Discovery of Regulators of Receptor Internalization by High Throughput Flow Cytometry


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Flow Cytometry Assay for Receptor Internalization Regulators

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Nonstandard Abbreviations Used In the Paper:

FAP, fluorogen activating protein; β2AR, beta 2 adrenergic receptor; FDA, food and drug administration; GPCR, G protein coupled receptor; ISO, isoproterenol; HCS, high-content screening; HTS, high-throughput screening; HTFC, high-throughput flow cytometry; PCL, Prestwick Chemical Library; TO, thiazole orange; ICI, ICI 118, 551; MCF, median channel fluorescence; MESF, mean equivalent soluble fluorescence; RV, response value; DHA, dihydroalprenolol; GFP, green fluorescent protein; SALB, salbutamol; FENO, fenoterol; ISOEth, isoetharine; SALM, salmeterol; (+)ISO, (+)-isoproterenol; (-)ISO, (-)-isoproterenol; CLEN, clenbuterol; TER, terbutaline; META, metaproterenol; RITO, ritodrine; ACE, acebutolol; BET, betaxolol; PIND, pindolol; BISO, bisoprolol; (S)ATE, (S)-atenolol; (R)ATE, (R)-atenolol; NAD, nadolol; ALP, alprenolol; (S)PRO, (S)-
propranolol; (R)PRO, (R)-propanolol; (+/-)PRO, 50/50 (+/-)-propranolol; TIM, timolol; METO, metoprolol; (+)LEV, (+)-levobunalol; (-)LEV, (-)-levobunalol; LAB, labetolol; OXP, oxprenol; PENB, penbutolol; PRON, propranolol; SOT, sotalol; CART, carteolol; XAM, xamoterol; PRAC, practolol; BAM, bambuterol; LEVON, levonordefrin; PROP, propafenone; ANI, anisomycin; NAF, naftopidil; DOM, domperidone; PIZ, pizotifen; MAP, maprotiline; BOL, boldine; METI, metixene; QUI, quipazine; LIDO, lidoflazine; BUC, bucindolol; CAV, carvedilol; CYC, cycloheximide
Abstract

We developed a platform combining fluorogen activating protein (FAP) technology with high-throughput flow cytometry to detect real-time protein trafficking to and from the plasma membrane in living cells. The hybrid platform allows drug discovery for trafficking receptors such as GPCRs, and has been validated using the β2 adrenergic receptor (β2AR) system. When a chemical library containing ~1,200 off-patent drugs was screened against cells expressing FAP-tagged β2AR, all 33 known β2AR active ligands in the library were successfully identified, together with a number of compounds that might regulate the receptor internalization in a non-traditional manner. Results indicate that the platform identifies ligands of target proteins regardless of the associated signaling pathway, which opens the door to the search for biased modulators of the receptor, and is also suitable for screening multiplexed targets for improved efficiency. The results revealed that ligands can be biased in the rate or the duration of receptor internalization, and that receptor internalization can be independent of the activation of the mitogen-activated protein kinase pathway.
Introduction

G protein coupled receptors (GPCRs) are the largest protein family in the human genome. They represent the most important class of drug targets (Lefkowitz, 2007). Nearly 30% of all FDA approved drugs target GPCRs, including 19 of the top 50 drugs sold in the US (Overington et al., 2006; Schlyer and Horuk, 2006). However, current drugs target just ~10% of the 357 non-olfactory GPCRs. Of those that are not current drug targets, about 100 are orphan receptors for which no endogenous ligand is known; the remaining almost certainly include therapeutically important targets that have not yet been exploited. Thus the search for new ligands of both liganded and orphan GPCRs is of considerable importance.

