

Enhanced Selectivity Screening of GPCR Ligands Using a Label-Free Cell Based Assay Technology

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Abstract: Drug discovery efforts advance in step with advancements in assay technologies, as new technologies provide new lenses through which biology can be viewed. The novel information gathered results in the better understanding of drug-target interactions leading to better decision making during the drug discovery process. One area of rapid development is within label-free technologies. Label-free technologies offer many distinct advantages to the drug discovery workflow. One such novel technology is the CellKey™ System, an impedance-based label-free live cell assay platform. The system is based on impedance technology and is a universal platform for the functional measurement of all classes of G-protein coupled receptors (GPCRs). Data are generated in a kinetic fashion on both endogenously expressed and transfected receptors in a wide variety of cell types. In the studies detailed here, we used the system to perform an enhanced selectivity screen of a small panel of compounds simultaneously against two unrelated GPCR targets signaling through different pathways. Utilizing both the quantitative measures of cellular activation and the qualitative information inherent in the rich output data, we gained knowledge not only about the relative selectivity of each compound across both targets, but also about the character of the interaction of each with the cellular target. In this manner, we successfully demonstrated proof of principle for using an impedance-based technology to perform selectivity analyses and to triage lead compounds in a simplified format.

Keywords: CellKey™ system, label-free, cell based assay, drug discovery, secondary screening, selectivity screening, endogenous GPCR, integrated cellular response.

INTRODUCTION

An essential step in the development of potent and selective drugs is the robust and informative secondary screening of lead compounds identified in primary screening campaigns. Ligand selectivity analysis is a secondary screening technique used to derive the potency of lead compounds on both related and unrelated receptor targets. The goal is to determine the degree to which lead compounds interact with unintended receptor targets or signaling pathways, as poor target selectivity and off-target effects have been implicated repeatedly in clinical side effects [1, 2]. Functional cell based assays are ideal for this approach, because they introduce biological complexity early in the drug discovery process, are much less expensive than *in vivo* testing, and meet the throughput needs of screening labs [3].

Label-free cell based assays are gaining acceptance in drug discovery as important and complimentary technologies to the prevalent labeled assays currently utilized [4-8]. Introducing label-free assays into the screening work flow is advantageous in many ways. They greatly simplify the assay development process by avoiding the laborious assembly of recombinant reporter systems and by removing labeling and loading protocols and their optimization. Additionally, by

eliminating the use of labels and recombinant readout systems, label free technologies are less prone to problems associated with such artificial systems [9-12]. When combined, these unique aspects create the potential for quickly identifying lead compounds which demonstrate greater activity in a native environment.

Here we describe the initial development of such a selectivity screen using the CellKey™ System, a label-free live cell assay platform. We employed the CellKey™ System to perform selectivity analysis of a small panel of diverse compounds on Chinese hamster ovary (CHO) cells expressing two different G-protein coupled receptors (GPCRs), the dopamine D3 receptor and the muscarinic acetylcholine receptor M₁ (muscarinic M₁) [13]. The goals of the studies were to identify potent and selective agonists and antagonists in a workflow that included hit confirmation, receptor specificity and potency ranking of the 25 compounds tested across both cell lines. Additionally, data generated on this system were compared to data generated on other cell-based second messenger assay platforms.

The CellKey™ System is a label-free, impedance assay technology based on cellular dielectric spectroscopy (CDS) (Fig. 1A) [14,15]. As such, there is no need for fluorescence, luminescence, colorimetric dyes or chimeric reporter proteins when performing assays. The assays are universal in that nearly all classes of GPCRs can be analyzed during a single experiment without changing assay parameters. The experiments detailed here make good use of this feature, as

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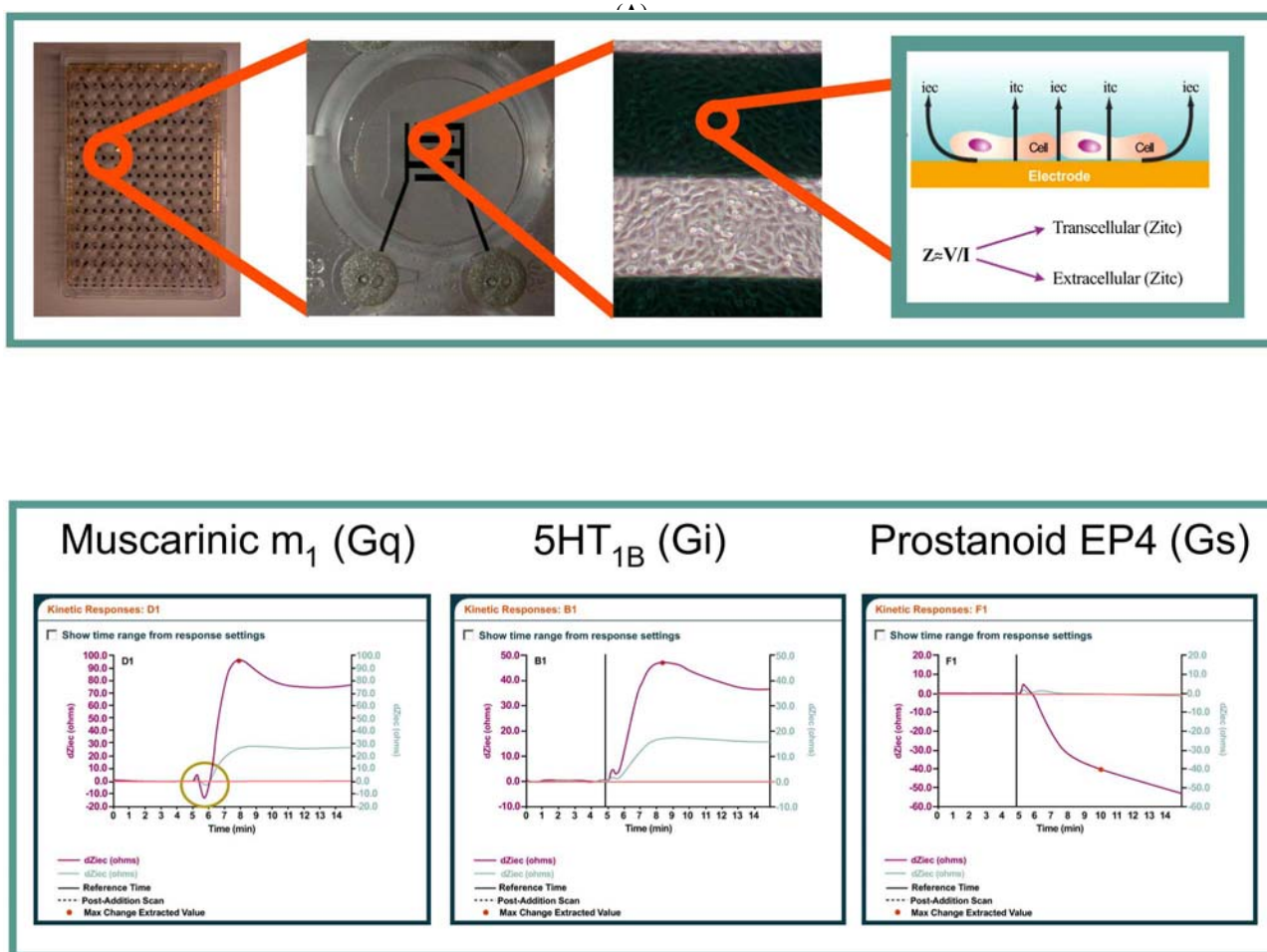


