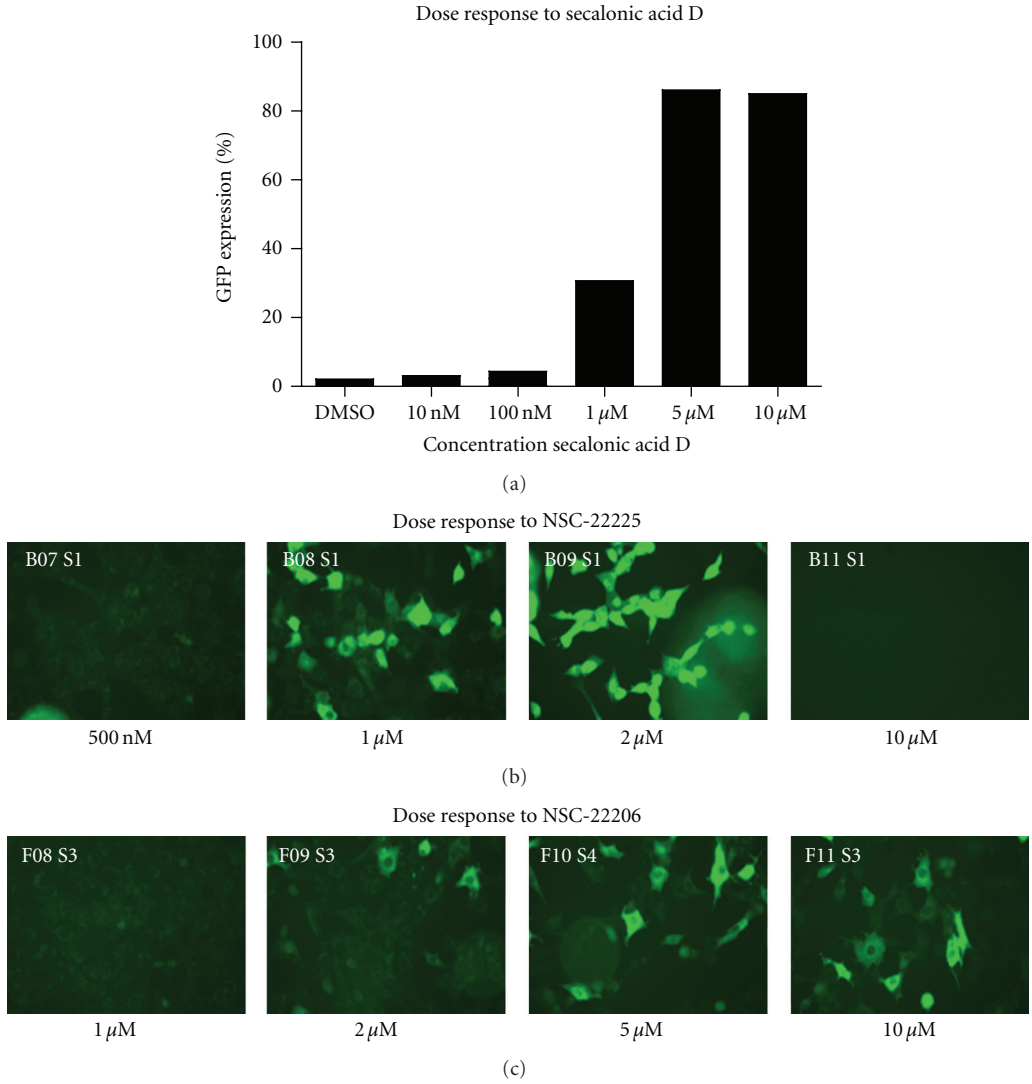
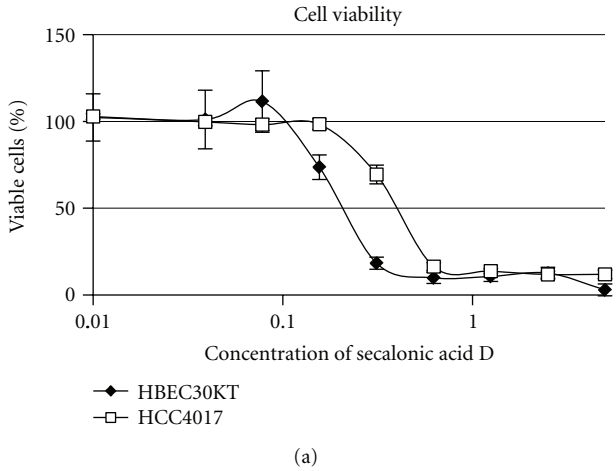