High-throughput screening (HTS) is often the most efficient first step to identify leads toward new ligands/drugs from large libraries of chemical compounds (Gribbon and Sewing, 2005). Traditional HTS methods employ automated plate readers for the measurement of absorbance, fluorescence intensity, fluorescence polarization, or luminescence. Technological advances have also permitted high-content screening (HCS) (Zanella et al., 2010), fluorescent label independent screening (Fang et al., 2008), and high-throughput flow cytometry (HTFC) (Ramirez et al., 2003). HCS throughput can be up to four to twelve minutes per 384 well plate for a maximum of 3 colors per single field scanning (Cell Voyager 6000, Yokogawa; Opera, Perkin Elmer; ArrayScan VTI, Thermo Fisher. YW personal communication). However, multiplexing remains challenging, and the frequently required multiple field scanning significantly increase the time for sample scanning and data analysis, which can be one of the reasons why only a few high-throughput primary screens performed using HCS were found in literature (Bickle, 2010). Instrument and reagent limitations, as well as difficulties in developing mechanistic biological assays have restricted label-free approaches from being a mainstream HTS platform (Moller and Slack, 2010). First introduced and validated by one of our laboratories (Ramirez et al., 2003), HTFC has been used primarily in bead-based and suspension cell-based assays,
including multiplex assays targeting GPCRs and GPCR related proteins (Edwards et al., 2007; Roman et al., 2009; Surviladze et al., 2010; Young et al., 2009). The throughput of HTFC is consistently in the range of ~10-12 min per 384 well plate, with no significant increase in the time necessary for data acquisition or analysis when using up to six multiplexed targets in each sample well (Surviladze et al., 2010).

Common approaches for GPCR screening include the direct measurement of fluorescent ligand binding to the receptor, or of downstream actions such as cAMP production, calcium mobilization or transcriptional activation. These approaches usually rely on measurement of subtle changes in signal intensity, or a second messenger signal that might not come from the targeted receptor’s activity.

An alternate downstream event indicative of receptor activation is receptor internalization. Activity-dependent GPCR internalization typically requires the activity of G protein receptor kinases and the translocation of β-arrestin from cytoplasm to plasma membrane upon agonist-dependent GPCR activation. Common assays including the luminescent based enzyme fragment complementation assay (Olson and Eglen, 2007), as well as the fluorescent based GFP-β-arrestin cluster assay and β-lactamase reporter gene expression assay (Barnea et al., 2008; Korn and Krausz, 2007; Lee et al., 2006).

Szent-Gyorgyi et al. described a number of new reporters called fluorogen activating proteins (FAP) that bind soluble small-molecule fluorogens. The fluorogens are non-fluorescent in solution, but acquire strong characteristic fluorescence when bound to the FAP (Szent-Gyorgyi et al., 2008). FAP have been fused to the N-terminus of the human β2 adrenergic receptor (β2AR) and the fusion proteins are functionally comparable to their wild type counterpart, with no apparent interference with ligand-receptor binding (Fisher et al., 2010).

We combined FAP technology with HTFC to develop a platform for directly monitoring ligand induced protein translocation in live cells. The high specificity of the FAP-fluorogen detection system ensures that the
fluorescent signal is specific for the target protein, while the HTFC system guarantees the throughput and multiplexing capability.

The platform was validated with the prototypical β2AR. We used the platform to screen against the Prestwick Chemical Library (PCL) to identify compounds that deplete cell surface β2AR, and compounds that stabilize cell surface β2AR. Hit molecules were confirmed by concentration-response assays followed with secondary assays. A set of time and concentration dependent experiments to investigate the relationship between several functionally selective β2AR ligands and receptor endocytosis/total receptor expression level have been performed. Several confirmed compounds that affected β2ARs in a concentration-dependent fashion, but had no known connection to the β2AR internalization pathway, are also under investigation.
Materials and Methods

Materials. Cell membrane impermeable fluorogen thiazole orange (TO1-2p, λ<sub>ex/em</sub> = 509/530nm) is synthesized in CMU as described earlier (Szent-Gyorgyi et al., 2008); plastic ware and chemicals are from Sigma except where stated otherwise.

Production of FAP-β2AR expressing Cells. A plasmid expressing the surface displayed fusion protein of FAP to the extracellular N-terminus of the human β2AR, was generated (Szent-Gyorgyi et al., 2008). Functional FAP-β2AR fusion protein was successfully expressed in NIH3T3 cells as reported (Fisher et al., 2010; Szent-Gyorgyi et al., 2008). A similar retroviral transfection protocol was applied to stably express FAP-β2AR in the U937 human monocytic cell line. U937 cells are known to show robust internalization of agonist-stimulated GPCRs and are well-suited for high-throughput flow cytometry assays, being a suspension cell line. Human single chain antibody AM2.2 (the FAP) binds the cell membrane-impermeable fluorogens thiazole orange derivative TO1-2p with high affinity and increases the fluorescent signal from this fluorogen over two thousand fold (Szent-Gyorgyi et al., 2008).

