Ranking of β_2 Adrenergic Receptor Agonists and Antagonists Using a Cell-Based GPCR Signaling Application

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Abstract

G-protein coupled receptor (GPCR) signaling has great importance in physiological development and various biological processes and hence has become a significant focus for various therapeutic groups in the area of drug discovery. As many of the various GPCR signaling pathways have been well characterized, a large number of chemical libraries are being synthesized targeting the various GPCRs either as agonists or antagonists. These compounds are usually ranked for potency against a specific GPCR based on homogeneous biochemical assays. The β_2 adrenergic receptor (β_2-AR) is a member of the GPCR family of receptors, linked to adenylyl cyclase. The activation of β_2-AR is regulated by the binding of β-arrestins to the phosphorylated receptor, leading to the internalization of the receptor. Cellomics Inc., has developed a cell based high content screening, GPCR Signaling BioApplication for the ArrayScan® HCS Reader that uses Norak's Transfluor™ technology and allows for the screening of compounds that affect the GPCR signaling cascade. This application quantitates GPCR activation by measuring translocation of a GPCR activated β_2-arrestin-GFP chimera in cells.

We screened a library of GPCR agonists and antagonists for their ability to activate β_2-AR in cells transfected with a β-arrestin-GFP chimera and β_2-AR, using the cell based high content screening assay. Dose response curves were then generated to determine EC_{50} and IC_{50} concentrations for the agonists and antagonists respectively. Our results showed that the compounds we screened for agonistic activity had EC_{50} concentrations ranging from 0.01– 225μM, while the compounds exhibiting antagonistic activity had IC_{50} concentrations ranging from 0.003- 0.160 μM. We were also able to show that the antagonistic potency of these compounds varied, depending on the agonist used in pre-incubating the cells. We have ranked these compounds for their agonist or antagonist potency and specificity towards the β_2-AR, based on their EC_{50} or IC_{50} concentration respectively. Our results show that ranking of various compounds as either an agonist, antagonist or a partial agonist can be rapidly done using this cell based high content screening assay. Our results also show that the cell based assay approach can be used to discriminate between isomeric forms of a compound.

Introduction

GPCRs are a super-family of integral membrane proteins that share a seven trans-membrane domain homology. These proteins are involved in transducing chemical/ biological messages across the cell membrane and between cells. The binding of a ligand to its GPCR activates the heterotrimeric G protein, which in turn activates a cascade of events that often leads to either inter-cellular or intra-cellular transduction of the biological signal (1, 2). GPCR signaling plays a vital role in a variety of biological functions such as neurotransmission, olfaction, mitosis, photo-transduction, cardiovascular function, sensory signaling such as perception of pain and hormone action (3). Due to their critical role in a wide variety of biological processes (3), and their availability on the surface of cells, GPCRs are important potential targets for treating various pathological conditions.

β-arrestins play a critical role in the process of receptor desensitization, internalization and also resensitization (4) of GPCR activity and are therefore natural indicators of GPCR activation. Following the activation of the GPCR, β-arrestins undergo a redistribution from their normal cytosolic location to the site of GPCRs on the cell membrane (4), thereby regulating the activity of the GPCRs. Thus, one method to study how GPCR activation is coupled o intracellular signaling, is the analysis of GFP-β-arrestin translocation using imaging methods.

Cellomics, Inc. has developed a High Content Screening (HCS) BioApplication to determine the activation of GPCRs (5). This application, when used with the ArrayScan HCS Reader, enables the spatial analysis of GFP-β-arrestin redistribution that results from GPCR activation. and trafficking, providing high content information at the cellular level. In the assays performed, cells expressing a GFP-β-arrestin chimera are used, such as the Transfluor™ technology available from Norak, Inc. The β2-adrenergic receptor (β2-AR), is a member of the GPCR family of receptors. This class of receptors are predominantly found in smooth muscles of vascular, bronchial, gastrointestinal tissues, as well as skeletal muscle and the liver (6). The activation of β2-AR is regulated by the binding of β-arrestins to the phosphorylated receptor, leading to the internalization of the receptor (7). Because of its physiological significance, the β2-AR has been well characterized and is the target of medicinal chemists for many years. This has resulted in the commercial availability of library of compounds that are either agonists or antagonists of the β2-AR.

We have used the Cellomics' GPCR Signaling BioApplication to study the effects of a few of these commercially available compounds on β2-AR as either agonists, partial agonists or antagonists of the GPCR. The compounds were ranked for agonistic or antagonistic activity based on EC_{50} or IC_{50} concentrations obtained from the GPCR Signaling BioApplication.

Assay Description

- Cellomics' HCS software modules (BioApplication) running on the ArrayScan HCS Reader, automatically quantitate changes in fluorescence intensities of fluorescently labeled targets in cells to provide individual cell and well level (population averages) data from cells in 96 or 384 well microplates.
- The Cellomics' GPCR Signaling BioApplication quantifies GPCR activation by classifying cells as being in one of four phases (Phase 0, Phase 1, Phase 2 and Phase 3), based on the sub-cellular localization of the GFP-β-arrestin chimera. See Figure 1 for a description of the various phases.

