NIH Chemical Genomics Center: Small-Molecule Screening for Investigating Fundamental Biological Questions

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The NCGC uses the latest industrial-scale technologies to perform quantitative HTS (qHTS), a dose-response screning paradigm (6), to enable cellular- and biochemical-based assays to identify chemical probes that modulate the activity of biological targets. All assay results are posted to the PubChem public database.

INTRODUCTION

The NIH Chemical Genomics Center (NCGC; www.NCGC.nih.gov), created in 2004, is part of the Molecular Libraries Screening Center Network (MLSCN), which is a network of screening centers created to discover small molecules for fundamental biological research as well to validate drug targets. NCGC optimizes high-throughput cellular-based and biochemical assays submitted by the biomedical research community. The Center performs automated HTS assays as well as chemical optimization on confirmed hits to produce chemical probes for distribution to public-sector researchers. Researchers can use these small molecule probes to interrogate basic biological pathways in their area of interest.

The NCGC is focused on ultrahigh-throughput screening (uHTS) and generation of chemical probe series, and the work of the NCGC will complement the ongoing screening programs of drug discovery and pharmaceutical companies. However, the goal of the NCGC is not to discover new block-buster drugs but to identify compounds that can be used to validate new drug targets or provide information about complex biological pathways. Researchers can submit assays to the NCGC through the MLSCN program. Proposed assays must be compatible with HTS platforms and address novel biological questions. Guidance for researchers on submitting assays is provided in the Molecular Library Initiative Program Announcement (1) on the NIH Roadmap Web site. Proposed assays undergo peer review prior to acceptance.

All of the results from the MLSCN's activities are placed in the public database PubChem (pubchem.ncbi.nlm.nih.gov), and information about probe compounds is available to all researchers, in both public and private sectors, for their use in studying biology and disease. Here we highlight a number of different assays the NCGC developed for their HTS efforts. Each of the assays described is identified by its PubChem BioAssay identifier (AID) number, and the full details of the assays, as well as the results, are available in PubChem.

NCGC ASSAY DEVELOPMENT GUIDANCE

An assay suitable for uHTS needs to be robust — producing results with minimal nonspecific variation. The NCGC offers an assay guidance manual on their Web site (2). The manual provides guidance to investigators who are interested in developing assays for the evaluation of compound collections to identify chemical probes that modulate the activity of biological targets. In this context, an assay is defined by a set of reagents that produce a detectable signal allowing a biological process to be quantified. Generally, the quality of an assay is defined by the robustness and reproducibility of the signal in the absence of test compounds or in the presence of inactive compounds. Robustness will depend on the type of signal measured (absorbance, fluorescence, luminescence, radioactivity, etc.), reagents, reaction conditions and the analytical and automation instrumentation that is used (2).

The NCGC assay guidance manual offers detailed information on the development of seven different categories of assays: 1) enzyme assays for identification of competitive, reversible inhibitors; 2) receptor binding assays in both filtration and scintillation proximity assay (SPA) formats for the analysis of receptor-ligand interactions in screening applications; 3) GTP S binding assays for the binding of GTP to the alpha subunit of heterotrimeric GTP binding proteins; 4) tissue culture or cell-based assays including proliferation assays, apoptosis assays, gene expression assays and activation assays; 5) cell-based ELISA or Western (cell-based ELISA or C-ELISA) assays, a moderate-throughput format for detecting and quantifying cellular proteins including post-translational modifications associated with cell activation; 6) FLIPR® (Fluorescence Imaging Plate Reader) assays for measuring GPCR and ion channel targets; and 7) immunoassays for quantifying an analyte when the concentration in a sample is unknown.

Promega has worked with NCGC and MLSCN to provide assay tools to help accomplish their goals. In this article, we highlight some examples of these assays.

CELL-VIABILITY ASSAYS

The NCGC developed a 1536-well cell-based assay for quantitative high-throughput screening (qHTS) against a number of cell lines to determine in vitro cytotoxicity of small molecules using the CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570), a homogeneous method to measure the number of viable cells in culture. The assay can be used to quantitate intracellular ATP, an indicator of metabolic activity, using the luciferase reaction. Luciferase catalyzes the oxidation of beetle luciferin to oxyluciferin and light in the presence of ATP. The luminescent signal is proportional to the amount of ATP present.