Cell Culture. AAM2 cells (U937 cells that stably express surface FAP-β2AR, with the FAP moiety able to bind to fluorogen TO1-2p) were maintained in sterile filtered RPMI 1640 media containing 10% heat inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, pH 7.4, 20 μg/mL ciprofloxacin, 2 mM L-glutamine, at 37°C in a water jacketed incubator with 5% CO<sub>2</sub> and 95% air (Forma Scientific, Marietta, OH) in tissue culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) at a maximum cell density of 400,000/mL until the day of harvest. For reliability purpose, FAP-β2AR expression in AAM2 cells were checked daily, and cells have over 30% of the population without detectable receptor expression were excluded from the experiment. Cells were typically counted and concentrated to 1 to 5×10<sup>6</sup>/mL before experiments.
Flow Cytometry Characterization of AAM2 Cells. For steady-state receptor expression experiments, AAM2 cells were kept in serum free RPMI 1640 medium in the presence or absence of β2AR full agonist isoproterenol (ISO) or compound of interest for up to 2 hrs before the addition of TO1-2p at 37°C. The fluorescence intensity was measured by flow cytometry immediately afterwards. For real-time kinetic experiments, AAM2 cells were kept in RPMI 1640 medium in a 37°C incubator in the presence or absence of 20 μM ISO for up to 90 min. The baseline cell autofluorescence was collected by flow cytometer for 30 sec before the addition of fluorogen. Light scatter and fluorescent signals were collected by either a FACScan flow cytometer (Becton-Dickinson, NJ) equipped with a 488 nm laser and a 530/30 band pass filter for FL1 channel or an Accuri C6 flow cytometer (Becton-Dickinson, NJ) equipped with 488 nm and 640 nm lasers and 530/50 band pass filter for FL1 channel. Total copies of surface FAP-β2AR were determined by comparing the fluorescent signal from FL1 channel from TO1-2p bound to cells and a set of FITC MESF beads (Becton-Dickinson, NJ).

Confocal Microscopic Imaging. Live cell confocal microscopy images were taken with a Zeiss 510 Meta inverse microscope equipped with four lasers (405 nm, 488 nm Argon, 543 nm, and 633 nm) and a 63 × water immersion objective. Resting cells or cells that have been pre-stimulated with the desired compounds for up to 120 min were added to a 37°C chambered coverglass system (Thermo Fisher, MA), after which up to 1 μM TO1-2p was added to the cells in the absence or presence of the compound of interest, and the fluorescent signals were collected using a 530/50 nm band pass filter.

High-Throughput Screening of the Prestwick Chemical Library. Flow cytometric measurements were performed using the HyperCyt® HTFC (Ramirez et al., 2003) platform. HTFC is a platform that uses a peristaltic pump in combination with an autosampler and a cytometer. The system enables measurement of microplate wells at rates in excess of one endpoint sample well per second. Used in multiwell format, the sampling probe of the autosampler moves from one well to the next as a peristaltic pump sequentially aspirates
sample particle suspensions from each well. Between wells, the continuously running pump draws a bubble of air into the sample line, to generate a series of bubble-separated samples for delivery to the flow cytometer. The data were collected at a sampling rate of forty ~1 µL samples per minute. Because of the nature of the described approach, agonist and antagonist screens were performed separately. AAM2 cells were harvested, centrifuged, and resuspended in serum free RPMI at 4×10^6 cells/mL and stored at 37°C in a humidified cell culture incubator until the time of the experiment.