Fig. (1). The CellKey™ System measures changes in the impedance (Z) of a cell layer to an applied voltage. Cells are seeded onto a CellKey™ Standard 96W microplate (A) that contains electrodes at the bottom of the wells. The instrument applies small voltages at 24 discrete frequencies, from 1 kHz to 10 MHz, once every 2 s. At low frequencies, these voltages induce extracellular currents (iec) that pass around individual cells in the layer. At high frequencies, they induce transcellular currents (itc) that penetrate the cellular membrane. Changes in impedance due to extracellular currents (dZiec) and to transcellular currents (dZitc) are reported kinetically for each well. When cells are exposed to a stimulus, such as a receptor ligand, signal transduction events occur that lead to cellular events which include changes in cell adherence, cell shape and volume, and cell-cell interaction. These cellular changes individually or collectively affect the flow of extracellular and transcellular current, and therefore, affect the magnitude and characteristics of the measured impedance. (A) CellKey™ Standard 96W microplate (left), an individual assay well with close-up of interdigitated gold electrodes (center left), and live cells plated on and between the electrode fingers (center right). A diagram highlighting the flow of extracellular (iec) and transcellular (itc) current is shown (right). (B) In response to ligand mediated activation of GPCRs, the system generates response profiles that are characteristic of $G_{\alpha s}$, $G_{\alpha q}$ and $G_{\alpha i}$ -coupled GPCRs. Examples are provided which illustrate the response profiles for the transfected muscarinic M_1 (typical $G_{\alpha q}$ -GPCR), endogenous serotonin 5HT_{1B} (typical $G_{\alpha i}$ -GPCR), and prostanoid EP4 receptors (typical $G_{\alpha s}$ -GPCR) in the context of CHO cells. $G_{\alpha i}$ -GPCRs typically demonstrate an increase in impedance, while $G_{\alpha s}$ -GPCRs exhibit a decrease in impedance following receptor activation. What distinguishes the $G_{\alpha q}$ -GPCRs from the $G_{\alpha i}$ -GPCRs is an initial transient dip in impedance (circled) that is followed by a later increase in impedance.

two different G protein-coupled GPCRs were studied simultaneously, often on the same plate. Additionally, the data can be analyzed to generate quantitative measures of cellular activation for many pharmacologically important parameters, including ligand-receptor specificity, detection of partial and inverse agonism, derivation of agonist and antagonist potencies, and Schild analysis [15,16]. In addition to quantifying cellular responses to ligand stimulus, the CellKey™ System generates unique response profiles that may indicate the signaling pathways accessed by stimulated cells (Fig. 1B) (Inset). Characteristic profiles of $G_{\alpha i}$ -, $G_{\alpha s}$ - and $G_{\alpha q}$ -GPCR activation are well documented [14-16], and

recent work has identified profiles for other families, such as integrin receptors (data not shown). Measurements obtained from each assay reflect integrated cellular responses to stimuli, and are tied to downstream events in receptor signaling cascades, particularly cytoskeletal rearrangement [16-20].

A very important feature of the CellKey™ System is its ability to routinely monitor the activation of both endogenously expressed and transfected cellular receptors. This target independent readout uniquely empowers the platform for selectivity screening applications. In practice, a

reference agonist is used to establish a standard response profile for the target receptor under study, which subsequently can be compared to all other response profiles generated. This enables the identification of compounds that interact with target cells in the expected manner, and compounds that do not, due to poor selectivity or off target effects. The magnitudes of cellular responses to ligand addition are dependent on input concentration and are quantified from the same data set that provides a qualitative look at the character of each response. In this way, from the earliest experiments in assay development, the data simultaneously generates both quantitative and qualitative information for the compounds and the target. This type of simultaneous analysis enables the identification and simple triage of non-selective compounds early in the drug discovery process. Ultimately, compounds that generate response profiles different than the standard are flagged for exclusion or further study, depending on the situation.

The studies detailed here offer a demonstration of the CellKey™ System's strengths when applied to selectivity screening in particular, and secondary screening applications in general.

MATERIALS & METHODS

CellKey™ System Specifications

As mentioned, the CellKey™ System, is a label-free, noninvasive, live cell assay technology, which is based on cellular dielectric spectroscopy [21, 22]. CDS measures the electrical impedance (Z) of cell layers positioned over electrodes patterned at the bottom of cell plate wells [23-25]. Alternating voltage is applied at a range of frequencies (V_f) and the resulting electrical currents (I_f) are measured. The impedance as a function of frequency (Z_f) can be represented by the equation $Z_f = V_f/I_f$ where V_f and I_f are complex numbers to preserve phase information. The cells and electrodes form an electrical circuit attached to an impedance analyzing system, which generates the voltage and measures the current (Fig. 1A). Basic biophysical principals indicate that impedance measurements acquired at low frequencies result from currents that flow around and between cells (extra-cellular current, or *iec*), while measurements acquired at high frequencies result from currents that flow across cell membranes (trans-cellular current, or *itc*) (Fig. 1B) [26, 27].

The CellKey™ System is comprised of a physical instrument, which fits conveniently on a laboratory bench top, a custom 96-well microplate (conforms to SBS standard footprint) and a comprehensive software package that controls all aspects of assay design, set up, execution and data analysis. The instrument contains an automated fluidics head which is capable of dispensing compounds to all 96-wells of the microplate, while the reader simultaneously collects data. Additionally, the fluidics head can be programmed easily to dispense compounds from four individual quadrants of 384-well compound plates, in order to accommodate libraries which are formatted in this manner. The reader is thermally controlled and can be set to perform assays from ambient temperature plus 4°C up to 37°C. The CellKey™ System is also designed to be effectively integrated into automated screening platforms. Two sliding decks are positioned on the front of the system to provide robotics access to the tip, compound plate, and

cell plate stations. Additionally, the instrument and software include hooks for automation scheduling systems.

All assays include a baseline measurement, typically of 30-s to 5 min duration, followed by ligand addition and cellular response measurement. Most assays require 15 min or less to complete per 96-well plate. As assays are label-free, there are no dye loading or labeling steps required, and cells are cultured in routine cell culture media without special components. All of the acquired data are captured and stored in data files that are about 10 MB in size (15 min assay with 2-s update rate). Data can be analyzed *via* the software and exported, automatically or manually, to spreadsheet programs or laboratory information management systems (LIMS).

Materials

The two Chinese hamster ovary (CHO) cell lines under study were obtained from Euroscreen (Gosselies, Belgium). One cell line expressed the human dopamine D3 receptor (CHO-D3) while the other expressed human muscarinic M₁ receptor (CHO-M₁). Cell culture reagents including Ham's F-12 nutrient mixture, Geneticin (G418), Zeocin, L-glutamine, Penicillin-Streptomycin, fetal bovine serum (FBS), and Versene were obtained from Invitrogen Corp. (Carlsbad, CA). Tissue culture flasks (225 cm²) and Costar microplates were purchased from Corning, Inc. (Corning, NY). Nonproprietary compounds were purchased from Sigma Chemical Co. (St. Louis, MO). The reference agonist for the human dopamine D3 receptor was dopamine (Sigma catalog #H8502) and was designated compound TH1. The reference agonist for the human muscarinic M₁ receptor was oxotremorine (Sigma catalog #O100) and was designated compound TH4. The cAMP assay kit, consisting of cell lysis reagent, EA reagent, ED-cAMP reagent, anti-cAMP antibody, and chemiluminescence β-galactosidase substrate reagents, was purchased from DiscoveRx Corp. (Fremont, CA). Coelenterazine h Luciferin was obtained from Assay Designs Inc. (Ann Arbor, MI).

Cell Culture

Both CHO_{k1}-based cell lines [28], expressing heterologous GPCRs were cultured in Ham's F12 media supplemented with 10% fetal bovine serum, penicillin/streptomycin and 20 mM L-glutamine in a 37°C humidified incubator containing 5% CO₂. Additionally, CHO-D3 cells were cultured in the presence of 0.4 mg/mL of Geneticin, while the CHO-M₁ cells were cultured in the presence of 0.25 mg/mL of Zeocin.