Experimental Procedure:

- Norak's Transfluor™ U_2OS cells co-expressing GFP-β-arrestin and β_2-AR (β_2-AR cells) were seeded on 96-well Packard micro-plates at a density of 2.5 X 10_4 cells/ well in complete Eagle's Minimal Essential Medium (EMEM). The cells were incubated for 18 to 24 hrs. at 37°C in 5% CO_2.
- For agonist experiments, β_2-AR cells were stimulated with agonists for 7 min. at 37°C in 5% CO_2 atmosphere.
- For antagonist experiments, cells were pre-incubated with antagonist for 20 min. followed by stimulation with agonist at 5–10 times EC_{50} concentrations at 37°C in 5% CO_2 atmosphere.
- Following compound treatment, cells were fixed with 8% para-formaldehyde for 30 min., washed with buffer and stained for 20 min. with a dye combination to label the nuclei and the cell membrane.
- Fixed, stained cells were scanned on an ArrayScan HCS Reader, running the GPCR Signaling BioApplication using a 20x objective.

Figure 1: Phase classification of GFP-β-arrestin in The GPCR Signaling BioApplication

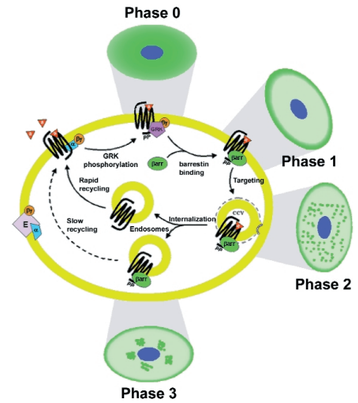

Figure 1: The Norak Transfluor technology monitors GPCR signaling by following the sub-cellular distribution of a GFP β-arrestin chimera. The Cellomics GPCR Signaling Application detects fluorescence within distinct sub-cellular domains and automatically classifies the GPCR activation state depending upon the localization of GFP β-arrestin. Phase 0: GFP β-arrestin distributed evenly throughout the cytoplasm; Phase 1: GFP β-arrestin accumulated primarily at the edge of the cell; Phase 2: GFP β-arrestin localized to early sorting endosomes; Phase 3: GFP-β-arrestin accumulated in large endosomal vesicles.

Transfluor is a trademark of Norak Biosciences, Inc. A separate license is required from Norak Biosciences, Inc. to make, use, sell offer for sale, or import Transfluor biology. (U.S. Patent Nos. 5,891,646 and 6,110,693, and other patents pending. Norak Biosciences 919-248-8000)

Results

Figure 2: Translocation of GFP-β arrestin mediated by β_2-AR agonists

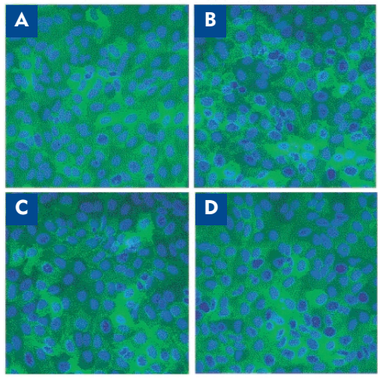

Figure 2: Translocation of GFP-β-arrestin in U_2OS cells treated with various compounds that activate the β_2-AR. (A) untreated, (B) 0.14 μM isoproterenol, (C) 0.14 μM cimeterol and (D) 0.14 μM substance P (4-11) fragment (a substance P receptor agonist-negative control) . Cells in 96 well plates were treated with the compounds as described previously. Fixed and stained cells were scanned on an ArrayScan HCS Reader running the Cellomics' GPCR Signaling BioApplication with a 20x objective. Images are two color composites of nuclear stain (blue) and the GFP-β-arrestin (green). Membrane marker (red) used to identify cell membrane is not shown. Cells treated with isoproterenol (panel B), a potent β_2-AR agonist shows high membrane localization of GFP-β-arrestin (Phase 2), while P (4-11) fragment (panel D), a substance P receptor agonist shows almost complete cytoplasmic (Phase 0) localization of the GFP-β-arrestin.

Figure 3: Dose response of β_2 adrenergic receptor activation by agonists

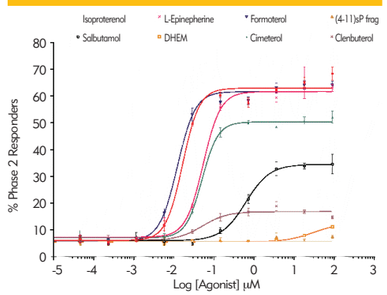

Figure 3: Dose response curves of various β_2-AR agonists in U_2OS cells. Cells were treated with the compounds for 7 minutes as detailed before. Fixed and stained cells were scanned on the ArrayScan HCS Reader using the GPCR Signaling BioApplication. Mean ± SEM of % Phase 2 responders (a well level output feature of the BioApplication) from 4 wells per concentration was plotted using Graphpad Prism.