For the agonist screen, 5 µL serum free RPMI or ISO in RPMI, 100 nL 1 mM compound, and 3 µL cells were added to microtiter plates sequentially using a NanoQuot microplate dispenser (BioTek Instruments, VT), a Biomek FXp laboratory automation work station (Beckman Coulter, CA) equipped with a pin tool, and the NanoQuot, respectively. Positive and negative control wells were included in each plate. Cells from negative control wells only received DMSO vehicle, Cells in positive control wells received 20 µM ISO. The assay plates were removed from the deck and incubated at 37°C in a humidified cell culture incubator for 90 min. TO1-2p was added in 3 µL of RPMI at a final concentration of 150 nM by the NanoQuot. The cells were immediately sampled using a HyperCyt autosampler platform, and the fluorescent signals from surface β2AR were detected by a Cyan ADP flow cytometer (Beckman Coulter). The concentration of compound in the assay was 12.5 µM during treatment, followed by addition of TO1-2p.

For the antagonist screen, 5 µL serum free RPMI or 10 µM (final concentration) β2AR antagonist ICI 118, 551 (ICI) in RPMI, 100 nL 1 mM compound, and 3 µL cells were added to microtiter plates. The plates were removed from the deck and incubated at 37°C in a humidified cell culture incubator for 30 min before the addition of 1 µM (final concentration) ISO in 3 µL of RPMI. In this screen, cells that received only DMSO as well as cells treated with 10 µM ICI followed by 1 µM ISO served as positive controls, and cells treated with 1 µM ISO alone served as negative controls. The assay plates were then incubated in the 37°C cell culture
incubator for 60 min more. After the addition of 150 nM (final concentration) TO1-2p in 3 µL of RPMI, the cells were immediately sampled using a HyperCyt autosampler platform, and the fluorescent signals from surface β2AR were detected by a Cyan ADP flow cytometer. The concentration of compound in the assay was 9 µM during treatment, followed by addition of TO1-2p.

“Hit” molecules were determined by the response value (RV), which represents the percent of surface β2AR depletion by agonist or preservation by antagonist, and can be calculated by Equation 1:

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RV = \frac{MCF_{NCntrl} - MCF_{Sample}}{MCF_{PCntrl} - MCF_{NCntrl}} \times 100
\]

Samples with a RV of 50 or higher were considered “hits”. Because of the high stability of this assay, the applied cut off filter exceeded 6 times the standard deviation of the controls.

The quality of the screen was evaluated by Z’, which is correlated with the MCF and standard deviation of both PCntrls and NCntrls. A Z’ of 0.5 or more generally indicates the HTS assay is reliable with a high degree of confidence that the hit molecules have biologically relevant activity (Zhang et al., 1999).

Concentration-responses of "hit" compounds between 2 ng/mL and 20 µg/mL (range from 27-142 µM) in half log increments were measured using a similar protocol described above.

Screening data were analyzed using HyperView software developed by Bruce Edwards, which is available from IntelliCyt (Albuquerque, NM). This software automatically resolves data clusters, and analyzes each bin to determine mean or median channel forward scatter, side scatter, and fluorescence intensity (MCF) and number of gated events from each well.

Membrane Preparation and Solubilization of β2AR-GFP. The procedure was published elsewhere (Simons et al., 2004; Simons et al., 2003). In brief, U937 cells expressing a β2AR-enhanced green fluorescent protein
(β2AR-GFP) construct on their surface were centrifuged at 1,200 rpm for 5 min and resuspended in cavitation buffer (10 mM HEPES, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, and 1× protease inhibitor cocktail 1 (AEBSF; Bovine Lung Aprotinin; E-64 Protease Inhibitor; Disodium EDTA; Leupeptin; from EMD Biosciences) at a density of 10^7/mL. The cells were placed in a pressure bomb and incubated at 450 psi for 20 min with nitrogen, after which the suspension was slowly released into a sample tube. The suspension was centrifuged at 1200 rpm for 5 min to remove unbroken cells and nuclei. Supernatant that contained suspended membrane was pelleted twice by centrifuging at 135,000×g for 30 min at 4°C, then the membrane pellet was resuspended in buffer (25 mM HEPES, pH 7.5, and 200 mM sucrose), aliquotted in 1×10^8 cell equivalents and stored at -80°C until the day of the experiment.