The CellTiter-Glo® Assay has been used to screen small molecules for cytotoxicity using a number of cell lines including renal proximal tubule cells that are derived from normal kidney cells freshly isolated from rat (PubChem AID 545), HEK293 cells (PubChem AID 427), Jurkat cells (PubChem AID 426), NIH/3T3 cells (PubChem AID 541), a kidney mesenchymal cell line that is derived from normal human renal glomeruli (PubChem AID 546), and several human lymphoblastoid cell lines (Coriell Institute for Medical Research; PubChem AID 963–989).

The CellTiter-Glo® Assay also has been adapted by other members of the MLSCN. The Penn Center for Molecular Discovery developed a 384-well assay for screening the cytotoxicity of doxorubicin (provided spiked in 30 random locations in a 384-well plate from Discovery Partners International (DPI) through the MLSCN) against human pulmonary artery cells (HPAECs), which are a target of the aerosol delivery of therapeutic agents (PubChem AID 370).

The Southern Research Molecular Libraries Screening Center developed a 384-well cell-based assay for measuring the cytopathic effect (CPE) induced in Madin Darby canine kidney (MDCK) cells by influenza virus infection (H5N1) using the CellTiter-Glo® Assay to detect the endpoint signal (3). The assay challenged MDCK cells with H5N1 virus at an MOI of 0.005 in the presence of the compounds to be screened. Cell survival was measured after 72 hours using the CellTiter-Glo® Reagent (PubChem AID 740).

CYP450 ASSAY

The P450 gene superfamily is involved in the metabolism and clearance of xenobiotics. For example, the NCGC used human CYP2C9 to measure the hydroxylation of deoxyluciferin (Luciferin-H; P450-Glo™ CYP2C9 Assay; Cat.# V8791) to luciferin. Luciferin was measured by luminescence after adding the luciferase detection reagent. The Luciferin-H concentration in this assay was equal to its Michaelis constant K_M for CYP2C9 (PubChem AID 883). As well, in a comprehensive profile of firefly luciferase activity associated with PubChem compounds, the NCGC noted that UltraGlo™ luciferase present in the CYP assay showed a marked resistance to typical luciferase inhibitors (ref B), so that decreases in luminescence could be reliably assigned to CYP activity. The assay has been adapted for other cytochrome P450 enzymes as well (Pubchem AIDs: 410, 884,885,899,891).

APOPTOSIS ASSAY

The NCGC used the Caspase-Glo® 3/7 (Cat.# G8090) assay in a 1536-well format to measure the amount of caspase-3/7 activity in rat kidney proximal tubule cells with complete culture medium following compound treatment for 16 hours (PubChem AID 667). The Caspase-Glo® 3/7 Assay is a homogeneous method that measures caspase-3 and caspase-7 activities in cultured cells. The assay is based on the cleavage of a peptide-aminoluciferin substrate. Following cleavage, the liberated aminoluciferin serves as the substrate of luciferase (provided in the assay reagent), which generates the light. The luminescent signal is proportional to the amount of caspase-3/7 activity present. The ability of library compounds to cause the activation of caspase-3/7 in the cell line is reflected by a concentration-dependent increase in luminescence signal.

IN VITRO CYTOTOXICITY ASSAY

NCGC developed a 1536-well cell-based assay for quantitative high-throughput screening (qHTS) against a number of human lymphoblastoid cell lines (purchased from Coriell Institute; PubChem AID 946, 947, 955, 960, 961, 962) to determine in vitro cytotoxicity of small molecules. The assay uses the MultiTox-Fluor Multiplex Cytotoxicity Assay (Cat.# G9200), a homogeneous method that simultaneously measures the relative number of live and dead cells in culture by detecting changes in cell membrane integrity using two fluorogenic peptide substrates. Viable cells are detected when the cell-permeant substrate (GF-AFC) enters a live cell and is cleaved by a specific protease, resulting in liberated fluorophores. This live-cell protease activity is restricted to viable cells and is proportional to cell number. Dead cells are determined by using the fluorogenic cell-impermeant peptide substrate (bis-AAF-R110), which is cleaved by a specific dead-cell protease leaking from damaged cell membranes. Library compounds are measured for their ability to cause acute toxicity in the cell line, as reflected by a concentration-dependent decrease in live-cell protease activity.

cAMP RESPONSIVE ELEMENT CELL-BASED LUCIFERASE ASSAY

Long-term memory (LTM) lasts for days or longer and may be due to the formation of new synapses or restructuring of existing synapses. Long-term, but not short-term memory requires gene transcription and synthesis of proteins. Cyclic-AMP Responsive Element Binding Protein (CREB) is one of the candidates that might play an important role for enhancement of some types of LTM. CREB, a family of transcription factors, regulates

Assay guidance

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7) immunoassays.

the transcription of genes containing cAMP Responsive Elements (CRE). CRE is a nucleotide sequence often found in the promoter regions of genes that are binding sites for CREB.

