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Cell-Based High-Throughput Screening Assay for Identification of G-Protein-Coupled Receptors Agonists and Antagonists.

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Abstract

The GPCRs are the target of a significant portion of all approved drugs and also represent an important parcel of potential drug targets for new indications. Activation of GPCRs can be detected by the utilization of functional cell-based assays like measuring transient intracellular calcium mobilization. The emergence of the fluorescent imaging plate reader and Ca^{2+}-sensitive fluorescent dyes has made the high-content screening of GPCR possible. Here was applied a dual automated FLIPR platform to testing a large collection of compounds to identification of both agonist and antagonists form a single screen of GPCR.

Key words: Ca^{2+} mobilization, GPCRs, FLIPR, fluorescent Ca^{2+} dye, high-throughput screening

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Introduction

G protein-coupled receptors (GPCRs) are the largest family of membrane receptors, which makes them very interesting targets for the development of new drugs [1]. More than 30% of the drugs currently available on the market are directed against this type of receptors [2].

After agonist binding, activated receptors suffer a conformational change that lead to the exchange of guanidine diphosphate (GDP) for guanidine triphosphate (GTP) on the α-subunit oh heterotrimeric G proteins (composed of α-, β-, and γ-subunits) which in turn, produce a conformational change that leads to the dissociation of Gα of the Gβγ units [3]. There are four G protein subfamilies based on the structural and functional similarity of their α subunit ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$) [4]. GPCRs mainly signal through the $G\alpha_{s/i}$ G-protein/cyclic adenosine monophosphate (cAMP) and $G\alpha_q$ G-protein/calcium to regulate a wide number of cellular functions [5].

In the early days of drug discovery, ligand binding assays were used to find compounds binding GPCRs, but the new technologies have permitted the introduction of functional cell-based assays in high throughput screening (HTS) campaigns, being the measurement of intracellular calcium mobilization the most widely used (Fig. 1A) [5]. GPCR that couple to $G\alpha_q$ induce a ligand-dependent increase in intracellular Ca^{2+}, but by the expression of a chimeric or a promiscuous G-protein, GPCRs can also be reconfigured to stimulate Ca^{2+} signalling [6].

The first readers had the disadvantage of being able to read only one well at the same time, what made little useful for the HTS. The appearance of the Fluorescent Imaging Plate Reader (FLIPR) and the cell-permeable Ca^{2+}-sensitive fluorescent dyes makes possible HTS in 384- or 1536-well plates, allowing the detection of agonist and antagonist in one assay (Fig. 1B) [7]. The FLIPR, and other similar instruments released in recent years, has an integrated pipettor which allows successive compound addictions to the assay plate [8].