CellKey™ System Assay Parameters

Both cell lines were cultured such that they never exceeded 90% confluence. In addition, cells were determined to respond optimally on the CellKey™ System when the population was harvested as it reached 90% confluence after 48 h in culture. The evening prior to experimentation, cells were released from tissue culture flasks using a 2 mM EDTA solution. In preparation for plating, harvested cells were washed once in assay buffer (Hanks Balanced Salt Solution supplemented with 20 mM HEPES buffer and 0.1% BSA (Sigma catalog #A0281)), counted by haemocytometer, and plated at the optimal densities determined by cell titration experiments (see results). Cells were plated onto CellKey™

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Standard 96W microplates at 135 μL /well, then allowed to rest on a non-vibrating, flat surface at room temperature for 25 min to promote even distribution across the well bottoms. Cell plates were incubated overnight in a 37°C humidified incubator containing 5% CO_2 . On the day of experimentation, cell plates were removed from the incubator and visually inspected by light microscopy for confluence and consistency across wells. Cells were determined to respond optimally on the system when the population was fully confluent (100%) in all wells. Cell plates were washed using the onboard fluidics head, which was programmed to replace culture media for assay buffer in three exchanges. 135 μL of assay buffer was left in the wells after the final exchange. The plates were then incubated at room temperature for 30 min to allow the cells to equilibrate to the new buffer environment.

Agonist Protocol

For agonist assays, 5 min of baseline measurements were recorded followed by addition of 15 μL of 10X ligands. Subsequently, 10 min of cellular activation were recorded at an update rate of 2 s per 96 wells. Data were analyzed using the CellKey™ software and exported into GraphPad Prism. Raw data were maximum-minimum values of dZiec expressed in ohms for the 10 min post ligand injection. For agonist CRC, all raw data were converted to Percent of Control (POC) values prior to EC_{50} analysis. POC was defined as $[100 \times (\text{test compound value} - \text{buffer control value}) / (\text{maximum agonist control value} - \text{buffer control value})]$.

Antagonist Protocol

For antagonist assays, the final buffer exchange in the wash protocol contained 1X antagonist in 135 μL . Cells were allowed to incubate in the presence of antagonist for 30 min before baseline measurements were recorded. This 30 min period served the dual purposes of allowing the cells to equilibrate to the new buffer environment and allowing the antagonists to bind receptor prior to agonist challenge. Five minutes of baseline measurements were recorded, followed by addition of 15 μL of 10X concentration of agonist at an EC_{80} dose. Subsequently, 10 min of cellular activation were recorded at an update rate of 2 s per 96 wells. Raw data were maximum-minimum values of dZiec expressed in ohms for the 10 min post ligand injection. For antagonist CRC, all raw data were converted to percent of control (POC) values prior to IC_{50} analysis. POC was defined as $[100 \times (\text{test compound value} - \text{buffer control value}) / (\text{EC}_{80} \text{ agonist control value} - \text{buffer control value})]$.

Sequential Agonist-Antagonist Screening Protocol

The basic wash protocol described above was performed leaving 135 μL of assay buffer in the wells after the final exchange. The plates were then incubated at room temperature for 30 min to allow the cells to equilibrate to the new buffer environment. A first experiment was performed to measure the agonistic activities of the panel of compounds. This included 5 min of baseline measurements followed by addition of 15 μL of 10X concentration of the compounds and 10 min measurement of receptor activation. The plate was then incubated on line for an additional 15 min, without acquiring data, before beginning a second

experiment. In this second experiment, a new baseline was read for 5 min followed by addition of 16.6 μL of 10X reference agonist at an EC_{80} dose. Subsequently, 10 min of cellular activation were recorded. In this way, the same cell plate was used to test both agonists and antagonists in a sequential manner.

cAMP Assay Parameters

Cytosolic cAMP levels in CHO-D3 cells were determined using DiscoverX cAMP assay kit in 384-well white plates (Costar 3705). CHO-D3 cells were cultured in growth medium at 37°C in an atmosphere of 5% CO_2 . Before each experiment, cells were harvested by Versene, centrifuged and washed once with PBS then suspended in a cell buffer (Ham's F12 medium, 30 mM HEPES, and 0.1% BSA) at 500,000 cells per mL. Compounds to be tested were diluted with the cell buffer to concentrations required. The assay was performed according to the cAMP assay kit protocol provided by DiscoverX. For agonistic response measurement, 20 μL of suspended cells (10,000 cells per well) was added to the well containing 5 μL of compound at various concentrations and incubated at room temperature for 30 min. This was followed by the addition of 5 μL forskolin in a final concentration of 30 μM and incubated at room temperature for 30 min. Lysis buffer containing anti-cAMP antibody in 10 μL was added to the cells, followed by adding 10 μL of ED reagent 60 min later and incubated at room temperature for another 60 min. Finally, 20 μL of EA and substrate reagents was added and after 2 h incubation at room temperature the luminescence signal was determined using a luminometer Leadseeker. In the antagonist studies, the dopamine D3 agonist, TH1 at a final concentration of 15 nM was included in the forskolin solution in the second addition. All raw data were converted to Percent of Control (POC) values prior to EC_{50} and IC_{50} analysis using XLFit within ActivityBase (ID Business Solution Inc., Emeryville, CA). POC was defined as $[100 \times (\text{test compound value} - \text{DMSO control value}) / (\text{agonist control value} - \text{DMSO control value})]$.

Ca^{2+} Flux Assay Parameters

Calcium flux was measured on an automated flash luminometer using a standard double-addition protocol in which compound was added prior to a reference agonist. This double-addition protocol detects agonist or antagonist effect of test compounds in the same well. Light emission due to Ca^{2+} activation of aequorin in the cells was captured at 5-s intervals for 1 min in both agonist and antagonist modes. The peak emitted light intensity within the 1-min period was used in subsequent calculation of the compound response.

One day prior to the experiment, CHO-M₁ cells, expressing apoaquorin, were seeded in 96-well clear-bottom black plates (Costar 3904) at a density of 25,000 cells per well in 80 μL growth medium, the plates were incubated at 37°C in an atmosphere of 5% CO_2 . On the day of experimentation, the medium was manually discarded and 60 μL of 10 μM coelenterazine solution diluted with the assay medium (Ham's F12 medium without phenol red, 15 mM HEPES pH7.5) was added. The plates were incubated at 34°C for 3 h. In the first compound addition, 20 μL of test compounds in various concentrations were added to the cells using a 96-well pipettor and the emitted light due to

cytosolic Ca^{2+} activation of aequorin was captured by the flash luminometer. After another 1.5 min, 120 μL of the muscarinic M_1 agonist, TH4 at a final concentration of 67 nM was added and the emitted light was captured for 1 min. All raw data were converted to Percent of Control (POC) values prior to EC_{50} and IC_{50} analysis using XLFit within ActivityBase (ID Business Solution Inc., Emeryville, CA). POC was defined as $[100 \times (\text{test compound value} - \text{DMSO control value}) / (\text{Reference Agonist control value} - \text{DMSO control value})]$.

RESULTS

To begin the process of developing an optimized selectivity screen on the CellKey™ System we investigated the specificity and selectivity of a set of 25 compounds (designated TH1-25) on two unrelated GPCRs, dopamine D3 and muscarinic M_1 . The compounds were selected such that a subset was intended to interact with the dopamine D3 receptor while a different subset was intended to interact with the muscarinic M_1 receptor. Of great interest was the manner in which the technology would report on the consequences of compounds interacting with unintended targets. All experiments were conducted under double-blind conditions, as neither the identity of the compounds nor the receptors were made known to the investigators during the period of the studies. Also, the CellKey™ System data generated in this study were compared to data gathered previously using cAMP and Ca^{2+} -aequorin assays.

Assay Development

The ideal CellKey™ assay depends on using a confluent monolayer of cells [16]. Both CHO cell lines expressing one of the aforementioned GPCRs were seeded across the cell plate at varying densities the night before the assay. Cells were challenged with a single 10 μM dose of the respective reference compound for each GPCR and the responses were measured. Compound TH1 (dopamine) was designated the reference agonist for the dopamine D3 receptor and compound TH4 (oxotremorine) was designated the reference compound for the muscarinic M_1 receptor. CHO-D3 cells generated a maximal response of 24.1 ohms at 60,000 cells per well (Fig. 2A). However, the optimal signal to background ratio of 6.8-fold was achieved at 40,000 cells per well. CHO- M_1 cells generated a maximal response of 52.8 ohms and a maximal signal to background ratio of 34-fold at 70,000 cells per well (Fig. 2B). For all subsequent experiments CHO-D3 cells were plated at 40,000 cells per well and CHO- M_1 cells were plated at 70,000 cells per well.

