Promega

High-Throughput Compound Profiling Using Tecan Instrumentation

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Abstract

Combining high-throughput, homogeneous screening assays and integrated automated instrumentation allows researchers to efficiently profile compounds that may be potential drugs. To assess if the compounds of interest are worth pursuing, multiple assays are required to examine cell responses (e.g., apoptosis) and effects on nontarget biochemical systems such as cytochrome P450 and kinases. Knowing how the drug affects its target and becoming aware of any potential cytotoxicity and other off-target effects are essential in the drug discovery decision-making process.

Automated instrumentation combined with high-throughput luminescent screening assays is an excellent tool for profiling compounds being considered for drug development.

Introduction

The drug discovery process requires the use of diverse assays to assess the various effects of compounds. Target-specific assays, as well as those that assess off-target effects and cytotoxicity, are all helpful in generating a broad profile of compound reactivity. Many times, results from diverse assays can drive lead selection and optimization for preclinical testing. With the demand to "fail fast, fail cheap", using a broad variety of assay types can help guide decisions regarding the drug-development pathway—which compounds to pursue or which to withdraw. In addition, results from profiling efforts can help improve compound library configurations so that compounds exhibiting broad toxicity or off-target activity can be removed.

Sophisticated liquid-handling and detection instrumentation can be used to generate profiling data. When cell dispensing, compound and reagent addition, and signal detection are all coupled onto one platform, the user has a turnkey solution for performing a variety of profiling assays within a completely integrated robotics system.

To demonstrate, we used Tecan instrumentation that is commonly found in screening facilities running assays in a 384-well format. This Tecan instrumentation was combined with 11 different homogeneous luminescent assays to generate a profile of compound activities. Table 1 summarizes the assays used in this study.

Table 1. The Luminescent Assay Profile Panel.

Assay	Assay Type	Parameter Measured
P450-Glo™ Screening Systems	Biochemical	Oxidation of specific substrates by cytochrome P450 CYP1A2, 2C9, 3A4, 2C19 or 2D6.
Kinase-Glo® Plus Assay	Biochemical	ATP remaining after a kinase reaction.
MAO-Glo™ Assay	Biochemical	Oxidation of an ester-luciferin substrate by monoamine oxidase.
Pgp-Glo™ Assay System	Biochemical	ATP remaining in solution following stimulation of P-glycoprotein ATPase activity.
CellTiter-Glo® Assay	Cell-Based	ATP contained within viable cells.
Caspase-Glo® 3/7 Assay	Cell-Based	Activity of caspase-3 and -7 in apoptotic cells via cleavage of a Z-DEVD-luciferin substrate.
Dual-Glo™ Assay System	Cell-Based	Firefly and *Renilla* luciferase reporter activity.

To generate the data for this study, 40 compounds were tested with each assay. Three representative compounds, PKI, H 89 and dihydrexidine, were chosen for discussion. The results generated were used to determine the IC_{50} or EC_{50} value for each respective assay and compound combination. IC_{50} refers to the concentration of a compound that inhibits a reaction by 50%, and EC_{50} is the concentration of an agonist that produces 50% of the maximum possible response for that agonist.

Automated Liquid Handling and High-Throughput Detection

The profiling assays run in this study were all performed on the Tecan Freedom EVO® 200 system. This includes an expandable eight-tip LiHa, a RoMa arm with gripper, an integrated Te-Mo™ multichannel pipetting system and the multifunctional GENios Pro™ plate reader. The Tecan system was used to dispense cells, dilute and transfer compounds to plates, add reagent, and read plates.

The scripts for performing the liquid handling and plate movements for each assay were written using the Tecan Freedom EVOware® standard software. Tecan Magellan™ software was used to operate the GENios Pro™ plate reader and export raw data results to Microsoft® Excel. Table 2 highlights the general assay protocol used for performing both cell-based and biochemical assays with this automated system.

Table 2. General High-Throughput Assay Protocol.