700 µL of 30 mM HEPES, pH 7.5, 100 mM KCl, 20 mM NaCl, 1 mM MgCl2 (HPSM) was added to an aliquot of thawed soluble membrane that typically consisted of 1×10^8 cell equivalents, and centrifuged at 13,500 × g for 15 min at 4°C to remove sucrose in the supernatant. The membrane pellet was then resuspended in 220 µL HPSM by passing through a 25G syringe 10 times, after which 25 µL 10% dodecyl maltoside (DOM) and 2.5 µL 100× protease inhibitor cocktail were added to the membrane. The mixture was kept in a cold room (4°C-7°C) for 2 hr under mild vortexing. The mixture was centrifuged at 13,500 ×g for 20 min to remove insoluble materials. Soluble receptor was used within 8 hr of solubilization.

**Dihydroalprenolol (DHA) Bead Competitive Binding Assay.** DHA beads were prepared as described (Simons et al., 2004; Simons et al., 2003). In brief, equal volumes of epoxy-activated Superdex-peptide beads (Amersham Biosciences) and 0.2 M dithiothreitol in 0.2 M NaHCO3 were mixed and incubated at 37°C for 4 hrs to create sulfhydryl-activated beads. The beads were then washed for 5 times, and 1 mL water, 40 mg of (-)-Alprenolol (ALP), and 10 µL of 10% ammonium persulfate were added to 1 mL of the beads. The reaction mixture was then flushed with a gentle stream of N2 for 5 minutes to remove oxygen, and kept capped under N2 with constant
mixing for 2 hrs at 37°C. The derivatized beads were then washed 5× each in water, 50% EtOH, EtOH, water, and HPSM to remove excess ALP, then stored as a 50% slurry in HPSM with 0.02% NaN₃ and 0.01% DOM at 4°C.

Typically, 10 µL of stock DHA beads were added to 400 µL HPSM plus 0.1% DOM, 0.1% BSA, and 0.05% Tween-20 under light vortexing or nutation in the cold room (4 - 7°C) for 30-60 min to reduce non-specific binding. The beads were then resuspended in 200 µL HPSM plus 0.1% DOM. A typical 10 µL binding assay consisted of 2 µL soluble receptor, 2 µL DHA bead, 2 µL test compounds or buffer, and 4 µL HPSM, 0.1%DOM. The mixture was prepared and allowed to incubate in a cold room under mild vortexing for 2 hr before measuring the forward scatter, side scatter, and fluorescence using an Accuri C6 flow cytometer. All experiments were repeated at least three times in duplicate.

**Kinetic Measurement of Surface and Total Receptor in Cells.** Typically, AAM2 cells or β2AR-GFP cell were harvested and resuspended in serum free RPMI at ~500,000 cells/mL for 1 or 18 hrs and kept in a 37°C tissue culture incubator until the time of experiment. Taking into account the initial 1:1 fusion of β2AR with GFP or the FAP tag, and the fused receptor was transduced into the cells with pseudo virus particle, we make the simplifying assumption that the signal from GFP or fluorogen-bound FAP represents intact β2AR, and that only intact FAP-β2AR will have access to the non-membrane permeable version of fluorogens under our experimental conditions. To measure the total amount of AM2.2-β2AR, AAM2 cells were pre-treated with 1 µM TO1-2p for 18 hrs with the assumption that all surface and internal FAP-β2AR were accessible to TO1-2p due to basal receptor recycling and probe redistribution. The relatively smaller fractional changes in the total β2AR-GFP signal as compared to the FAP-β2AR signal suggest the possible contribution of β2AR-GFP located in non-trafficking compartments.

At the time of experiment, cells were exposed to selected drugs and kept in a 37°C tissue culture incubator. An aliquot of cells were transferred to sample tubes on ice immediately after adding the drugs and after 30, 60, 90,
120, 180, 240, 300 or 360 min after drug treatment. For the measurement of total receptor numbers, the fluorescent signal from β2AR-GFP cells and AAM2 cells that have been pre-treated with 1 μM TO1-2p were measured immediately by an Accuri C6 flow cytometer. In the case of surface receptors, AAM2 cells were allowed to cool on ice for a minimum of 15 min before the addition of 1μM TO1-2p, and flow cytometry measurement was performed 15 min after exposing to TO1-2p.
**Results**