These instruments allow the reading of the full plate once per second, allowing the recording of Ca^{2+} kinetics, which is critical to measure the fast response, observed in the GPCRs (Fig. 1C). The kinetics data make possible obtain more information that a single-read endpoint assay, providing activation fingerprint for the GPCRs, although the signal description parameters commonly used in HTS are either the maximum peak height (PH) or the area under the response curve (AUC), which are normalized using the control wells [9].
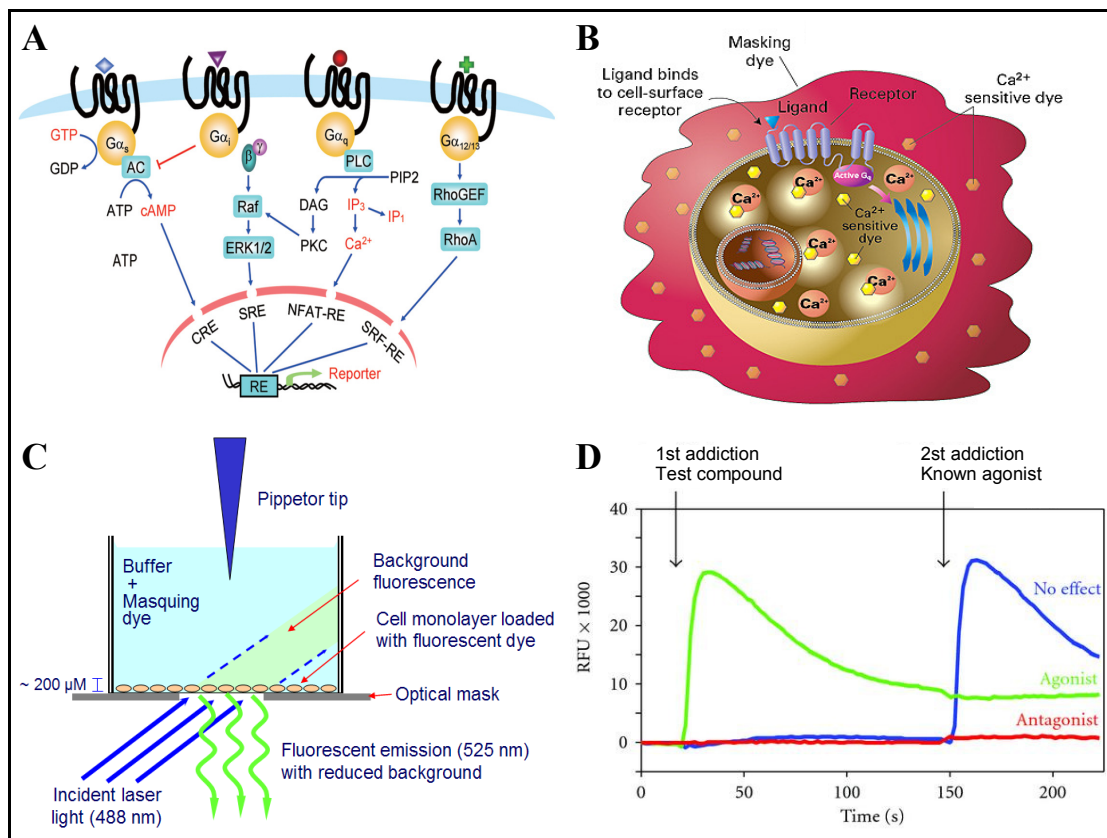
Figure 1. *A. Receptor binding and G-protein-dependent assays.* Schematic representation of receptor binding and major pathways activated by different G proteins. Red indicates the detection points of commonly used HTS assays. *B. Calcium mobilization assay.* The calcium sensitive dye is taken into the cytoplasm of the cell during incubation. The masking dye remains outside the cell and blocks background fluorescence. Upon ligand binding to the receptor, calcium is released into the cytoplasm of the cell. The dye binds to the intracellular calcium and becomes fluorescent. *C. FLIPR features.* FLIPR system combines multiwell liquid addiction, simultaneous data acquisition and optic system for illumination and fluorescence detection. *D. Assay format for the identification of an agonist and antagonist from a single screen.* Test compound is added first followed by a second addition of a known agonist. An agonist will induce a FLIPR response immediately upon the addition of a test compound. An antagonist will cause a pharmacological blockade of the GPCR resulting in attenuation or complete lack of response to a known agonist. In the event of no receptor engagement, a normal agonist response will be observed from known agonist addition.

The introduction of simple statistical measurements, such as the Z prime factor (Z'), has facilitated creating a common tool to compare the quality of different assays [10]. In an ideal test assay, the active compounds must have a completely distinguishable behaviour from that of inactive compounds, which should allow its discrimination. The maximum and minimum control values must be reproducible and widely separated from each other and there would be no false positives or false negatives [11]. For a more detailed description on terminology related to HTS, see the glossary of terms used in biomolecular screening following IUPAC recommendations [12].

Materials and methods

U-2 OS/Ga chimera stable cell line and BacMam Virus generation.