The 1536-well format, cell-based assay uses the luciferase reporter gene under the control of the cAMP responsive element found in the GloResponse™ CRE-*luc2P* HEK293 Cell Line (Cat.# E8500) to measure forskolin-induced signaling (PubChem AID 907). The CREB family of transcription factors is one of the candidates that might play an important role for enhancement of some types of long-term memory. In this assay, the selected compounds were measured for their ability to modulate positively the action of EC10 NKH 477, a water-soluble forskolin, on reporter gene activity.

DUAL-REPORTER NF-KAPPA-B ASSAYS

Nuclear factor kappa-B (NF-κB) plays an important role in normal B cell development and survival. Diffuse large B cell lymphoma (DLBCL) is the most commonly observed type of non-Hodgkin's lymphoma. Gene expression analysis has identified an activated B cell-like subtype of DLBCL (ABC-DLBCL) that expresses known NF-κB target genes. In ABC-DLBCL cell lines this is due to high constitutive activity of I-κB kinase (IKK), a key regulator of NF-κB. Low molecular weight molecules that inhibit IKK have been shown to be selectively toxic for ABC-DLBCL cell lines. However, targets upstream of IKK have been largely unexplored, and so nodes upstream of IKK in the NF-κB pathway remain untested due to the lack of chemical probes.

The NCGC optimizes high-throughput cellular- and model organism-based assays to collect information that will help researchers characterize the activity of small-molecule chemical probes in biological systems.

This cell-sensor assay for I-κB-α stabilization was developed with NIH investigator Dr. R. Eric Davis in Dr. Louis M. Staudt's laboratory (5; PubChem AID 445). The assay uses the Chroma-Glo™ Luciferase Assay System (Cat.# E4920), which contains green- (CBG68) and red- (CBR) emitting beetle luciferases. The green luciferase is fused to I-κB-α, and the red luciferase is present in its native state. Both luciferase genes are stably present in the OCI-Ly3 human ABC-DLBCL cell line, but their expression is under the control of tetracycline-inducible promoters. Following the addition of doxycycline to induce expression of the luciferases, the green luminescence in control cultures rises only slowly because the CBG fusion partner I-κB-α is targeted by IKK activity for rapid degradation. In contrast, green luminescence rises rapidly in the presence of proteasome inhibitors or IKK inhibitors. The rise in red luminescence of CBR is not affected by proteasome or IKK inhibitors and serves as normalization for cell number and nonspecific effects.

H358 CELL-VIABILITY SECONDARY ASSAY: qHTS ASSAY FOR EPIGENETIC MODULATORS

NCGC describes secondary assays as assays that follow the primary assays and are used to confirm the biological activity of chemical entities identified in the primary assays. These assays can include selectivity and specificity assays (2). The NCGC used the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Cat.# G3582) as a 96-well, cell-based secondary assay (PubChem AID 1043) to help characterize selected activities identified in the qHTS of the Locus Derepression (LDR) assay (PubChem AID 597). The LDR assay detects the derepression of a GFP reporter that is stably integrated in a region of the genome of murine c127i mammary cells that is presumably silenced. Selected LDR activities were assayed for killing of H358 non-small cell lung carcinoma (NSCLC) cells. Certain epigenetic drugs, like histone deacetylase inhibitors, can block cancer cell growth. H358 cell viability was measured using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, which detects the ability of metabolically active cells to reduce a tetrazolium salt substrate (MTS) into a soluble formazan product that can be measured by colorimetry.

SUMMARY

As part of the MLSCN, the NCGC is helping the research community capitalize on the wealth of information provided by the Human Genome Project. The center, standing at the cross-section of chemistry and biology, uses the latest industrial-scale technologies and ultrahigh-throughput biological assays to collect information that will help researchers characterize the activity of small molecule chemical probes in their biological systems. Promega will continue to work with NCGC and MLSCN to provide assay tools to help achieve these goals.

REFERENCES

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