*Visualization of Receptor Internalization.* AAM2s are U937 cells that stably express surface FAP-β2AR, with the FAP moiety pointing outwardly into the medium. The FAP specifically binds the fluorogen TO and derivatives to exhibit fluorescence. Confocal microscopic images of live AAM2 cells are shown in Figure 1. In each case, the receptor was visualized using the membrane-impermeable fluorogen TO1-2p. Immediately after the addition of TO1-2p (Fig. 1a), strong surface fluorescence is observed. In contrast, when the same cells were treated for 60 min with the receptor agonist ISO and then exposed to TO1-2p (Fig. 1b), very little fluorescence was observed, presumably because the receptor had been internalized and was no longer available to contact the fluorogen at the cell surface. Last, when the cells were exposed to TO1-2p together with ISO for 60 min, fluorescent signal was observed in the interior compartment of cells (Fig. 1c). These results indicate that TO1-2p had indeed bound to the FAP moiety and then co-internalized with the activated receptor. The order and timing of addition of fluorogen and agonist, as shown in the diagrams below each micrograph, were critical to the experimental design and outcome. Thus, we could detect either just the surface displayed FAP-β2AR or the surface plus internalized receptors in a cell, by adding fluorogen and receptor agonist at appropriate times.

*Functional Characterization of Receptor Internalization by Flow Cytometry.* Figure 2 presents quantitative data obtained by flow cytometry for AAM2 cells. The kinetics of TO1-2p binding to untreated and activated cells is shown in Figure 2a. After collecting baseline fluorescence for 30 seconds, 150 nM TO1-2p was added to the cells. Fluorescent signal from resting cells appeared almost immediately, with a \( \tau / 2 < 5 \) s, where \( \tau / 2 \) represents the time when half of the FAPs are occupied with TO1-2p. No detectable signal over cell autofluorescence was recorded from wild type U937 cells, indicating that the binding between TO1-2p and FAP is highly specific. Little signal was detected from ISO pre-stimulated cells, which is consistent with the imaging results (Fig. 1b),
thus is confirming that the majority of the cell surface receptors are internalized as a consequence of ISO stimulation.

Equilibrium binding data between TO1-2p and the AAM2 cells (Fig. 2b) shows that the fluorogen binds to FAP-β2AR with an affinity of ~2 nM, which is consistent with that between TO1-2p and FAP-β2AR expressed in NIH 3T3 cells (Szent-Gyorgyi et al., 2008).

The effective concentration for ISO induced receptor internalization was determined by exposing the cells to ISO for 90 min before the addition of TO1-2p (Fig. 2c). The measured EC₀ value for receptor internalization was ~0.8 µM, which is comparable to the reported binding affinity between ISO and β2AR (Baker et al., 2003a; Copik et al., 2009; Hoffmann et al., 2004).

Receptor antagonists can prevent agonist induced receptor internalization. The high affinity β2AR antagonist ICI (Hoffmann et al., 2004) was chosen for this study. Cells were treated with different concentrations of ISO alone or in the presence of 10 µM ICI for up to 120 min. ISO alone progressively reduced the number of surface displayed receptors in a time and ligand concentration dependent fashion (Fig. 2d). Approximately 15%, 60%, and 90% of surface displayed receptors were depleted after 60 min of 0.2, 1 and 20 µM ISO activation, respectively. The measured half-time of 20 µM ISO induced FAP-β2AR internalization was ~11 min. However, in the presence of 10 µM ICI, even 20 µM ISO had no impact on the surface displayed FAP-β2AR. The absolute amount of surface FAP-β2AR can be calculated by converting the median channel fluorescence (MCF) value to MESF (mean equivalent soluble fluorescence) (Supplementary Equation 1). Results from this series of experiments indicated that the FAP-β2AR fusion protein and the wild type counterpart functioned similarly, and that TO1-2p showed virtually no non-specific binding to the cells.
HTS of the Prestwick Chemical Library. The feasibility and biological reliability of the platform was evaluated by screening against the PCL. PCL consists of 1,200 off-patent drugs, 34 of which are known to target the β2AR, including 10 agonists, 23 antagonists and a prodrug that was not expected to be an active ligand under our experimental condition (Tunek and Svensson, 1988). Agonist and antagonist screens were performed separately due to the differing nature of the approaches, and illustrative results for each are shown (Fig. 3). Cells were treated with 12.5 µM of each compound for the agonist screen, and 9 µM of each compound for antagonist screen.