Agonist Screening

Once demonstrating that the cells responded to administration of reference agonists, we then proceeded with hit confirmation experiments using all 25 compounds. These compounds were applied at 10 μM concentration to both CHO-D3 and CHO- M_1 cells (Fig. 3A, B), respectively. Hits were defined as compounds inducing cellular responses significantly greater than a threshold set at the buffer mean plus three times the standard deviation (+3X SD). Several compounds were observed to specifically activate the CHO-D3 cells (TH1, TH2 and TH21) and the CHO- M_1 cells (TH4 and TH9). The data are summarized in Table 1.

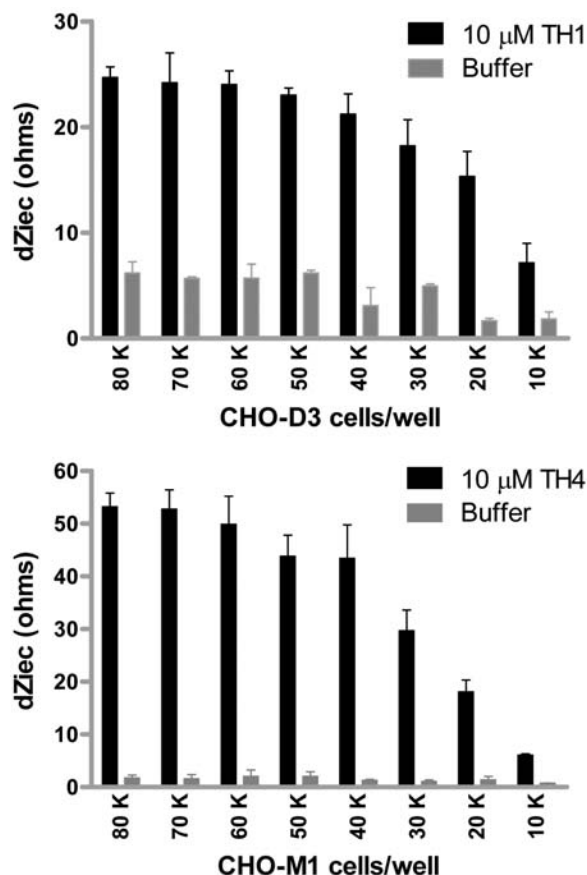


Fig. (2). Cell titration experiments for CHO-D3 and CHO- M_1 cell lines. Varied cell densities were plated onto CellKey™ Standard 96W microplates the evening prior to experimentation. Cells were then tested with 10 μM reference agonist and buffer to determine which cell densities generated the highest signal to background ratio. Data are mean dZlec $\pm$ SD, $n = 4$.

For subsequent use in the antagonist screen described below we derived the potencies of the designated reference agonists, TH1 and TH4. We performed concentration response curves (CRC) in quadruplicate across a range from 1 μM to 1 pM for TH1 and across a range from 100 μM to 1 pM for TH4 (Fig. 4A, B), respectively. The resulting EC_{50} value for TH1 was determined to be 1.0 nM, which was similar to the 1.9 nM value determined by cAMP assay (Fig. 4C). The resulting EC_{50} value for TH4 was determined to be 18.0 nM, which was consistent with the 18.8 nM value determined by Ca^{2+} -aequorin assay (Fig. 4D). The EC_{80} values for TH1 and TH4 were determined by CellKey™ assays to be 3.0 nM and 75 nM, respectively, and these concentrations were used as the challenge doses in the antagonist studies described below.

CellKey™ Response Profiles

To investigate the selectivity of the compounds in greater detail, we looked at the unique qualitative aspects of the CellKey™ response profile for each compound tested. The response profile is a kinetic graph of a cell line's response to activation by a compound [16]. This cellular behavior is driven by the signaling pathways activated as the cell surface receptor binds compound. As such, compounds that

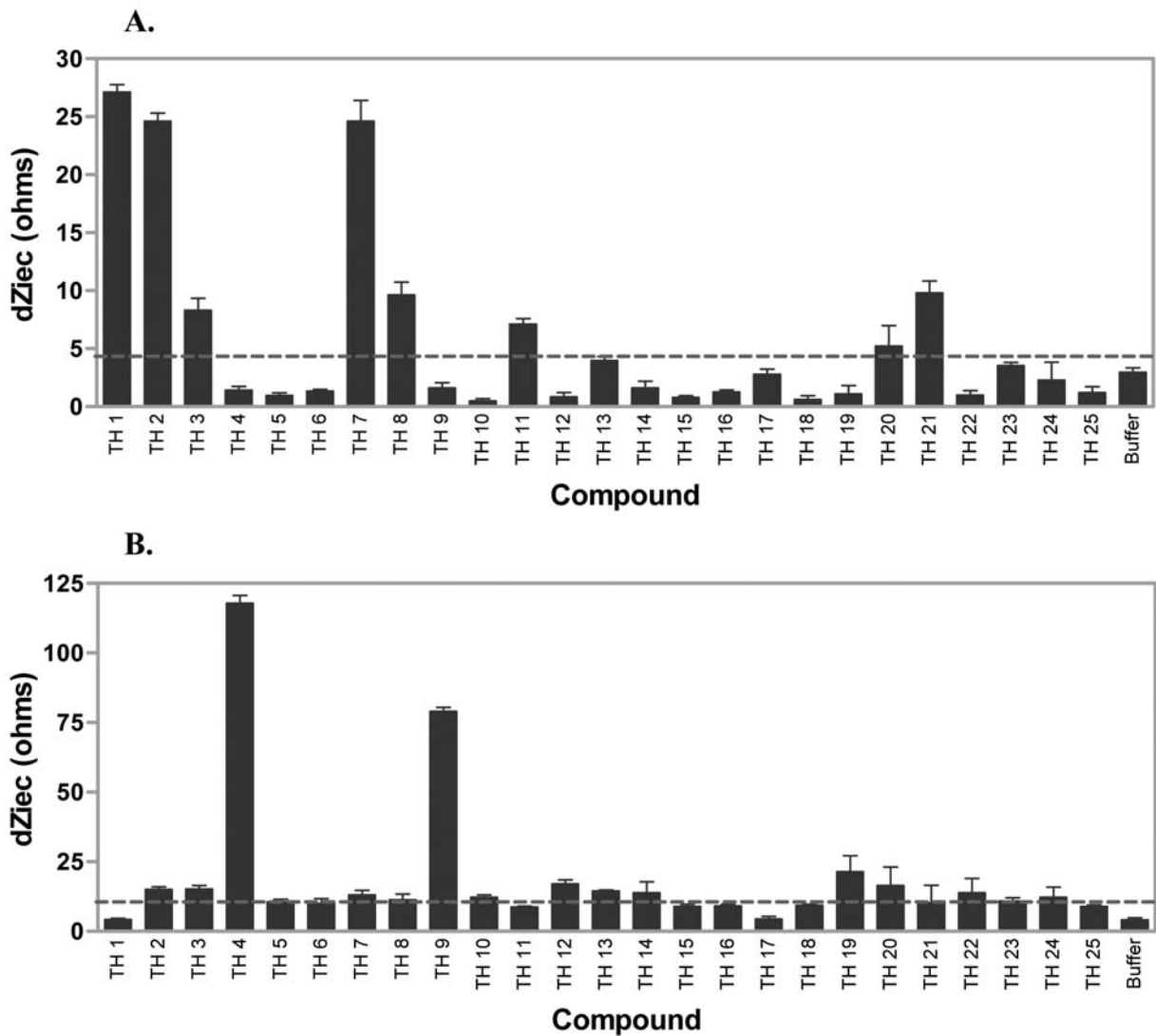


Fig. (3). Agonist screen on CHO-D3 and CHO-M₁ cells. In agonist hit confirmation experiments, CHO-D3 (**A**) and CHO-M₁ (**B**) cells were treated with single doses (10 μM) of TH1 to TH25, and cellular activation was monitored. Hits were defined as compounds inducing cellular responses significantly greater than a threshold set at the buffer mean + 3X SD (dotted line).

