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# [2] High-Content Fluorescence-Based Screening for Epigenetic Modulators

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## Abstract

Epigenetic processes have gained a great amount of attention in recent years, particularly due to the influence they exert on gene transcription. Several human diseases, including cancer, have been linked to aberrant epigenetic pathways. Consequently, the cellular enzymes that mediate epigenetic events, including histone deacetylases and DNA methyltransferases, have become prime molecular targets for therapeutic intervention. The effective and specific chemical inhibition of these activities is a top priority in cancer research and appears to have therapeutic potential. This chapter describes the development of mammalian cell-based fluorescent assays to screen for epigenetic modulators using an innovative combination of approaches. Detailed protocols for the use of the assays in drug screens,

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as well as for the initial characterization of hits, are provided. Furthermore, options for evaluating the mechanism of action of these compounds are presented and principles to govern the choice of hit compounds for the development of leads are discussed.

## Introduction

Proper physiological function of organs requires the correct temporal and spatial expression of genes and the regulation of their expression levels. Chromosomal aberrations, mutations of transcription factors, and changes in chromatin structure are among the events that can give rise to abnormal silencing or abnormal activation of gene expression. Chromatin architecture is in large part determined by chemical modifications of DNA sequences and of histones, the proteins on which DNA is wound. The methylation status of DNA, as well as the acetylation, methylation, and phosphorylation status of histone proteins, determines the level of chromatin compaction in the cell and, thus, the accessibility of transcription factors to their target genes. In general, unmethylated DNA and acetylated histones at promoter sites are permissive to transcriptional activity, whereas methylated DNA and hypoacetylated histones prevent transcription. The expression and/or function of the enzymes that mediate histone acetylation, histone deacetylation (HDACs), and DNA methylation (DNMTs) is often altered in human cancers (Dhordain *et al.*, 1998; Kitabayashi *et al.*, 2001; Melki *et al.*, 1998; Mizuno *et al.*, 2001; Shigeno *et al.*, 2004; Warrell *et al.*, 1998), consequently affecting the regulation of transcriptional processes and thus cellular growth and function.

Several compounds have been identified that inhibit the methylation or deacetylation pathways mediated by these enzymes. These compounds have had immediate application in the treatment of cancers because of their ability to reactivate aberrantly silenced tumor suppressor genes (Cote *et al.*, 2002; Sandor *et al.*, 2002; Suzuki *et al.*, 2002; Vigushin and Coombes, 2002). A number of these inhibitors are being evaluated in current clinical trials (Kelly *et al.*, 2005; McLaughlin and La Thangue, 2004; Ryan *et al.*, 2005; Villar-Garea and Esteller, 2004), but the search for structurally diverse inhibitors with improved pharmacological properties and selectivity continues (Haggarty *et al.*, 2003; Hennessy *et al.*, 2003). Most efforts to date to screen for such drugs have used *in vitro* approaches or have looked for compounds capable of overinducing already transcriptionally active genes. This chapter discusses the development of a fluorescence-based cellular assay to identify small molecules based on their ability to reactivate the expression of a silenced reporter gene. The principles that should guide the development of similar cell-based assays, the

actual techniques that can be used to develop and use these systems in drug screens, and detailed protocols for hit analysis are provided.

## Epigenetic Regulators of Gene Expression as Drug Targets

The mechanism by which HDACs and DNMTs generally repress transcription has been partly elucidated over the last few years. Methylation of DNA at CpG islands (genomic regions of statistically high CG dinucleotide density, found near or on gene promoters) is an uncommon event in active euchromatin. Rather, cellular regulatory cascades or pathological signaling pathways specifically target particular promoter sequences, leading to their methylation. The methylated DNA then becomes a docking site for methyl-binding proteins (Magdinier and Wolffe, 2001). These, in turn, recruit transcriptional repressor complexes, containing histone deacetylases. HDACs deacetylate local histones, causing the compaction of chromatin, thus hindering the access of transcription factors to promoter sequences. HDACs can also exert their transcriptional inhibitory activity in a DNA methylation-independent manner. In either case, the resulting chromatin compaction, if aberrant, deregulates transcriptional control and can lead to unchecked growth or proliferation due to the silencing of tumor suppressor genes or to functionally equivalent events (Konishi *et al.*, 2002; Nakamura *et al.*, 2001; Roman-Gomez *et al.*, 2002). It follows that chemical inhibition of HDACs and/or of DNMTs should allow for the reactivation of silenced genes, potentially restoring transcriptional balance to the cell.