Biochemical Assays	Cell-Based Assays
1. Add water and DMSO to a deep-well, 96-well assay plate.	1. Dispense cells* to 384-well plates with Te-Mo™ System.
2. Perform serial 1:3 dilutions of test compounds with Te-Mo™ System on Freedom EVO® 200.	2. Add media and DMSO to a deep-well, 96-well assay plate.
3. Transfer test compounds** in quadruplicate to 384-well assay plates with Te-Mo™ System.	3. Perform serial 1:3 dilutions of test compounds with Te-Mo™ System on Freedom EVO® 200.
4. Dispense enzyme, substrate and other assay components with Te-Mo™ System.	4. Transfer test compounds** in quadruplicate to 384-well assay plates with Te-Mo™ System.
5. Move plates to shaker with RoMa arm.	5. Move plates to shaker with RoMa arm.
6. Incubate. **Note:** All assays were performed at 22°C with the exception of Pgp-Glo™ and P450-Glo™ CYP2C9 assays, which were performed at 37°C.	6. Incubate plates at 37°C with 5% CO_2 (Jurkat), or 37°C with 10% CO_2 (HEK 293).
7. Add detection reagent to plates with Te-Mo™ System.	7. Add detection reagent to plates with Te-Mo™ System.
8. Move plates to shaker with RoMa arm.	8. Move plates to shaker with RoMa arm.
9. Transfer plates to GENios Pro™ plate reader with RoMa arm.	9. Transfer plates to GENios Pro™ plate reader with RoMa arm.

*Jurkat cells were dispensed and used immediately. HEK 293 cells were dispensed and allowed to equilibrate and attach overnight before use.

**Final concentration range of dihydrexidine and H 89 compounds was 100µM to 0µM. The final concentration range for PKI was 1µM to 0µM.

Cell-Based Assays

Two different cell lines were used with cell-based assays for cell viability, apoptosis and a dual-reporter system. For the CellTiter-Glo® and Caspase-Glo® 3/7 Assays, Jurkat cells were plated at a density of 5,000 cells per well in a 384-well plate. Following cell dispensing, test compounds were diluted and added to the wells as described in Table 2, and plates were incubated at 37°C with 5% CO_2 for either 4 or 18 hours. The two incubation time points allowed monitoring of short-term and long-term cytotoxicity, respectively. To monitor cytotoxicity, the CellTiter-Glo® Reagent was added to the wells of the plate following the 4-hour or 18-hour incubation, and luminescence was recorded 10 minutes later. The same process was used for the Caspase-Glo® 3/7 Reagent, but the incubation was extended to 30 minutes post-reagent addition before recording luminescence.

For the Dual-Glo™ Luciferase Assay System, a HEK 293 cell line stably transfected with firefly and *Renilla* luciferase was used to assess off-target effects on the G-protein coupled receptor (GPCR) dopamine receptor D1 (DRD1) via the $G\alpha_s$ signaling pathway. The stable cell line was established using two plasmids: One expresses the firefly luciferase gene under the control of cAMP response element (CRE)/promoter and a hygromycin selectable marker; the other plasmid expresses the GPCR DRD1 and a *Renilla* luciferase-neomycin selectable marker fusion. The effects of compounds were determined by measuring firefly and *Renill*a luciferase signals, and the firefly luciferase signal was normalized with the *Renilla* luciferase signal. An agonist to the GPCR DRD1 will result in an increase in firefly luciferase luminescence as the concentration of the test compound increases. An antagonist will show the opposite result over the same concentration range.

The cells were plated at a density of 5,000 cells per well in a 384-well plate and allowed to adhere overnight at 37°C with 10% CO_2 before the compound was added. For the agonist assay, cells were incubated at 37°C with 10% CO_2 with compound for 4 hours. The Dual-Glo™ Luciferase Reagent was then added and followed by a 10-minute incubation and subsequent measurement of firefly luciferase luminescence. After the firefly reading, the Dual-Glo™ Stop & Glo® Reagent was added to the same wells, incubated for 10 minutes, and the *Renilla* luciferase luminescence was recorded. For the DRD1 antagonist assay, test compounds were added and incubated for 15 minutes. One micromolar SKF 38393, a DRD1 agonist, was added to each well, and plates were incubated for 4 hours as described above. The Dual-Glo™ Luciferase Assay was then performed as described for the agonist assay.

Biochemical Assays

The P450-Glo™ Assays were performed to assess inhibition of cytochrome P450 enzymes by the test compounds. CYP1A2, 2C9, 2C19, 2D6 and 3A4 assays were performed according to Technical Bulletin #TB340. After adding the test compound, cytochrome P450 enzyme and P450-Glo™ Substrate to each well, the 384-well plate was incubated for 10 minutes at room temperature. An NADPH regenerating solution was then added to initiate the reaction, and plates were incubated an additional 60 minutes at room temperature. After the second incubation, the Luciferin Detection Reagent was added, and luminescence was recorded.