Table 1. Summary of Hits from Agonist and Antagonist Screens on CHO-D3 and CHO-M₁. Selective and Non-Selective (*Italics*) Hits are Tabulated. Data were Compiled from Experiments Detailed in Figs. (3) and (6)

	Dopamine D3	Muscarinic M ₁
Agonists	TH1, TH2, <i>TH7</i> , <i>TH8</i> , TH21	TH4, TH9
Antagonists	<i>TH3</i> , TH10, <i>TH11</i> , TH18, <i>TH20</i> , <i>TH22</i>	<i>TH3</i> , TH6, <i>TH7</i> , TH8, <i>TH11</i> , TH12, TH13, TH14, <i>TH20</i> , <i>TH22</i> , TH23, TH24, TH25

selectively bind and activate a particular cell surface receptor should have similar response profiles. Fig. (5) displays examples of the response profiles induced by two selective compounds (TH1 and TH4) and one non-selective

compound (TH3). The data are highly reproducible in terms of kinetics and magnitude of response (each plot represents three independent wells within a single assay plate, error bars are ±SD). The response profile observed upon activation of dopamine D3 receptor with compound TH1 indicates a G_{αi}-coupled GPCR pathway (for background information on response profiles please refer to inset (Fig. 1)). Compound TH1 did not induce a cellular response in the CHO-M₁ cells demonstrating that the cellular response observed in CHO-D3 cells was dependent on the presence of dopamine D3 receptor. The response profile observed upon activation of muscarinic M₁ receptor with compound TH4 indicates a G_{αq}-coupled GPCR pathway (refer to Fig. (1)). Compound TH4 did not induce a cellular response in the CHO-D3 cells indicating that the cellular response observed in CHO-M₁ cells was dependent on the presence of muscarinic M₁ receptor. Compound TH3 was active in both cell lines and induced similar response profiles in both CHO-D3 and CHO-M₁ cells. This response profile was distinct from buffer

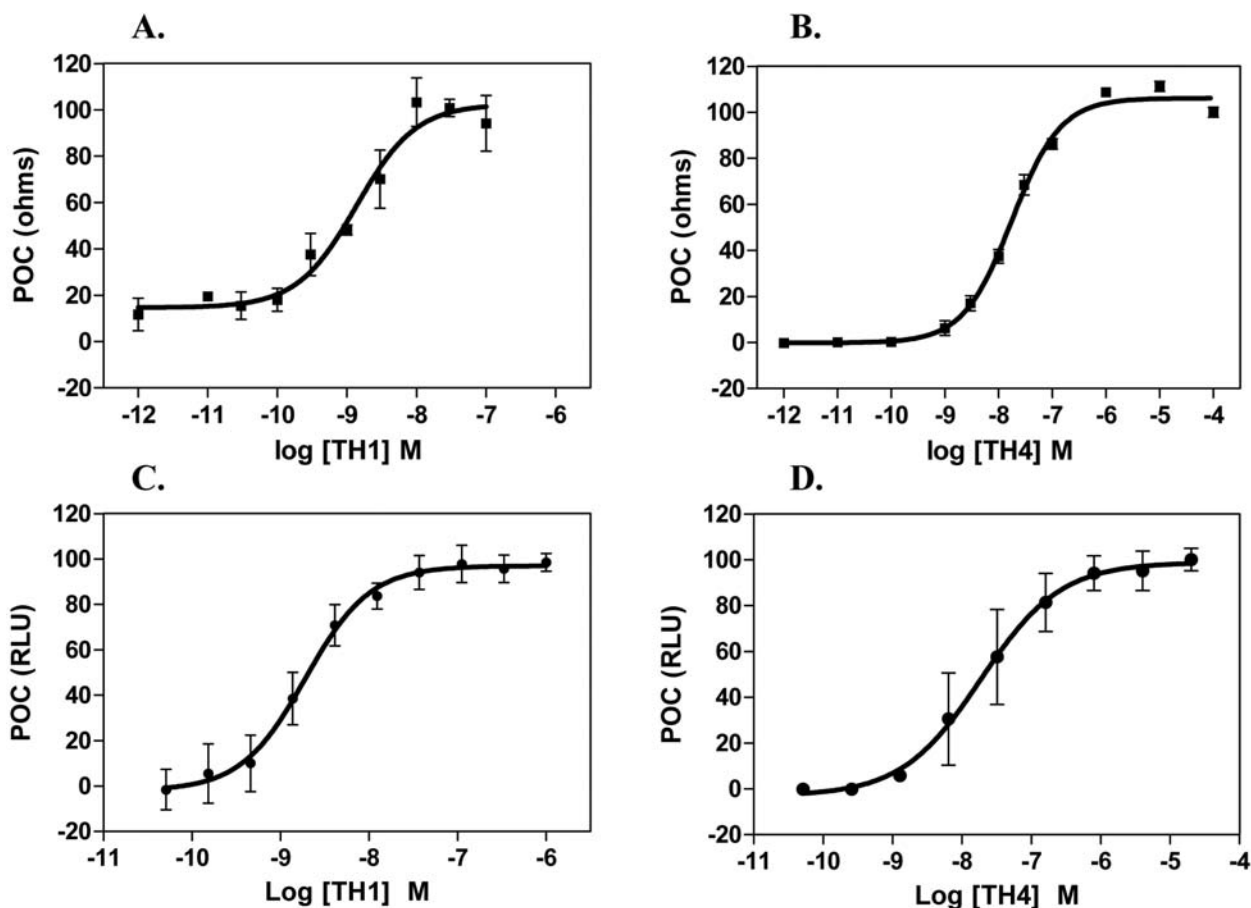


Fig. (4). Concentration response curves of dopamine D3 receptor and muscarinic M₁ receptor reference agonists. CRC for dopamine D3 receptor agonist TH1 and muscarinic m1 receptor agonist TH4 were produced using impedance assay, cAMP assay or Ca²⁺-aequorin assay. The EC₅₀ values for TH1 and TH4 as determined by the impedance assay were 1.0 nM (A) and 18 nM (B), respectively. Responses are normalized to the highest doses of ligands (n=4, error bars indicate $\pm$ SD). The EC₅₀ of TH1 as determined by cAMP assay was 1.9 nM (C). The EC₅₀ of TH4 as determined by Ca²⁺-aequorin assay was 18.8 nM (D). Responses were normalized to the highest dose of each compound and expressed as percent of control (n = 4 error bars indicate $\pm$ SD).

controls and from the profiles induced by the selective compounds for dopamine D3 receptor and muscarinic M₁ receptor. These data indicate that the compound was not acting exclusively *via* one of the transfected receptors of interest. More likely, compound TH3 activated an endogenous receptor(s) expressed in the CHO cell background in addition to one of the transfected receptors. This observation is discussed further below.

Antagonist Screening

We also sought to identify which of the 25 compounds might be selective antagonists for the two different GPCRs. Using the CellKey™ System, we applied all 25 compounds at a maximal dose of 10 μ M to each of the CHO cell lines. After 30 min of incubation, the reference agonist for each cell line was applied at an EC₈₀ concentration and the resulting cellular activation was monitored for 10 min. Data were plotted as percent of positive control (EC₈₀ dose of compound TH1 or TH4 in the absence of pre-treatment) and are displayed in Fig. (6). Compounds that demonstrated agonist activity in the previous screen (Fig. 3), and thus subsequently appeared as antagonists due to desensitization of the receptors are indicated by grey bars. This experiment

identified several selective antagonists for the dopamine D3 receptor (TH10 and TH18) and the muscarinic M₁ receptor (TH6, TH12, TH13, TH14, TH23, TH24, TH25), but non-selective antagonists were identified as well (TH3, TH8 and TH11). These results are summarized in Table 1. It is worth noting here that sequential agonist and antagonist screens were performed using the same plate of cells as described in the materials and methods section. This is important demonstration of the assay design flexibility enabled by a non-invasive, label-free assay platform.

Antagonist screening experiments were performed using cAMP and Ca²⁺-aequorin assays (data not shown) and results were in good agreement with CellKey™ data except for TH8, which was identified as a weak agonist in the impedance assay but not in the cAMP assay for dopamine D3 receptor.