Several compounds have been shown to inhibit the activity of HDACs, such as the hydroxamic acid trichostatin A, the short chain fatty acid butyrate, the epoxide apicidin, and the depsipeptide FR901228. Compounds that inhibit DNMTs have also been developed based on the structure of cytidine, the DNA base methylated by this enzyme family. These include 5-aza-2′-deoxycytidine (also known as decitabine), azacytidine, and zebularine. These agents reactivate the expression of aberrantly silenced tumor suppressor or differentiation genes and/or downregulate (presumably by indirect mechanisms) the expression of cell proliferation or oncogenes in human cancer cells (Cote *et al.*, 2002; Sandor *et al.*, 2002; Suzuki *et al.*, 2002; Vigushin and Coombes, 2002). In general, these inhibitors do not produce global transcriptional changes, and normal cells can overcome the transient G2/M arrest that is sometimes induced by treatment (Johnstone, 2002). Early clinical trials have shown some promise in cancer treatment (Kelly *et al.*, 2005; McLaughlin and La Thangue, 2004; Ryan *et al.*, 2005; Villar-Garea and Esteller, 2004), yet a wider structural variety of inhibitors is necessary to address drug absorption, distribution, stability, efficacy, and toxicity, as well as variations in patient responses.

## Rationale for the Development of Cell-Based Assays to Screen for Epigenetic Modulators

Because of their potential usefulness as therapeutics and as research tools, we sought to develop a system to identify novel inhibitors of HDACs and DNMTs that would have unique advantages over existing methods and to establish a standard protocol for developing such assays. Our optimal design includes the following requirements: (1) the system should be mammalian cell based for maximal biological relevance, (2) it should allow simultaneous screening for small molecule inhibitors of both HDACs and DNMTs, (3) it should measure a biological event closely related to the events one seeks to target in cancer cells (i.e., gene silencing), and (4) it should produce a signal that is easy to measure and amenable to automation. This design is represented in Fig. 1.

Our design rationale is based on several points. Because their functions overlap, a system that involves the reversal of transcriptional repression could be used to interfere with either DNA methylation or histone deacetylation pathways. To achieve this, we could stably introduce a reporter gene into mammalian cells together with a selection marker and screen for cells that, while retaining selection, showed no reporter expression. We decided to use the CMV promoter to drive our reporter gene because it is known to be a strong and constitutive promoter. In this way, the lack of

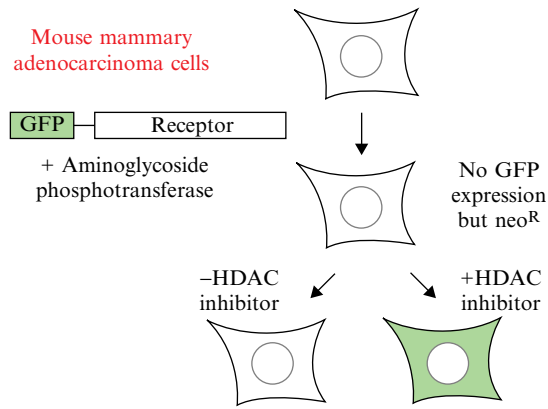


FIG. 1. Schematic of the strategy used in the development of the mammalian cell-based assay. Briefly, C127-derived cells were cotransfected with an expression vector for a GFP-tagged protein driven by a constitutively active CMV promoter and with a gene for neomycin resistance. After selection, clones that survived in the presence of the antibiotic but did not express GFP were expanded. GFP expression was obtained by treatment with inhibitors of HDACs or DNMTs.