Table 1: EC_{50} concentrations for % Phase 2 responders of β_2-AR agonists

Agonists	EC_{50} (μM)
Isoproterenol	0.011
Formoteroi	0.012
Cimeterol	0.050
Cienbuteroi	0.051
L-Epinepherine	0.054
Salbutamol	0.619
Dihydroegocristine Mesylate (DHEM)	30.61
Nylidrin	224.6
Substance P (4-11) fragment	No activity

Table 1: Ranking of β_2-AR agonists (most potent are at the top and least potent are at the bottom of the table) based on EC_{50} concentration for % Phase 2 responders. Compounds such as isoproterenol and formoterol show the most potency, while DHEM and nylidrin which are partial agonists show least potency (see Figure 3). The table was compiled based on the dose response of the compounds shown in Figure 3. EC_{50} concentrations were determined using Graphpad Prism.

Figure 4: Inhibition of GFP β-arrestin translocation by β_2 adrenergic receptor antagonists

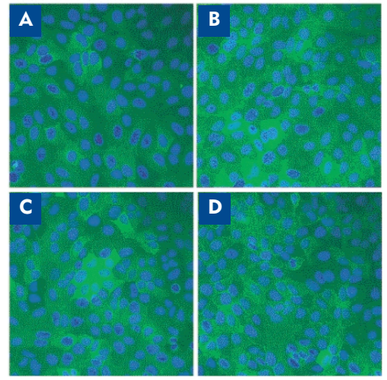

Figure 4: Inhibition of GFP-β-arrestin translocation induced by isoproterenol in U_2OS cells treated with compounds that are β_2-AR antagonists. (A) 16 nM (S)-propranolol + 100 nM isoproterenol, (B) 16 nM (R)-propranolol + 100 nM isoproterenol, (C) 16 nM ICI-118,551 + 100 nM L-epinepherine and (D) 16 nM ICI- 89,406 + 100 nM L-epinepherine. The more potent antagonist, (S)-isopropranolol shows mostly cytoplasmic localization (Phase 0; panel A) of the GFP-β-arrestin, compared to the less potent (R)-isopranolol (panel B). Cells were treated with the compounds as described earlier. Images were acquired from an ArrayScan HCS Reader running the Cellomics' GPCR Signaling BioApplication with a 20x objective. Images are two color composite showing nuclei (blue) and GFP-β-arrestin (green).

Figure 5: Dose response of antagonists inhibition of β_2-AR activation by isoproterenol

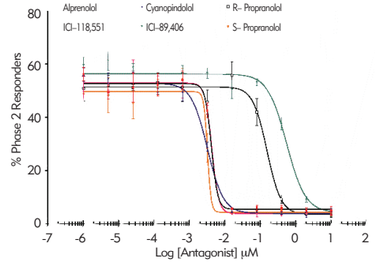

Figure 5: Dose response curves for antagonism of various compounds against β_2-AR activation by isoproterenol. Cells were treated with varying doses of the antagonists for 20 min. prior to a 7 min stimulation with isoproterenol as described previously. Mean ± SEM of % Phase 2 responders from four wells per concentration was plotted using Graphpad Prism. Notice the two orders of magnitude difference in the plots between (S)-propranolol and (R)-propranolol isomers.

Table 2: IC_{50} concentrations for various β_2-AR antagonists

Antagonist	IC_{50} μM (Rank)			
	Agonist			
	Isoproterenol	Formoterol	L-Epinepherine	Cimeterol
Alprenolol	0.004 (2)	0.040 (4)	0.004 (1)	0.003 (2)
Cyanopindolol	0.003 (1)	0.015 (1)	0.004 (1)	0.002 (1)
ICI-89,406	0.554 (4)	ND	0.102 (3)	0.114 (5)
ICI-118,551	0.004 (2)	0.025 (3)	0.010 (2)	0.004 (3)
(R)-Propranolo	0.156 (3)	ND	0.041 (4)	0.044 (4)
(S)-Propranolol	0.003 (1)	0.018 (2)	0.004 (1)	0.002 (1)

Table 2: Ranking of β_2-AR antagonists against different β_2-AR agonists. The rank of each antagonist against an agonist is shown in parenthesis beside the IC_{50} concentration for that compound. IC_{50} values obtained using Graphpad Prism.

Summary

- We have used the Cellomics' GPCR Signaling BioApplication to rapidly and efficiently screen a library of focused compounds for agonistic and antagonistic activity against β_2-AR.
- Compounds that vary widely in their activation profile of β_2-AR can be successfully screened with this application.
- The GPCR Signaling BioApplication is sensitive enough to differentiate between agonists or antagonists that differ in their activity only very minimally, and also successfully differentiate isomers of the same compound.
- Differences in antagonistic efficiency against varying agonists can be quickly ascertained.

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