Figure 5: Characterization of assay hits. Dose-response experiments were performed with secalonic acid D, or NSC-159631 (a) and NSC-22225 (b), a related molecule NSC-22206 (c), which differs from NSC-22225 only by the metal salt (Cd for NSC-22225 and Hg for NSC-22206). In all three cases, GFP expression was dose dependent.

salt, mercury, but not the metal salt of NSC-22225, cadmium, contributes to the inhibition, potentially by replacing zinc in the HDAC catalytic site or by unspecifically binding cysteine thiols [35]. Indeed, 10 μM mercury chloride on its own also inhibited HDAC activity in vitro although no GFP induction was seen in LDR cells (data not shown). To confirm the relevance of HDAC inhibition by NSC-22206 *in vivo*, we treated human lung cancer cells with this compound as well as with TSA as a positive control and secalonic acid as a negative control. Consistent with the HDAC activity data, cells treated with either 200 nM TSA or 10 μM NSC-22206 showed increased levels of acetylated histone 3 and no changes in methylated histones (Figure 6(d) and Supplementary Figure 2B). In contrast, 0.5 μM secalonic acid D, which inhibited cancer cell viability, had no effect on histone acetylation, yet surprisingly it did affect the global levels of histone 3 methylation in the same experiment (Supplementary Figure 2B) . This suggests that the ability of secalonic acid D to kill cells may be related to its cell cycle effects as mentioned above in combination with its global effects on histone methylation. In contrast, the effects of NSC-22206 on global histone acetylation are not sufficient to inhibit the viability of cancer cells. The possibility that in combination with other compounds our hits may be selective against cancer remains open to further investigation.

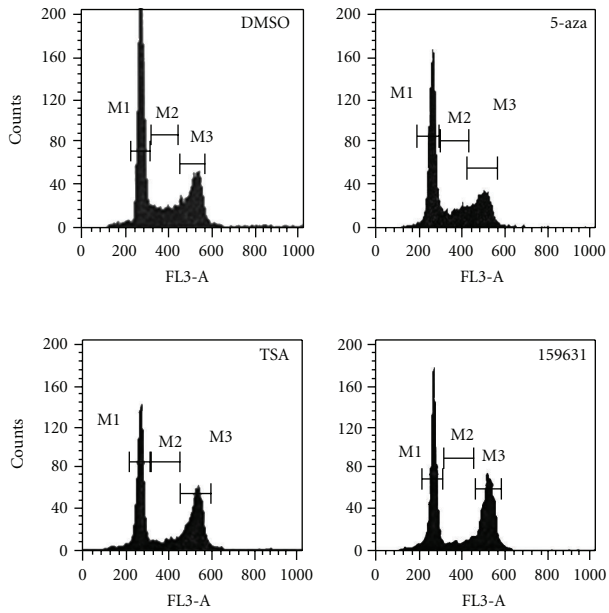
4. Conclusions

We have developed a cell-based system to identify compounds that potentially could affect a broad spectrum of epigenetic targets. Using this LDR assay to screen the NCI's structural diversity library, we have found four new epigenetic chemical modulators whose structure varies from known compounds in this class. Mechanistically, each small