U-2 OS (human osteosarcoma ATCC HTB-96; ATCC, Manassas, VA) stably expressing chimeric $G\alpha_q$-protein was generated by the Biological Reagent Assay Development Division (BRAD). The cells are frozen and sent to the department of Screening and Compound Profiling (S&CP) where the assay was conducted. Recombinant GPCR BacMam was generated according to established protocols by BRAD.

Transduction of U-2 OS/Gα Cells.

Cells are thawed according to established protocols. U-2 OS/Gα are re-suspend in DMEM/F12 with 10% FBS to a final density of 250,000 cells/ml. BacMam are added with a titer of $7.4 \cdot 10^8$/ml. 50 μL of cells were plated in 384-well clear black bottomed poly-d-lysine tissue culture treated plates (12.500 cells/well). Cells were placed in an incubator at 37°C, 5% CO_2, 95% humidity for 18-24 h to allow for GPCR expression.

Small-molecule library used for HTS and compound plates.

Compound 384-well plates were prepared by Sample Management Technologies Department (SMTech) with 250 nL of the compounds dissolved in DMSO (dimethyl sulfoxide) at 1 mM using an Echo 555 acoustic liquid handler (Labcyte Inc.) and stored at 4 ºC. The small-molecule library of the Centro de Investigación Básica (CIB) at *GlaxoSmithKline (*GSK) in Tres Cantos was screened. The CIB library compound library contains approximately 2.5 million compounds. Column 6 was plated as unstimulated negative control, containing only DMSO and in the column 18 was added 30 mM of a known agonist as a positive control.

FLIPR functional studies.

The HTS campaign was conducted using a fully automated assay system with dual FLIPR[384] fluorescence imagers (Molecular Devices). On the day of assay, cell plates were removed from the incubator, and culture medium was removed and replaced with a cell staining buffer containing calcium-sensitive dye prepared following manufacturer's instructions (Calcium assay kit; Molecular Devices). Cells were dye-loaded with 20 μL of cell staining buffer at 37°C for 1 h. The dye is excited at the 488 nm wavelength with an Argon ion laser and a 515 nm emission filter was used. In each experiment baseline fluorescence after dye loading was adjusted to ≈15,000 fluorescence counts by adjusting the strength of the laser or changing the exposure time. Compounds were dissolved in 20 μL Tyrodes Buffer (Sigma) supplemented with 20 mM HEPES and 12 mM sodium bicarbonate. For the double addition protocols, initial readings by the first FLIPR (3 images at 1 sec intervals) of the cell plate were taken to assess loading uniformity. Following addition of 10 μL from the compounds plate to the cell plate at a final assay concentration of 4.1 μM with a 0.5% DMSO, 30 images were recorded at 1 sec intervals and then 30 at 2 sec intervals. This first read was used to determine direct agonist activation of the GPCR by the test compounds. Plates were then returned to the 37°C incubator for 30 to allow system to equilibrate. After the compound equilibration time, 10 μL of known agonist at a final concentration of 16 μM (EC_{80}) was added by the second FLIPR in all wells and 30 readings were taken at 1 sec intervals and then 30 at 2 sec intervals. This second read was used to determine direct antagonist inactivation of the GPCR by the test compounds. Ca^{2+} mobilization was monitored as an increase in relative fluorescence units (RFU), by the FLIPRs and the AUC as well as difference between maximum and minimum RFU was calculated. ScreenWorks 3.1 software (Molecular Devices) is used for data collection. Data analysis was performed using ActivityBase (ID Business Solutions).

Statistical analysis.

Assay performance was judged by calculating the Z′ factor value and coefficient of variation (CV). $Z′=1-3(SD_{c+}+SD_{c-})/|M_{c+}-M_{c-}|$; $CV=SD_{c+}/|M_{c+}-M_{c-}|$. Signal-to-background ratio (S/B) was also used as a reference to evaluate the performance of the assay: $S/B = M_{c+}/M_{c-}$. SD_{c+} and SD_{c-} represent standard deviation (SD) for the positive control and negative control, respectively. M_{c+} and M_{c-} represent the mean for the positive control and negative control, respectively. The Z' was calculated from the measurements from the 8 positive and 8 negative control wells located in each plate. Only plates with a Z' < 0.4 were accepted.