promoter activity would likely be the result of a repressive chromatin structure on or near the promoter rather than the lack of a stimulatory signal (such as a hormone or a particular transcription factor or a signal arising during a stage of the cell cycle). Similarly, the reversal of repression by candidate compounds would likely be the consequence of changes in chromatin architecture rather than the induction of unrelated stimulatory signals. We then chose a green fluorescent protein (GFP)-fusion protein as our reporter to facilitate its detection by automated fluorescence microscopy. The methods that follow describe the principles and techniques applied in the development and use of this cell-based system, which can serve as a template for developing similar cell-based assays for use in drug screen applications.

## Methodological Considerations

### *Choice of Cell Line, Reporter System, and Selection Marker for High Content Transcriptional Assays*

The choice of cell line should be based on the following principles: (1) the cell line should express various HDACs and/or DNMTs, (2) it should be responsive to chemical epigenetic modulators, that is, exhibiting histone hyperacetylation when treated with known HDAC inhibitors, (3) it should not express high levels of multidrug resistance pumps or drug efflux transporters, (4) it should exhibit a morphology that is amenable to imaging, that is, flatter cells that spread well on a surface and are relatively homogeneous and, (5) it should grow under standard conditions that can facilitate miniaturization (for high-throughput applications). All other things being equal, the user may also want to consider the advantages of using a human cell line for greater relevance in drug screening, although our experience has been very positive using C127 mouse mammary adenocarcinoma cells.

While there are currently many reporter systems available for cellular transcriptional studies, we chose GFP over other reporters, such as CAT or luciferase, because of the following advantages. GFP expression can be measured in live cells, and its monitoring does not require cell lysis or the addition of reagents to the cells (Hager, 1999; Martinez *et al*., 2005). Fluorescence detection is simple, friendly to automation, and requires only an initial investment in equipment without the ongoing cost of substrates. Furthermore, the use of GFP as a reporter allows the user to query the experimental results at any time before the intended end point of the study without irreversibly interfering with biological processes. This permits added flexibility in experimental design. In terms of promoter choice, we strongly recommend use of the CMV promoter to drive the reporter

gene. The assay presented here is based on silencing of the integrated promoter and thus this promoter must be constitutive and strong to be useful in this application. Any selection marker can be used; we recommend neomycin because of its availability and the low cost of G418 compared to other selection antibiotics.

### *Development and Basic Characterization of Cell-Based Fluorescent Assays to Screen for Epigenetic Modulators*

Once the mammalian cell line, promoter–reporter gene construct, and antibiotic resistance gene are chosen, most cells can be stably transfected using the following procedure.

1. Exponentially growing mammalian cells are transfected with expression vectors for a GFP-tagged construct (or other reporter) driven by a strong promoter (we recommend the CMV promoter) and for aminoglycoside phosphotransferase, which confers neomycin resistance, in a 20:1 molar ratio using Lipofectamine 2000. Fugene 4 is an effective alternate reagent for transfection. The transfected cells are then incubated under standard conditions.
2. Forty-eight to 72 h posttransfection, the cells are split 1:5 or 1:10 into separate dishes and allowed to reattach.
3. Once attached, the cells are exposed to selection media consisting of growth media supplemented with 1 mg/ml G418. The amount of G418 may have to be optimized depending on the cell line used. Most manufacturers of G418 have sample killing curves on their web sites that can serve as reference.
4. The selection is continued for 2 to 3 weeks with selection media being replaced every other day.
5. Once the majority of the cells are dead, colony formation is monitored by inspection and the corresponding areas are marked on the bottom of the dish.
6. When colonies reach approximately 1 to 3 mm in diameter, they are detached with 1 to 2 $\mu$l of trypsin and placed directly into wells of a 24-well dish already containing fresh selection media.
7. Antibiotic-resistant colonies are progressively expanded and monitored for fluorescence under a fluorescent microscope or plate reader. Colonies that are consistently negative for GFP expression are further expanded and some of these are chosen for characterization. Note: depending on the type of fluorescent microscope and objective or plate reader being used, plates where the colonies are growing may need to have glass or optic plastic bottom surfaces for optimal imaging or monitoring of GFP expression.