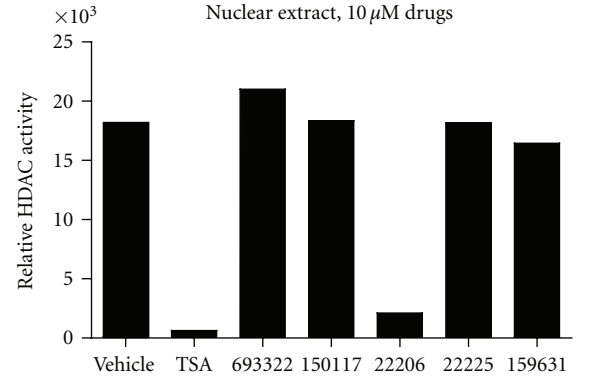


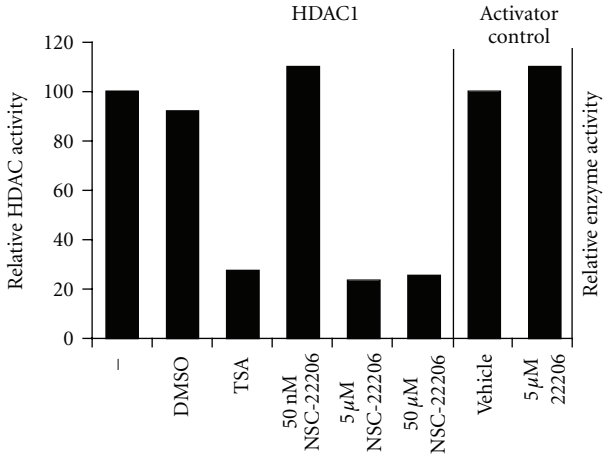
(a)

H358 cells	G0/G1	S	G2/M
DMSO	56%	14%	26%
5-aza	55%	17%	25%
TSA	50%	9%	39%
NSC-159631	51%	7%	39%

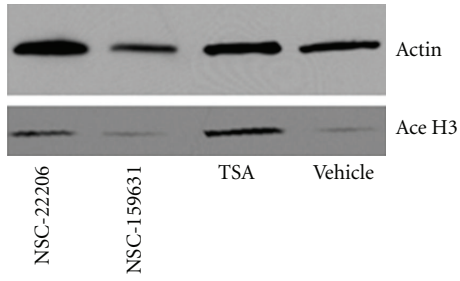


(b)





(c)



(d)

Figure 6: Characterization of LDR assay hits. (a) MTS Assay was performed with increasing doses of Secalonic acid D on a matched pair of lung cells: the lung cancer line HCC4017 and the normal line HBEC30KT. Secalonic acid D did not show selectivity for cancer. (b) Cells were treated with vehicle or drug and sorted by cell-cycle phase after propidium iodide staining. TSA (200 nM) and NSC-15931 (5 μM) both caused cells to accumulate in G2/M phase as compared to vehicle or 5-azadeoxycytidine (1 μM). Values do not add to 100% because of the small fraction of sub G0/G1 cells. (c) *In vitro* HDAC activity assays were performed on nuclear extracts treated with 10uM hit compounds (left panel). Purified active HDAC1 activity was assayed in the presence of increasing concentrations of NSC-22206, which inhibited HDAC1 (right panel) but did not inhibit the coupled reaction (right side of panel)." (d) H358 lung cancer cells were treated with drugs for 24 h and analyzed by Western Blot for changes in global histone modifications. NSC-22206 (10 μM) and TSA (200 nM) both caused an increase in levels of acetylated histone 3 whereas NSC-159631 (0.5 μM) did not.

Table 2: Characterization of the anticancer properties of hits. Cell viability assays were performed with hits from the assay, and IC_{50} values were obtained for several nonsmall cell lung cancer cell lines and several melanoma cell lines (bold).

Drug ID	Cell Line	$IC_{50}(\mu M)$
NSC-22225/NSC-22206	H1395	>20
	H1437	>10
	H358	>10
	Mel270	**>5**
	OCM3	**>5**
NSC-150117	H1395	2.3
	H1437	>10
	H358	1.9
	Mel270	**1.7**
	OCM1	**3.4**
	OCM3	**2.2**
	Omm1	**0.5**
	Omm2.3	**1.7**
NSC-693322	H1395	>10
	H1437	>10
	H358	8.7
	Mel270	**1.5**
	Mel285	**1.7**
	OCM1	**1.4**
	OCM3	**2.9**
	Omm1	**0.1**
	Omm2.3	**0.3**
NSC-159631	H1395	0.4
	H1437	0.7
	H358	0.6
	HCC4018	0.4
	Mel270	**0.5**
	Mel285	**0.8**
	OCM1	**0.7**
	OCM3	**0.3**
	Omm2.3	**0.5**

molecule hit exhibits unique properties in their ability to block cell cycle progression, inhibit HDACs, alter global histone acetylation, and induce cancer cell death. These chemical modulators may now be explored to define their specific molecular targets and to identify further their anticancer applications alone or in combination with existing anticancer drugs.

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