Results and discussion

Assay development and library screen.

A dual-addiction assay format that could allow both agonists and antagonists to be distinguished in a single screen was developed. The diagram in Figure 1D describes the two step protocol where addition of the test compound (addition 1) is followed by the addition of known agonist (addition 2). Upon ligand binding to the receptor, Ca^{2+} is released into the cytoplasm of the cell. The calcium sensitive dye is taken into the cytoplasm of the cell during incubation. The kit's masking technology remains outside the cell and blocks background fluorescence. The dye binds to the intracellular calcium and becomes fluorescent. A Ca^{2+} mobilization response to the addition of a test compound would indicate its agonist activity. Besides, if the compound behaves as an antagonist, not only will there be an absence of response to this test compound, it will also decrease the effects of the following addition of known agonist. If there is no receptor inhibition by the test compound, a normal agonist response will be observed to a known agonist.

Assay validation.

The validation of the test consists of a series of steps necessary to ensure good assay robustness, performance reproducibility over the course of the screen and ability to discriminate active from nonactive compounds. This process is composed of 4 steps, the first of which applies to all assays transferred from an external laboratory. Step 0 consists of the initial test of the reagents that are going to be used in the HTS process. Step 1 is about the design of the operational procedure or workflow of the

campaign. The objectives of steps 2 and 3 include the validation of HTS hardware and process but also the testing of statistical parameters of the assay as the values to determine the baseline values to monitor the quality of the primary trial, check the reproducibility of the assay, as well as the ability to distinguish correctly hits from no hits [13]. In step 3 was assayed a pilot screen of a small set of the compound collection in triplicate to estimate screening performance (Fig. 2A). In this step 28 plates were tested in triplicate in different days and on random order. This small collection includes a representation of a great diversity of chemotypes present in the GSK collection. The workflow was tested in a fully automated platform with 2 readers on line. Throughput was 180 plates/day in a 10 h operation time. Using a 45% activation threshold, the calculated hit rate was 0.7% for agonists and a 45% inhibition threshold submitted a 0.5% hit rate.
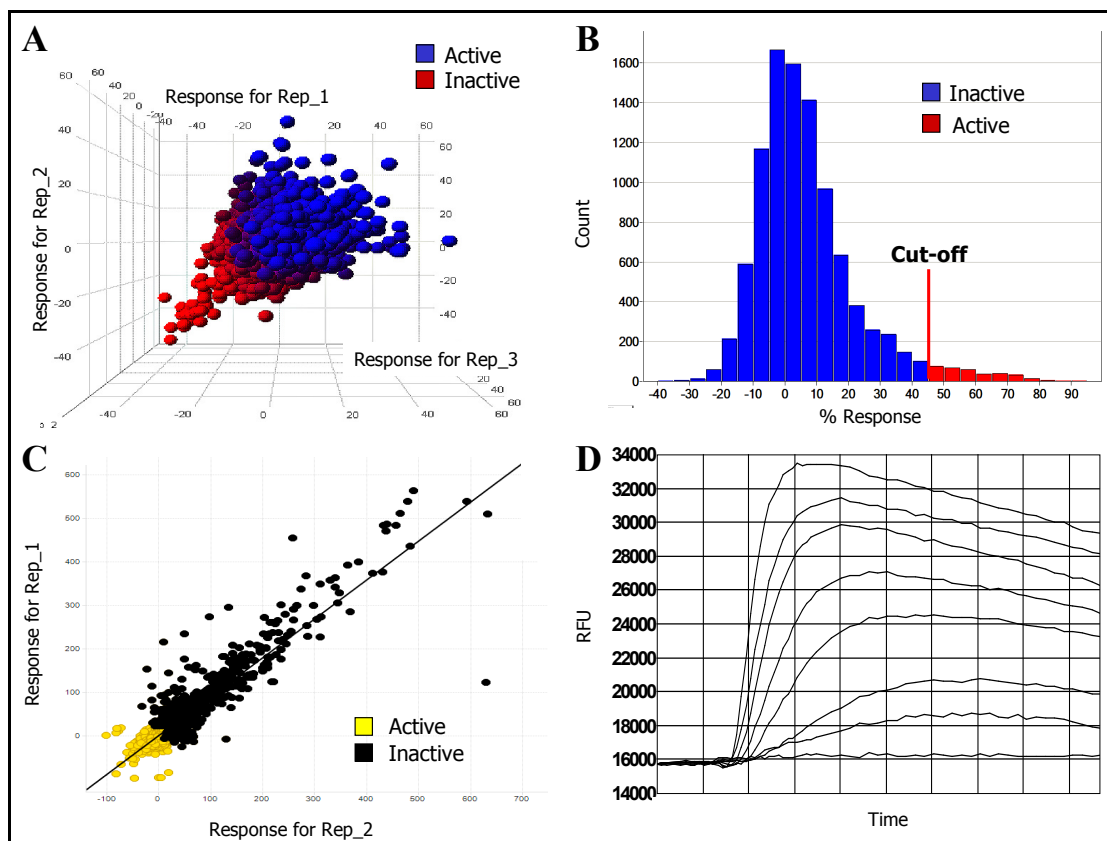