Once neomycin-resistant clones with a stable GFP-negative phenotype are obtained, they should be characterized for their response to known epigenetic modulators such as known inhibitors of HDACs or DNMTs. This can be done as follows.

1. Logarithmically growing stable cell lines are plated on glass bottom chamber slides (e.g., Labtek II from Nalge-Nunc) at about 30 to 40% confluency and are allowed to attach overnight.

2. Cells are then treated with known epigenetic modulators for 6–18 h and the induction of GFP expression is monitored by fluorescence microscopy on a fluorescent plate reader or by FACS analysis. Although the concentrations of HDAC and DNMT inhibitors may have to be optimized, we have found that 50 ng/ml of TSA, 25 m*M* sodium butyrate or 1 $\mu M$ 5-aza-deoxycytidine give excellent GFP expression. Note: 5-aza-deoxycytidine is very unstable. Only use stocks freshly made the day of the induction. In our hands, frozen stocks of TSA and sodium butyrate are stable for 1 to 3 months at –20°.

3. Based on the GFP expression profile of the cell lines, one can determine which cell lines show the more robust and homogeneous response, which can then be characterized further. An example of a robust and homogeneous response is shown in Fig. 2. It is important to freeze down a number of stocks of early passages of the chosen cell lines. Note: stable cell lines should be monitored regularly to ensure the stability of the negative-GFP phenotype. After prolonged culture, some cells may begin to

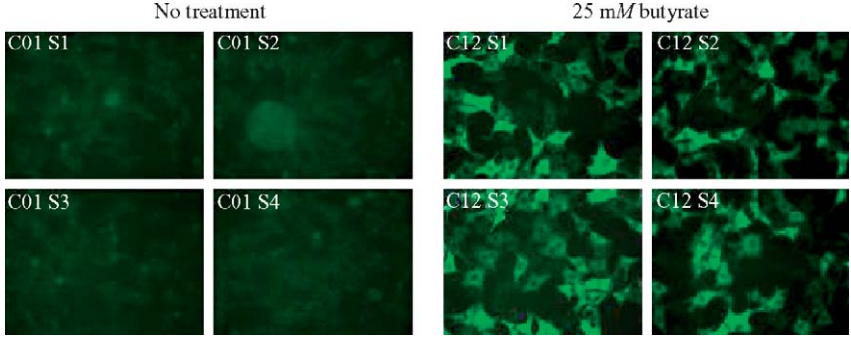


FIG. 2. Robust and homogeneous cellular response to inhibitors. Cells were plated on glass-bottom 96-well plates and the next day they were treated with 25 m*M* sodium butyrate or with 0.5% DMSO vehicle (labeled no treatment). After overnight incubation, cells were fixed and imaged using a Discovery-1 automated system (Molecular Devices). Four areas per well were imaged showing clear fluorescent signal only in wells that were treated with HDAC inhibitor. Induction of GFP expression by sodium butyrate is the positive control for the assay and is the basis for identification of hit compounds.

spontaneously express GFP. These cells will not be useful for screening purposes and thus should be discarded.

Basic molecular characterization of the stable cell line(s) that shows a clear and constant negative GFP phenotype and a robust response to known epigenetic modulators can then proceed. This characterization can include the following.

1. ChIP analysis of histone acetylation status at the integrated CMV promoter.
2. Southern blot analysis to determine the approximate copy number of inserts integrated in the genome.
3. Metaphase DNA spreads combined with fluorescence *in situ* hybridization (FISH) to determine the chromosomal location of insertions.
4. GFP induction dose–response curves and $EC_{50}$ calculations to determine the potency and efficacy of the various chemical epigenetic modulators.
5. FACS analysis of cells induced with maximal doses of chemical epigenetic modulators to determine the total percentage of cells that respond by expressing GFP. For automated screening purposes the cell line of choice should have greater than a 60% response rate.
6. Before proceeding on to primary screening, the cell line of choice should exhibit, in addition to the qualities described already, reproducibility of induction by the various drugs. Several frozen stocks of varying passages should be tested to ensure reproducibility, robustness, and at least moderate homogeneity in the response. Subcloning of cells may be necessary if these qualities are not maintained over time.