Potency Ranking of Antagonists

Using the EC₈₀ concentrations of the reference agonists we challenged cells pre-incubated with various concentrations of four different antagonists for each receptor target. For dopamine D3 receptor we derived the potencies of the four most efficacious antagonists identified in the

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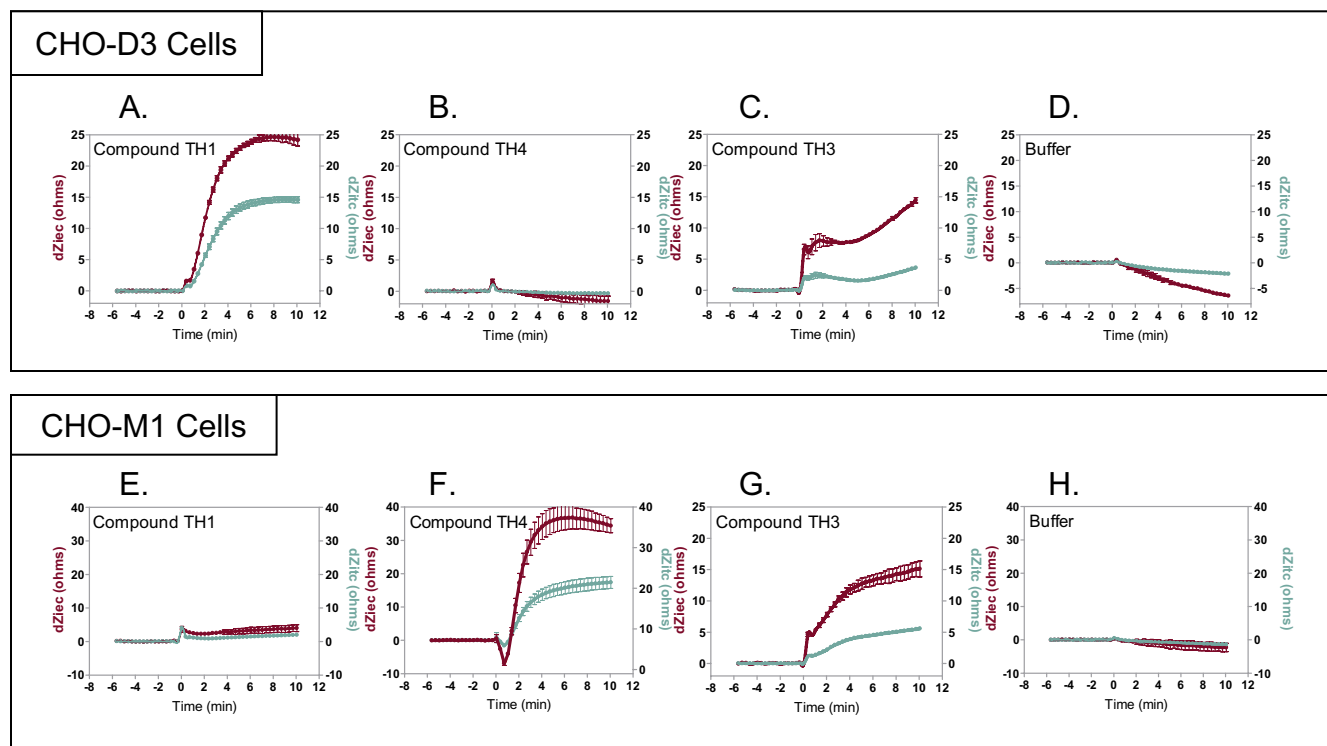


Fig. (5). CellKey™ response profile data: receptor specificity and selectivity. Response profile data were used to investigate the signaling pathways triggered by activation of dopamine D3 receptor and muscarinic M₁ receptor, to demonstrate the target specificity of each compound and to indicate the selectivity of compounds TH1 to TH25. Examples of the CellKey™ response profiles induced by two selective compounds, TH1 (dopamine) and TH4 (oxotremorine), and one non-selective compound, TH3, are shown. The response profile observed upon activation of dopamine D3 receptor with 10 μ M compound TH1 (A) indicates a G α_i -coupled GPCR pathway. Compound TH1 did not induce a cellular response in the CHO-M₁ cells (E), thus demonstrating selectivity for dopamine D3 receptor. The response profile observed upon activation of muscarinic M₁ receptor with 10 μ M compound TH4 (F) indicates a G α_q -coupled GPCR pathway. Compound TH4 did not induce a cellular response in CHO-D3 cells (B), thus demonstrating selectivity for muscarinic M₁ receptor. Compound TH3 was active in both cell lines and induced response profiles which were unlike those induced by the reference agonist TH1 and TH4 on CHO-D3 and CHO-M₁ (C, G) and were distinct from buffer controls (D, H). These data indicate that compound TH3 was not exclusively acting *via* one of the transfected receptors of interest. (n = 4, error bars are $\pm$ SD).

antagonist screen and compared the resulting values to cAMP (Fig. 7). IC₅₀ data for dopamine D3 receptor antagonists are tabulated in Table 2. For the muscarinic M₁ receptor, we derived the potencies of the four most efficacious antagonists identified in the antagonist screen and compared the resulting values to Ca²⁺-aequorin data (Fig. 8). IC₅₀ data for muscarinic M₁ receptor antagonists are tabulated in Table 2. The rank order of potencies of antagonists were consistent across platforms, however for these receptor targets and compounds, the sensitivity of the CellKey™ System matched or was greater than that of cAMP assay and was significantly greater than the Ca²⁺-aequorin assay.

DISCUSSION

The CellKey™ System is a novel cell-based assay platform for screening cell-surface receptors. Using this system, we tested a small panel of 25 compounds on two differently-coupled GPCRs expressed individually on CHO cells to identify hits for each receptor, evaluate the selectivity of those hits, and define the potency of selected hits. By applying the full set of compounds to both cell lines,

we effectively utilized each cell line as a null expressing, or receptor specificity control for the other. By exploiting the system's ability to monitor activation of both endogenous and transfected receptors, we were able to identify compounds that interacted non-selectively with other receptors, whether they were exogenously or endogenously expressed. Furthermore, we utilized the universal nature of impedance assays to study the two GPCRs simultaneously. In this way we demonstrated the advantages of using a single assay modality instead of resorting to two independent platforms based on the regulation of cAMP and intracellular Ca²⁺-flux.

The label-free, non-invasive nature of the CellKey™ System enables streamlined assay development. Indeed, in a single day's experimentation on one plate for each cell line, we were able to select appropriate conditions with which to run these assays for the receptors studied (Fig. 2). Using these simple parameters, we applied each of the 25 compounds to both cell lines expressing either dopamine D3 receptor or muscarinic M₁ receptor. The agonist hits identified are displayed in Fig. (3), and summarized in Table 1. Additionally, we determined the potencies of the reference