The Kinase-Glo® Plus Assay was performed to examine target activity for protein kinase A (PKA). The kinase reaction components consisted of PKA, Kemptide Peptide Substrate (Cat.# V5601) and 10µM ATP. Following a 10-minute reaction, the Kinase-Glo® Plus Reagent was added, and luminescence was recorded.

The MAO-Glo™ Assay was performed to assess compound effects on monoamine oxidase A (MAO A) activity. MAO A was incubated for 60 minutes at room temperature with test compound and substrate. The Luciferin Detection Reagent was then added, and luminescence was recorded.

The Pgp-Glo™ Assay was used to assess compound effects on the drug transport enzyme P-glycoprotein (Pgp). Compounds that are substrates for transport by

Pgp stimulate its ATPase activity. Following the instructions in Technical Bulletin #TB341, test compounds were incubated with P-glycoprotein, ATP and substrate for 40 minutes at 37°C, followed by addition of the ATP Detection Reagent and luminescence detection.

The IC_{50} and EC_{50} values were calculated with GraphPad Prism® version 4.0 software. In Table 3, the results have been color-coded for easy visual interpretation of data patterns. These results show that the three test compounds inhibit PKA enzyme activity at less than 10µM, but the cytotoxicity and off-target activity profiles differ for each. For example, if PKA was a drug target, and we were looking for the best compound for lead development and optimization, our profile results show that PKI would be the best choice since it is the only compound that is specific for the PKA target. Unlike H 89 and dihydrexidine, PKI does not appear to be toxic or induce apoptosis in a cell-based system. Nor does PKI inhibit cytochrome P450; in contrast, the H 89 compound inhibits several cytochrome P450 assays at less than 10µM. In addition, PKI does not exhibit off-target activity, as no effect was seen with the GPCR DRD1 assays.

Table 3. Summary of Cell-Based and Biochemical Assay Profiles for PKI, H 89 and Dihydrexidine. The data below were generated as described in the text for biochemical and cell-based assays. Key to the data is at the bottom of the table.

Assay	PKI	H 89	Dihydrexidine
P450-Glo™ Assay: CYP1A2			
P450-Glo™ Assay: CYP2C9			
P450-Glo™ Assay: CYP3A4			
P450-Glo™ Assay: CYP2C19			
P450-Glo™ Assay: CYP2D6			
Pgp-Glo™ Assay: P-glycoprotein			
MAO-Glo™ Assay: monoamine oxidase A			
Kinase-Glo® Plus Assay: PKA			
CellTiter-Glo® Assay: 4 hours cell viability			
CellTiter-Glo® Assay: 18 hours cell viability			
Caspase-Glo® 3/7 Assay: 4 hours apoptosis			
Caspase-Glo® 3/7 Assay: 18 hours apoptosis			
Dual-Glo™ Assay: GPCR DRD1 agonist			
Dual-Glo™ Assay: GPCR DRD1 antagonist			

No Effect
IC_{50} <10µM
EC_{50} >0.1µM
Stimulation >threefold

Conclusions

Using a panel of different assay types, it is possible to obtain a general pattern of compound activity. By measuring IC_{50} or EC_{50} dose responses, as opposed to single-point screening, we acquired more accurate drug potency data in both cell-based and biochemical assay systems. Target-specific effects can be seen as well as off-target and cytotoxic events.

Generating these large amounts of data would not be possible without the use of high-throughput, instrumentation (e.g., Tecan Freedom EVO® system described here) and suitably designed assays. When used together, Promega reagents and Tecan instrumentation offer an integrated solution to high-throughput users looking to generate large amounts of profiling data for their compounds of interest.

Ordering Information

Product	Size	Cat.#
Caspase-Glo® 3/7 Assay†	10ml*	G8091
CellTiter-Glo® Luminescent Cell Viability Assay	10ml*	G7570
Dual-Glo™ Luciferase Assay System	10ml*	E2920
Kinase-Glo® Plus Luminescent Kinase Assay	10ml*	V3771
MAO-Glo™ Assay	200 assays*	V1401
P450-Glo™ Screening Systems		
CYP1A2	1,000 assays	V9770
CYP2C9	1,000 assays	V9790
CYP3A4	1,000 assays	V9800
CYP2C19	1,000 assays	V9880
CYP2D6	1,000 assays	V9890
Pgp-Glo™ Assay System	10ml	V3591
Pgp-Glo™ Assay System with P-glycoprotein	10ml	V3601

†For Laboratory Use.

*Other sizes available.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

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