Note: while some of the aforementioned characterization is not strictly necessary for drug screening purposes, we strongly recommend that the cell line be studied as noted in 1, 4, 5, and 6 *before* any screening is done.

### *Setup and Optimization of Primary Screens*

Depending on the imaging platform or reader to be used and on the cell type, the first step is to examine how well the cells grow on various kinds of multiwell plates, including doubling time, and how they tolerate assay conditions, as well as the quality of the image or readout acquired using the different plates. The goal is to establish the best conditions for both cell growth and imaging/readout quality. Using the Discovery-1 imaging system from Molecular Devices, we found that optimal cell adhesion and image quality were obtained with Nunc glass-bottom 96-well plates. These plates were selected for use in the screen due to the clarity of acquired images and the ability of the cells to grow on the glass and remain attached after

repeated washings. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4 m*M* L-glutamine, 1 m*M* sodium pyruvate, 1% penicillin/streptomycin, 1 mg/ml G418, and 10% fetal bovine serum for the entirety of the assay. Optimal growth conditions for the assay may need to be established for each cell type.

Before proceeding further, cells should be tested for their tolerance to the standard vehicle in which the compounds in the library are dissolved. This is typically dimethyl sulfoxide (DMSO). DMSO tolerance can be evaluated in a 96-well plate format using a sulforhodamine staining protocol for cell viability. The DMSO tolerance threshold is critical in determining compound dilution protocols and in deciding on the final concentrations to be tested in the screen. The cells are treated with increasing doses of DMSO for 48 h. Then the cells are fixed, stained, and quantitated with sulforhodamine B staining as detailed next.

1. Plate cells into 96-well Nunc plastic plates at 60,000 cells/well in complete media at 100 $\mu$l/well. Add increasing doses of DMSO diluted in growth media in a 50-$\mu$l total volume to each well for final concentrations of 0, 0.05, 0.1, 0.25, 0.5, 1, 2, 5, and 10% (repeat each concentration in at least 3–4 wells). Allow the cells to grow for 48 h in the presence of DMSO under standard conditions. Each well contains a 150-$\mu$l volume at this point in the procedure.
2. Fix the cells by adding 50 $\mu$l/well of 40% cold trichloroacetic acid (TCA) for 30 min at room temperature (for final concentration of 10% TCA).
3. Decant the media containing TCA and rinse the plates five times with water. Air dry the plates.
4. Add 100 $\mu$l SRB (0.4% sulforhodamine B [SRB], 1% acetic acid) per well and stain for 1 h at room temperature.
5. Rinse the plates five times with 1% acetic acid. Air dry the plates (must be completely dry).
6. Extract SRB with 100 $\mu$l of 10 m*M* Tris base per well.
7. Shake and read at 520 nm using a SpectraMax 250 plate reader from Molecular Devices or similar equipment.

As can be seen in Fig. 3, our cells were able to survive for 48 h in up to 1% DMSO. Consequently, the assay was designed so that compounds were added for a 0.5% final DMSO concentration.

Among known inhibitors of epigenetic pathways active in our system, sodium butyrate was selected as a positive control compound for the screen due to its stability in cell culture, low cytotoxicity, and availability. Various doses in the millimolar range were tested and examined for GFP expression on the imaging system. A dose of 25 m*M* sodium butyrate for 24 h was