Figure 2. *A. Assay Validation*. Correlation plot for the 3 replicates of the validation collection. Compounds with more of 45% effect in 2 repetitions are defined as actives. *B. Distribution of total agonist compound activities in the HTS campaign.* The hit cut-off was set at 45 % response (3 times the standard deviation), generating a 0.7% hit rate. *C. Hits Confirmation*. Secondary screen of candidate GPCR agonists using parental cell line in the Ca^{2+} mobilization assay with the objective of removing compounds not interesting for the project. *D. Example of dose dependent response induced by a candidate GPCR agonist as measured in Ca^{2+} mobilization assay.*

The quality of assay results was monitored by determination of the Z' factor for each assay plate. The Z' factor remained greater than 0.7 for all plates, indicating the robustness and the suitability of the assay test, so the assay was validated.

Primary screening.

Figure 2B shows the primary screen of nearly 2.2 million compounds identified ≈14.000 compounds that behaved as agonists and ≈10.000 as antagonists. Compounds with more than 45% activation or 45% inhibition of GPCR at a concentration of 5 µM were designated as "hits" and subjected to further verification of activity. The cut-off for active agonist and antagonist was based on the mean effect of the minimum control and 3 times their SD.

Hit Confirmation.

Over-expression of chimeric G-proteins as well as GPCRs may cause artefacts, possibly caused by the interference of these proteins with multiple signal transduction pathways. Thus, the selected primary hits were "cherry-picked" and rescreened against the parental cell line, which lacks the chimeric G-protein and the receptor of interest, in order to discriminate compounds with endogenous activity. The assay was

performed in duplicate on different days. The agonist confirmation hit response cut-off was set at 10% activation response (Fig. 2C).

Compounds titration.

The potency of a compound is indicative of the amount of it needed to obtain a certain effect. The potency of the agonist and antagonist compounds confirmed was the determined by assaying compounds as 11-point titrations (Fig. 2D). These compounds had a potency range, expressed as the EC50, of 0.1 to 5 μM.

Lead generation.

Once obtained a list of confirmed hits, along with supporting information on their potency and chemical features, it is decided which of them will continue in a lead generation program. It is very important set a quality assessment to include compounds with good drug-like properties to prevent attritions in a posterior development phase. For this it is necessary from the beginning to determine the characteristics of chemical integrity, structure-activity-relationship (SAR) as well as bio-physicochemical and absorption, distribution, metabolism and excretion (ADME) properties required [14].

Acknowledgements

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