Primary Agonist Screen: FAP-Receptor Internalization. A time dependent cytometer display from a single 384 well plate collected by HTFC is shown in Figure 3a. For easy viewing purposes, fluorescent readouts from the first 26 wells are shown in the insert. Cells from each well of the plate were delivered sequentially to the cytometer and separated by air bubbles that contain no cells. As shown in the graph, each colored cluster represents cells from an individual well of the microtiter plate, the small gaps represent air bubbles in between samples, and the large gap toward the end of the insert is the indication of the end of the row because the last two wells in each row were kept sample free intentionally. Each dot in the graph represent signal from an individual cell, and the pseudo color represent the density of cells that emit the same level of fluorescent signal at any given time point, where blue represents the highest cell density and red the lowest. Analyzed data from a single row (row N) are shown in expanded form in Figure 3b, where each square represents the averaged cell fluorescence from an individual well. Clear separation between signals from positive control well N1 (point 1 in Fig. 3b; 12.5 µM ISO) and negative control well N2 (point 2 in Fig. 3b; DMSO vehicle) was observed. Signal from most of the sample wells was similar to the negative control, however, cells in some wells showed a significant decrease in fluorescence, such as the one from well N11 (point 3 in Fig. 3b). The compound in that well was the well-known β2AR agonist Salmeterol (Advair™(Keating and McCormack, 2007; Tashkin and Fabbri, 2010)). The PCL screen targeting β2AR agonists was performed twice in duplicate, and the average Z'
of all 16 plates was 0.72.

Hit molecules were determined by the response value (RV), calculated by Equation 1 in Methods, which represents the percent of surface β2AR depletion. Samples with a RV of 50 or higher were considered as hits. Because of the high stability of this assay, the applied cut off filter exceeded 6 times the standard deviation of the controls. A total of 23 hits were identified, of which 8 were known high-potency agonists for β2AR (first 8 compounds in Table 1).

All 23 hits from the primary screen, plus two additional β2AR agonists and the pro-drug that did not appear among the hits as expected, were selected for a concentration-response screen for confirmation and potency determination, followed by secondary assays to validate their target and cellular effects.

Primary Antagonist Screen: Prevention of FAP-Receptor Internalization. The cytometer display of the antagonist screen against the same PCL plate as in Figure 3a is shown in Figure 3c, and processed data from row N is shown in Figure 3d. The separation between signals from positive control wells N1 and N23 (points 1 and 1’ in Fig. 3d; 9 µM ICI + 1µM ISO, and vehicle) and negative control well N2 (point 2 in Fig. 3d; 1 µM ISO) indicated that ICI completely inhibited ISO-induced receptor internalization. As in the agonist screen, signals from the majority of the wells were similar to those from the negative control wells. Cells from well N17 (point 3 in Fig. 3d) showed a significant increase in fluorescence, due to the β2AR antagonist Timolol (Baker, 2005). RV in this assay represents the percent of stabilized surface β2AR, and samples with RV >50 were scored as hits. By this criterion, the elevated fluorescent signal due to the compound in well N15 (point 4 in Fig. 3b) was not scored as a hit; the compound was prazosin hydrochloride, an α-adrenergic receptor antagonist(Centurion et al., 2006) that displays some cross reactivity to β-adrenoceptors.
Similar to the agonist screen, the antagonist screen was performed twice in duplicate and yielded an average $Z'$ of 0.68. A total of 62 hits were identified, including all 23 known beta blockers in the library. Other compounds that made the list included some weak $\beta_2$AR antagonists as well as compounds that had no known connection with the $\beta_2$AR.