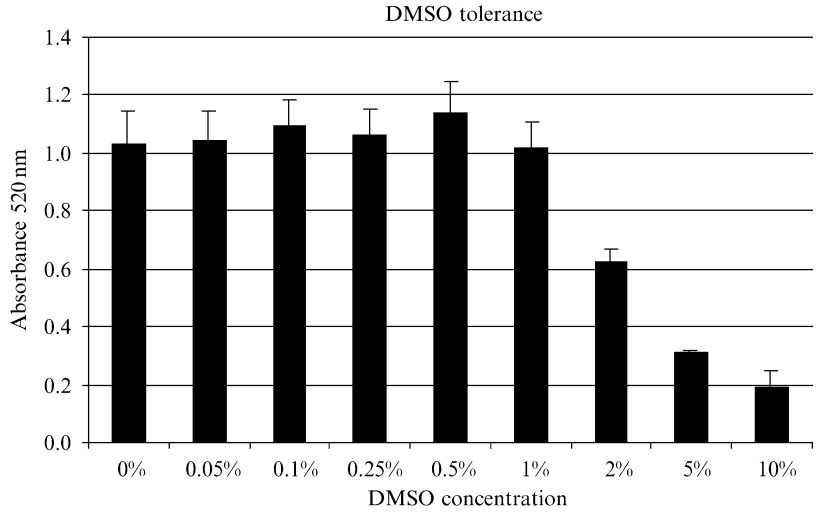


FIG. 3. DMSO tolerance assay. C127-derived cells were treated with increasing amounts of DMSO for 48 h and cell viability was measured by staining with SRB. These cells are able to tolerate 1% DMSO for 48 h.

routinely utilized in the assay (see Fig. 2). In our case, because the GFP signal was not bright enough for reliable measurement on a fluorescence plate reader, the images were acquired on the Discovery-1 imaging system, which has greater sensitivity. Each system should be optimized to obtain strong positive control signals and a large dynamic range of fluorescence detection. To facilitate location of the focal plane on the imaging system, nuclei are stained using Hoechst 33342 (2 $\mu$g/ml). Hits can be identified by visual inspection of the images for GFP expression, or by automatic intensity measurements, depending on the robustness of the assay, and the degree of automation used.

We suggest starting with small validation libraries such as the Library of Pharmacologically Active Compounds (Sigma) before other chemical and natural products libraries are screened in the assay. Libraries can be selected based on screening rationale. For example, the presence of chemical diversity is conducive for the discovery of novel compounds, whereas libraries of structurally related compounds may aid in finding analogs of existing drugs with improved pharmacokinetics. Compounds are added to the plates containing the cells using liquid-handling systems such as the Biomek FX (Beckman Coulter). The FX program may need to be modified for attached cells to ensure that the monolayer is not disrupted during the addition of compounds. Pipette tip height above the bottom of the well,

speed of addition, mixing volume, and speed of mixing may need to be optimized to maintain the integrity of the cell monolayer during compound addition.

Screening for epigenetic modulators is as follows.

1. On day one, seed cells at 70,000 cells/well into Nunc glass-bottom 96-well plates (100 $\mu$l/well) in complete media. Grow the cells overnight at 37°, 5% $CO_2$ , 95% air in a humidified incubator. Note: the cell number may need to be adjusted according to cell type and doubling time.
2. On the following morning, treat the 96-well plate as follows for 24 h.
   a. Column 1 (negative control): add 50 $\mu$l of 1.5% DMSO solution in complete media for 0.5% final DMSO concentration.
   b. Column 12 (positive control): add 50 $\mu$l of 75 m*M* butyrate for 25 m*M* final concentration.
   c. Columns 2–11: add 30 $\mu$l complete media + 20 $\mu$l compounds from chemical or natural products library for 0.5% final DMSO concentration and 5 $\mu M$ concentration of compound. Compounds are added to the plates using the Biomek FX liquid handling system from Beckman Coulter or similar device.
3. On the third day, after 24 h of drug treatment, fix the cells in media containing compounds and stain nuclei by adding 4% final formaldehyde and 2 $\mu$g/ml final Hoechst 33342 for 1 h. Wash the plates three times with 200 $\mu$l of phosphate-buffered saline and add a final volume of 100 $\mu$l/well for imaging. Acquire images or read on a plate reader. For the Discovery-1 imaging system, we recommend using a 20× Nikon Plan Fluor objective and exposing for 600 ms in the fluorescein isothiocyanate (FITC) channel and 30 ms in the DAPI channel. Four sites per well can be acquired for each GFP and Hoechst signal for thorough querying of each well. It is best to autofocus on the DAPI channel first, acquire these images, and then image in the FITC channel without refocusing. Note: fixing the cells and staining nuclei are optional. Depending on the imaging system used, these steps can enhance image quality greatly.