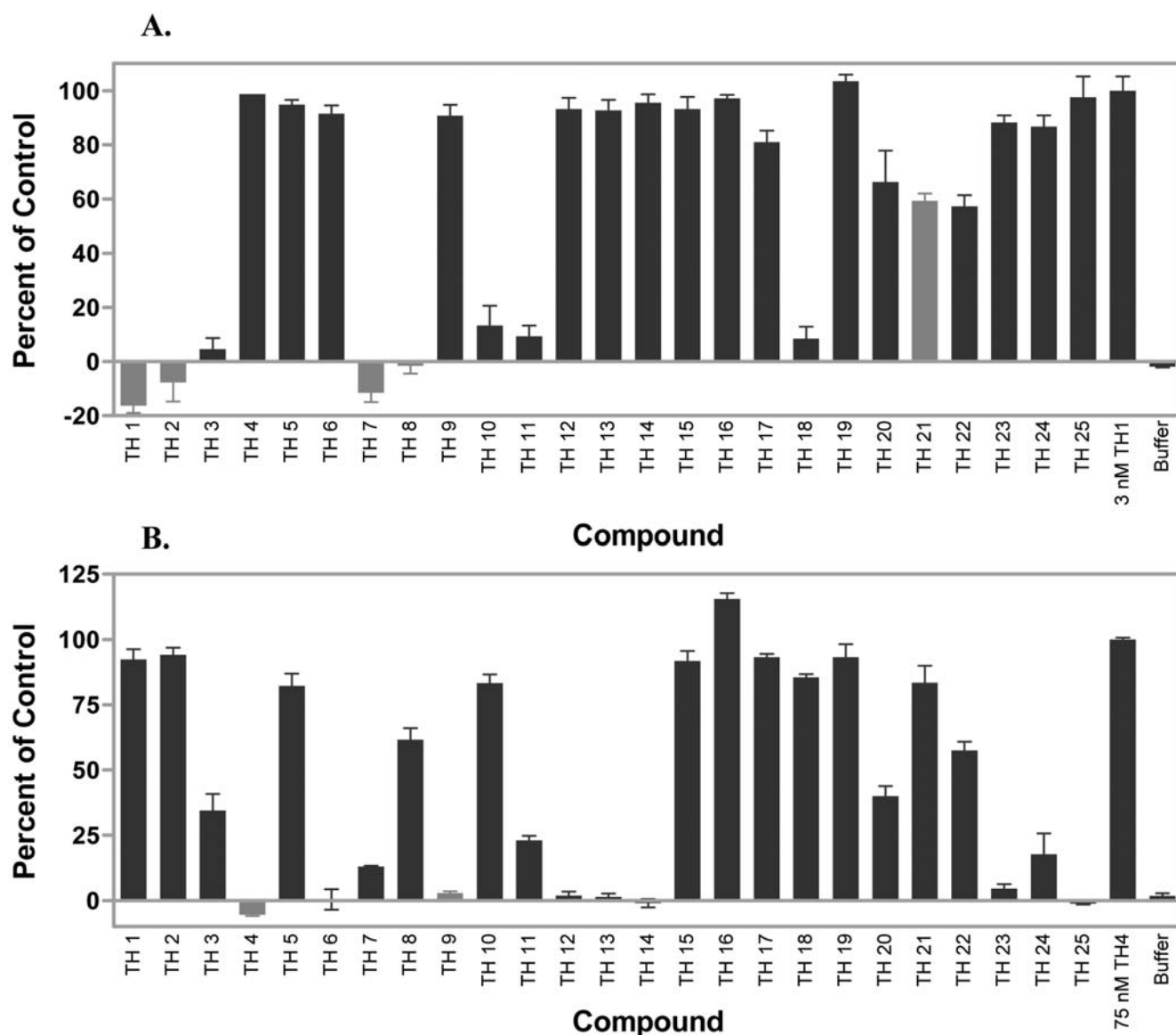


Fig. (6). Antagonist screen on CHO-D3 and CHO-M₁ cells. In antagonist hit confirmation experiments, CHO-D3 (A) and CHO-M₁ (B) cells were pre-treated with 10 μ M TH1 to TH25 then challenged with the EC₈₀ dose of TH1 (A) or TH4 (B) as indicated. Cellular activation was monitored and data were plotted as percent of positive control (EC₈₀ dose of reference agonist in the absence of pre-treatment). Gray bars indicate compounds that were shown to have agonist activity in the previous agonist screen. (n = 3, error bars indicate $\pm$ SD).

agonists on their respective targets (Fig. 4) and used this information to derive the EC₈₀ doses to be applied in the antagonist screen (Fig. 6).

The kinetic read-out of the system, visualized by the response profile, reflects the integrated cellular response to stimulation of receptor by a ligand. This is in contrast to other technologies that assay a single event, or mediator, in a signaling cascade (e.g., cAMP and Ca²⁺) [29]. Therefore, each response, as detected by the CellKey™ System, is a summation of all the intracellular pathways that ultimately result in modulation of the cytoskeleton. Since differently coupled receptors activate different signaling pathways, their activation results in distinct response profiles. Experiments not shown here have been performed to link the activities of key cellular pathway intermediates to the gross features of the response profiles. For example, the biochemical

modulators, BAPTA-AM and phorbol 12-myristate 13-acetate (PMA), when applied to cells prior to activation with a G_{αq}-coupled GPCRs, modulate the observed early dip in impedance and later rise in impedance, respectively. In this way it has been determined that the early dip in impedance is related to intracellular Ca²⁺ release and the later rise in impedance is related to protein kinase C (PKC) activation. By using these and other biochemical pathway modulators, the signaling cascades of the major GPCR families have been analyzed. Thus, the kinetic features of the CellKey™ response profiles can be used as a starting point for understanding the pathways activated, yet the natural complexity of GPCR signaling dictates that modulators be included to fully develop assays.

Important to this study are the response profiles generated by activation of G_{αi}- and G_{αq}-coupled GPCRs

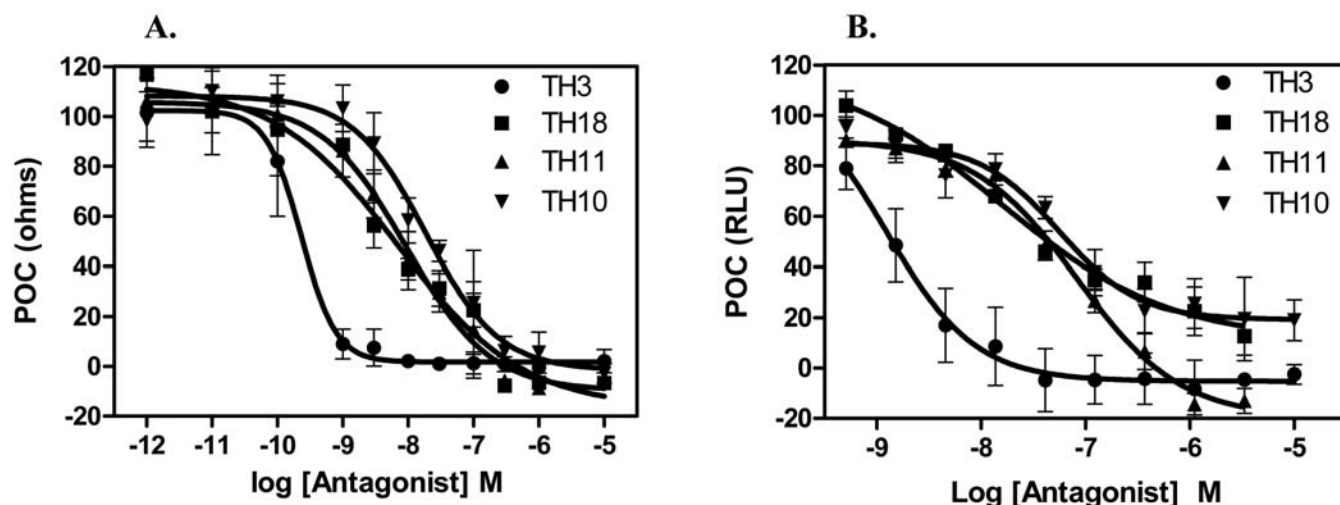


Fig. (7). Concentration response curves of four dopamine D3 receptor antagonists. CRC for four dopamine D3 receptor selective antagonists were produced. IC_{50} values for four selective antagonists identified in Fig. (5) were derived by the CellKey™ System (A) and cAMP (B) assays. The EC_{80} of TH1 was used as the challenge dose in the antagonist assays. Responses are normalized to the highest doses of ligands ($n=4$, error bars indicate $\pm SD$).

expressed in CHO cells. As discussed in Fig. (1), $G_{\alpha i}$ -coupled response profiles feature an immediate and sustained increase in impedance after ligand addition, while $G_{\alpha q}$ -coupled response profiles dip in impedance then rise. Response profiles that do not conform to these basic features are generated by receptor signaling cascades other than those under study, either due to mixed/complex coupling or to the activation of multiple receptor pathways simultaneously. Therefore, inherent to the CellKey™ response profiles is a measure of compound selectivity. Matching the unique qualitative data of the response profiles, with quantitative measures of receptor activation, we have created an enhanced selectivity screening approach. To demonstrate this technique, we compared the response profiles of the reference agonists to others in the sample set (Fig. 5). The reference compounds generated distinctive response profiles only on the cell line expressing their target receptor, indicating the reference agonists were in fact stimulating the intended receptors. Some compounds generated cellular responses and response profiles that were similar regardless of the cell line to which they were applied, indicating that these compounds were non-selectively activating receptors expressed on these cells. The CellKey™ system identified the non-selective nature of compound TH3 where Ca^{2+} -aequorin assays did not (data not shown). This is due to the ability of the system to monitor cellular activation in a pathway independent manner, and likely to its greater sensitivity. This experiment confirmed that the unique CellKey™ response profiles enabled the discrimination between selective and non-selective compounds. Further experimentation would need to be performed to determine the precise nature of the interaction between compound TH3 and the receptors under study. Indeed, it would be interesting to test compound TH3 on untransfected CHO1 cells to determine the degree to which endogenous receptors expressed in the CHO background contributed to the overall response.

