A Duplicated Calcium and β-Arrestin Assay Protocol for Human Histamine H1 Receptor PathHunter CHO-K1 HRH1 β-Arrestin Cell Line

Thierry Calmels1, Sabine Rouanet1, Jean-Marc D’Angelo2, Andrew Green3*

1 BioProject, 30, rue des Francs-Bourgeois, 75003 Paris, France; 3 Hamamatsu Photonics France S.A.R.L., 19, rue du Saule Trapu, Parc du Moulin de Massy 91300 Massy – France
2 DiscoverX Corporation, Fremont, CA 94538 * Corresponding Author: agreen@discoverx.com

Abstract
The screening and functional characterization of novel ligands for G-protein-coupled receptors (GPCRs) remains a key activity in drug discovery process. Furthermore, an increasing awareness of the subtleties of receptor signaling and potential for ligand bias requires multiple endpoints to be monitored. Efforts to reduce costs and improve media as per recommendation also remain a key consideration. In this poster, we describe efforts to develop a protocol for the Histamine H1 receptor employing the DiscoverX PathHunter® β-Arrestin clonal cell line. We have adapted existing protocols such that from a single plate we can monitor calcium signaling with fluorescent dyes sensitive to intracellular Ca2+ ion flux in real time, followed by the recruitment of β-arrestin as an endpoint assay. Protocol development will be discussed and successful implementation of the final assay conditions will be exemplified with a characterization of H1 agonists and antagonist pharmacology.

GPCR-Mediated β-Arrestin and Calcium Signaling Dual Assay Principle

GPCR-mediated β-arrestin recruitment and calcium signaling pathways in the CHO Histamine H1 PathHunter β-arrestin cell line.

Methods

Cell Culture
PathHunter CHO-K1 HRH1 β-arrestin cell line (Part Number 93-0503C2) co-expressing the ProLink™ (PK) tagged GPCR and the Enzyme Acceptor (EA) tagged β-arrestin-2, were used. Cells were maintained in AssayComplete™ media as per recommendation. Media as well as media was supplemented with 5% CO2 at 37°C. All cultures were incubated in 1% CO2 at 37°C, 5% CO2.

Calcium Signaling Assay
The Fluor-4 AM dye solution was prepared as per manufacturer’s protocol. Test compounds, agonists or antagonists (10 nM stock solution) were diluted in AssayComplete Cell Plating (CP) 2 Reagent or in 1X HBSS-Cal5+Mg2+ and 20 mM HEPES buffer (pH 7.4). Cell culture medium was removed and replaced with 100 µL of Fluor-4 dye solution. Cells were then incubated for 30 - 45 minutes in 5% CO2 incubator at 37°C. Cells were washed with 200 µL of the 1X HBSS Buffer to remove excess dye and re-suspended in 80 µL of CP 2 Reagent. At this step, 10 µL of 10X agonist solution (agonist mode) or 10 µL of CP 2 Reagent (agonist mode) were added to each well. Plates were incubated in the dark for 30 minutes at RT for esterase activity and antagonist binding.

The plate was placed in the FDS55/Cell (Hamamatsu), 10 µL of 10X agonist solution (Histamine at 300 nM final concentration) were injected (3 mm height, aspiration rate at 40 µL/s) at a dispense rate of 10 µL/s. Signal Detection and Data Analysis
Fluorescence was measured for 90 seconds (1 lecture), binning at 2 X 2, exposure time 200 ms (sensitivity 2), Ex 480/Em 540. Responses were calculated as the difference between maximum and minimum fluorescence counts (Fmax - Fmin). Results were normalized and were presented as a % of maximal response of histamine agonist.

β-arrestin Assay
β-arrestin assay is started by agonist injection during the calcium assay. Cells were incubated for additional 90 minutes in 5% CO2 incubator at 37°C.

Working Detection Solution was prepared by mixing 19 parts of Cell Assay Buffer, 5-parts of Substrate Reagent 1, and 1-part Substrate Reagent 2 (included in the PathHunter detection kit). 50 µL of the solution was added to the assay plate and incubated in dark for 1 hour at RT prior to chemiluminescent signal detection.

Signal Detection and Data Analysis
The chemiluminescent signal was read on the FDS55/Cell (Hamamatsu) in the luminescence mode: 10 samples, 10 ± internal, sensitivity 5. All curves fitted using unconstrained four parameters logistic fit using GraphPad Prism software.

Results were normalized and were presented as a % of maximal response of histamine agonist.

Optimizing the assay conditions to facilitate dual readouts from a single well. Cells were seeded in black/clear bottom TC-treated plates and assays read in standard luminometers. 1 FDS55/Cell Plating (Hamamatsu) and 1. Microliter” Microplate reader (Perkin Elmer)

Assay of binding and antagonist binding. Histamine concentration response curves were compared in standard loading conditions to confirm CP 2 for both binding and loading in CP 2 to establish compatibility with the β-arrestin recruitment assay protocol. CP 2 can be used to support a calcium signaling study with Flu-4 dye, and the same plate and culture conditions are compatible with β-arrestin measurement.

Study of Histamine H1 Agonist Pharmacology Using Dual Protocol

Summary
- β-arrestin cell lines expressing H1 receptor provided robust responses by detecting HA-induced β-arrestin recruitment
- To measure both fluorescence (Ca2+) and luminescence (β-arrestin), use of black clear-bottom microplate as well as CP 2 plating medium is highly recommended
- Single-plate dual Ca2+ and β-arrestin assay have been implemented for hHR1 expressing β-arrestin cell line: Strong fluorescence (Calcium) as well luminescence (β-arrestin) signals have been recorded
- Histamine H1 antagonists profiling can be obtained regarding EC50 of histamine, up to 100 µM.

POTENCY RANKING OF GPCR MODULATORS FOR CALCIUM SIGNAL AND β-ARRESTIN PATHWAYS

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