A number of compounds may exhibit autofluorescence, which can produce false-positive hits in the screen. In order to circumvent this issue, compounds should be screened in parallel against the untransfected parental cell line. Hits identified in the assay can be compared with the parental cell line to determine if the compound was a hit or a false-positive fluorescent compound.

Cherry picking is used to confirm hit compounds by selecting hits from the original compound plate and transferring to a plate containing only hits from the primary screen for retesting. Hits from the primary screen can be cherry picked using the Multiprobe robotic handling system

from Packard and reevaluated in the assay to confirm activity. After hit compounds are confirmed with cherry-picking experiments, the compounds are tested in secondary and tertiary assays. We recommend running retesting experiments at least twice for validation of the primary hits.

*Evaluation of Hit Compounds*

A screening assay represents only the very first step in discovery. It is essential following any screen to counterscreen the confirmed hits in assays that complement the initial screening assay in order to establish specificity. In the case of the current assay, the primary goal is to determine if the hit compound actually hits target enzymes in epigenetic pathways (e.g., HDACs or DNMTs). This is established by conducting cell-free enzymatic assays (see later).

A second consideration when prioritizing hits is to consider the chemical and pharmaceutical properties of the hit. In a perfect world, all library compounds would have desirable ''drug-like'' properties; however, most libraries contain compounds that are less than ideal as molecular probes or drug leads. The most common type of undesirable compound are those with reactive functional groups capable of binding covalently to target molecules, at sulfhydryl groups of cysteine residues, $\beta$-amino group of lysines, or other sites within biological macromolecules. Because such sites occur on nearly all proteins, the activity of compounds with reactive functional groups observed in a single assay is likely to be nonspecific in nature, as the compound may react with many proteins. With a cell-based assay, reactive compounds pose less of a problem than in cell-free biochemical assays; however, they do turn up. One example of this found in our screening campaign was a platinum complex. A second class of undesirable compound is those that are broadly toxic, such as perhalogenated organics derived from pesticides, a group that we also encountered as active with our assay.

Drug likeness has been defined by Lipinski's well-known rule of five, which is aimed at identifying synthetic molecules that will have a good chance of being orally bioavailable drug candidates. These rules require that a compound have less than 5 hydrogen bond donors, less than 10 hydrogen bond acceptors, a log $P$ of $<5$, and a molecular mass of $<500$ Da (Lipinski *et al.*, 2001). However, the rules explicitly exempt natural products due to the built-in evolutionary pressures that demand a measure of bioavailability to be useful toxins and modulators or to serve other ecological roles. We chose to ignore the rule of five in evaluation of hits from this assay, as we were willing to accept compounds as molecular probes, for which the physicochemical requirements are less stringent than for drugs. Nonetheless, many of the hits fell within the limits of the rule of five.

Once a hit has been confirmed, it is desirable to explore the chemical space surrounding its structure to determine if a lead series of analogs can be developed. This can be done in the case of NCI structural diversity set compounds by substructure searches of the full NCI repository to identify a number of related compounds, which can then be tested in the primary assay. Commercially available analogs can also be identified using ChemNavigator software (http://www.chemnavigator.com), which facilitates procurement of compounds from a wide variety of commercial sources. If a limited number of samples are screened, it is possible to analyze the structural relationships of hit compounds by inspection. If a larger number of hits require analysis, a software package such as Leadscope (http://www.leadscope.com) may be used to cluster hits by structure.