We also used this label-free platform to identify and characterize antagonists in this compound set for each GPCR. For each receptor, several antagonists were identified (Fig. 5). We further characterized the most efficacious antagonists by defining their potency and compared our results to results generated from either a cAMP assay or a Ca^{2+} -aequorin assay. For the $G_{\alpha i}$ -coupled dopamine D3 receptor, the impedance assay results indicated that some antagonists for this receptor were significantly more potent than that determined by cAMP assay (Table 2). This was also true for the $G_{\alpha q}$ -coupled muscarinic M_1 receptor (Fig. 6).

At this time, it is unclear why the impedance assay was consistently detecting antagonists with greater potency than as determined by other assays. However, there are a few plausible explanations. First, CellKey™ assays represent a direct measurement of cellular responses manifested by receptor activation, whereas other assays (cAMP modulation or Ca^{2+} -flux) utilize platforms that rely on forskolin stimulation of the signaling event, in the case of cAMP assays, or the expression of a reporter gene, in the case of Ca^{2+} -aequorin. Additionally, impedance data reflect the integrated activity of a receptor across the global cellular signaling pathways activated. It is possible that by interrogating only a single point in a pathway activated by a GPCR (e.g., cAMP modulation or Ca^{2+} -flux) the true potency of a compound is misinterpreted, since that particular pathway intermediate may not reflect the global change imparted on the cell after receptor stimulation [30]. It is also somewhat possible that these observations are specific to the two receptors tested in this study.

These studies provide the first look at an enhanced selectivity screening approach, which combines two techniques that are typically broken into separate components. First is the derivation of the relative potencies of compounds across related targets or subtypes within a family. Second, is a determination of the off-target effects

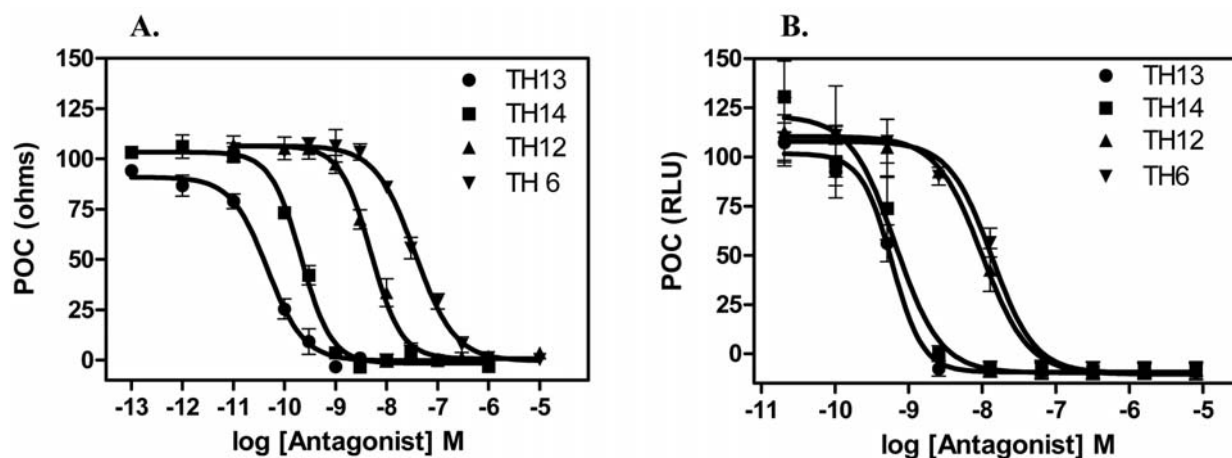


Fig. (8). Concentration response curves (CRC) of four muscarinic M_1 receptor selective antagonists. CRC were produced for a panel of four muscarinic M_1 receptor selective antagonists identified in Fig. (5). IC_{50} values were derived by (A) the CellKey™ System and (B) Ca^{2+} -aequorin assays. The EC_{80} of TH4 (Fig. 7A) was used as the challenge dose in the antagonist assays. (C) As can be seen, the potency ranking of antagonists was similar on both assay platforms, demonstrating that impedance assays generate robust data that are comparable to other assays. Data are normalized to the cellular response induced by treatment with the EC_{80} of TH4 in the absence of antagonist. (n = 4 CellKey™ System CRC; n = 3 Ca^{2+} -aequorin CRC; error bars indicate $\pm$ SD).

across a panel of unrelated receptors [31, 32]. Prior to these studies, the processes of deriving relative selectivity of compounds and identification of off target effects were functionally uncoupled and performed at greater effort and expense [33]. By the very nature of the information rich readout, the CellKey™ System therefore, simultaneously provides information on the potency and efficacy of a lead compound and a measure of its selectivity. This critical information is most valuable at the early stages of drug development and helps to inform decisions in later phases that are more costly and difficult to perform [34].

Table 2. Potencies of Selective Dopamine D3 Receptor and Muscarinic M_1 Receptor Antagonists. IC_{50} Values Derived by Impedance Assay, cAMP Assay and Ca^{2+} -Aequorin Assay for Each Receptor Target. Data were Generated from Concentration Response Curves Displayed in Fig. (7) for the Dopamine D3 Receptor and in Fig. (8) for Muscarinic M_1 Receptor

Antagonist	Impedance IC_{50}	cAMP IC_{50}	Ca^{2+} -Aequorin IC_{50}
TH3	0.23 nM	2.7 nM	-
TH18	8.1 nM	14.7 nM	-
TH11	9.2 nM	12.1 nM	-
TH10	18.5 nM	65.3 nM	-
TH13	0.46 nM	-	0.59 nM
TH14	0.21 nM	-	0.66 nM
TH12	4.9 nM	-	9.9 nM
TH6	36 nM	-	130 nM

In conclusion, we have shown that common applications in secondary screening, such as hit confirmation, receptor specificity, and pharmacological profiling are easily enabled using the CellKey™ System. Importantly, in a single experiment the unique response profiles were able to

generate hit data for target receptors, indicate the possible G-protein coupling mechanism of those receptors and compile information about compound selectivity. Additionally, these experiments yielded robust pharmacological data that are comparable to typical cAMP and Ca^{2+} -flux measurements, but indicated a greater degree of potency than was determined by either single point assays. However, potency ranking of antagonists was maintained between the three assay technologies. The label-free and universal nature of the system made assay development very simple relative to other cellular assay technologies [35]. These studies provide proof-of-concept that label-free technologies offer distinct benefits to drug discovery efforts, and should aid in the identification of novel, selective lead compounds earlier in the drug discovery process.

CONFLICTS OF INTEREST

Ryan McGuinness, John Proctor and Debra Gallant are employees of MDS Analytical Technologies, manufacturer of the CellKey™ System.

ABBREVIATIONS

CDS	=	Cellular dielectric spectroscopy
CHO	=	Chinese hamster ovary
GPCR	=	G-protein coupled receptor
LIMS	=	Laboratory information management systems
PTKR	=	Protein tyrosine kinase receptor
POC	=	Percent of control
dZiec	=	Difference in impedance related to extracellular current
dZite	=	Difference in impedance related to transcellular current
CRC	=	Concentration response curve
EC_{50}	=	50% Effective concentration
IC_{50}	=	50% Inhibitory concentration

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