### *Available Secondary Screens for the Evaluation of Candidate Epigenetic Modulators*

Once the candidate compounds have been confirmed in cherry-picking experiments and/or characterized as described earlier, before going on to perform mechanistic secondary assays, we recommend that the hits be tested on a different clone than the one used in the primary screens, that is, one of the original cell clones that was characterized as GFP negative, antibiotic resistant, that responded to known HDAC or DNMT inhibitors but which was not selected for use in the screen. This will ensure that the compounds have a transcriptional effect independent of the insertion site of the GFP construct, of copy number, etc.

Secondary mechanistic assays can then proceed. Among the available secondary assays, the ones listed here are particularly relevant. We limit ourselves to brief descriptions of each assay as detailed protocols can be found elsewhere and fall outside the scope of this chapter.

1. Western analysis of global histone acetylation. Candidate epigenetic modulators can be assayed for their ability to cause global increases in histone acetylation by treating cells overnight with the individual compounds and appropriate positive and negative controls and then performing Western analysis on the total cell lysates with antibodies specific to the various modifications of histones 3 and 4. Although many antibodies against modified histone 3 and 4 are available commercially, our experience with Upstate antibodies has been very good.

2. ChIP to measure histone acetylation at the integrated silenced promoter. As has been described by others (El Osta and Wolffe, 2001; Lambert and Nordeen, 2001), chromatin immunoprecipitation assays can be useful in identifying the modification status of particular histones at a given genomic loci. Cells can be treated with classical epigenetic

modulators as described earlier in this chapter and with candidate compounds in parallel for various lengths of time, as determined by GFP induction time courses, taking into consideration that histone modifications presumably are a relatively early event in the induction process. Standard ChIP assays can then be performed using primers specific for the promoter of interest, for example, CMV, or the promoter of a silenced tumor suppressor gene for the polymerase chain reaction (PCR).

3. *In vitro* HDAC inhibition assays. Several active, purified recombinant human HDACS, including HDAC 8, Sirt 1, 2, and 3, are currently available from Biomol, Upstate, and other companies. In addition, several investigators have developed protocols for the purification of active enzymes (Li *et al.*, 2004; Schultz *et al.*, 2004). Active, purified recombinant proteins can be combined with labeled hyperacetylated histone substrates or commercial fluorophore conjugated substrates, and the activity of the enzymes can be monitored in the presence or absence of candidate inhibitors or control drugs.

4. *In vitro* DNMT inhibition assays. These are performed using purified recombinant mammalian DNMTs or bacterial methylases using appropriate oligonucleotide substrates in the presence of candidate or control inhibitors (Brueckner *et al.*, 2005;Yokochi and Robertson, 2004). Presumably, this assay will only detect inhibitors that do not require incorporation into DNA, that is, those that can inhibit soluble DNMTs.

5. Reexpression of silenced tumor suppressor genes in human cancer cell lines. The RNA expression levels of a silent tumor suppressor gene (by RT-PCR) or its protein expression levels (by Western blotting) can be measured after treatment with candidate or control compounds.

6. Demethylation of hypermethylated promoters in human cancer cell lines. The methylation status of aberrantly hypermethylated promoters can be measured by bisulfite sequencing or by methylation-specific PCR after treatment of cells with candidate or control compounds.

Hits that are demonstrated to act via acetylation or methylation mechanisms should be then submitted for chemical optimization and developed further as drug leads. Evaluation of how the compounds may interact with detoxification pathways should be a component of lead development.

## General Conclusions and Perspectives

The methods described in this chapter provide a simple, relatively economic way of developing a fluorescence-based mammalian cell assay for the identification of novel epigenetic modulators. The detailed protocols provided for the basic characterization of hits can be useful not only in analyzing epigenetic modulators, but also in dealing with hits from other

assay systems. Furthermore, the principles upon which the assay is built can be directly applied as described in this chapter or can be used to develop assay variations such as to screen for inducers or silencers of a particular promoter. Combining the power of GFP technology with the biology of the cell can give rise to creative avenues to search for solutions to cellular conundrums and therapeutic needs.

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