**High Throughput Concentration-Response Assay.** HT concentration-response assays were performed with all hit compounds for activity confirmation and potency assignment. Concentration-response curves from 6 representative hit compounds are shown in Figure 4, including agonists (a-c) and antagonists (d-f). Hits identified from the primary agonist screen include low affinity $\beta_2$AR ligands, such as the $\alpha_2$AR selective agonist levonordefrin (LEVON, Fig. 4a), that induced $\beta_2$AR internalization with an expectedly weak EC$_{50}$ of ~50 $\mu$M. Interestingly, some hit molecules that have no known connection to $\beta_2$AR also reduced cell surface expression of $\beta_2$AR in a concentration dependent manner, such as the protein synthesis inhibitor anisomycin (ANI, measured EC$_{50}$ ~35 nM, Fig. 4b), and the calcium channel blocker pimozide (Patmore et al., 1989) (measured EC$_{50}$ ~20 $\mu$M, Fig. 4c). Similarly, in the antagonist screen, propafenone (PROP) (Harron and Brogden, 1987), a class Ic anti-arrhythmic medication that has weak $\beta_2$AR antagonist activity, prevented ISO-induced $\beta_2$AR internalization with an IC$_{50}$ of 0.2 $\mu$M (Fig. 4d). Several compounds that have not been reported as $\beta_2$AR ligands - such as naftopidil (NAF) and pizotifen (PIZ) (Fig. 4e and f, respectively) - also prevent ISO induced receptor internalization with sub-micromolar to micromolar potency.

Selected measures of both screens are summarized in Table 1.

**Validation Assays.** Two secondary assays - a competitive binding assay and confocal microscopy - were used to further validate the hits identified in the primary screens.
**β2AR-DHA Competitive Binding Assay.** This assay is based on the direct binding of solubilized β2AR-GFP, a fusion of β2AR and enhanced green fluorescent protein, to beads derivatized with dihydroalprenolol (DHA) (Simons et al., 2003). The fluorescent readout was from bead-bound β2AR-GFP. When a β2AR agonist or antagonist competes with alprenolol (ALP, the presumed ligand structure after linkage of DHA to the beads) for binding to the receptor, a decrease in bead fluorescence is expected (Fig. 5a). Concentration-response curves for several agonists and antagonists are shown in Figures 5b and 5c. The EC$_{50}$/IC$_{50}$ values, or potencies, of β2AR hits from the primary PCL screens measured with this bead-based assay were typically in close agreement with the measured potency of receptor internalization (agonists), or potency of prevention of internalization (antagonists), as well as with literature reported K$_d$ values for β2AR binding affinities. Summary data for all 33 known β2AR ligands identified as hits in the primary screen, plus the prodrug bambuterol and eight of the “unexpected” hits, are shown in Table 1.

Most of the compounds identified from primary screens and concentration-response confirmation were found to induce β2AR internalization (or prevent ISO-induced β2AR internalization) and couple to β2AR with similar EC$_{50}$ values. Interestingly, some compounds had significantly different potencies (>100×) measured by these two assays. Among these were ANI (identified from the agonist screen), and NAF, DOM or PIZ (identified from the antagonist screen). These compounds all induce receptor internalization (or prevent ISO-induced internalization) at a significantly lower concentration than the concentration at which they compete with bead-borne DHA for binding to the receptor. The situation was reversed for maprotiline (MAP, agonist) and boldine (BOL, antagonist). The conflicting results suggest that these compounds do not compete with the same binding site on β2AR as ALP does, but might be non-canonical regulators of β2AR, compounds that induce receptor internalization in a receptor-biased manner or compounds that non-specifically downgrade the surface receptor expression.
Live Cell Confocal Microscopic Imaging of AAM2 and β2AR-GFP Transfected Cells. To further investigate the compounds that showed apparent inconsistency in affinity when measured by the two different methods, we monitored the behavior of both FAP-β2AR cells and β2AR-GFP cells in response to these compounds by confocal microscopy. For AAM2 cells, saturating TO1-2p was added to the cells together with the compounds. Fluorescence micrographs (Fig. 6) were obtained from untreated FAP-β2AR and β2AR-GFP cells (a,g), cells stimulated with 20 μM ISO (b,h), cells exposed to both 10 μM ALP and 1 μM ISO (e,k), and cells incubated in 10 μM active molecules ANI (c,i), LEVON (d,h), or NAF plus 1μM ISO (f,l), respectively. Although identified as active in the β2AR agonist concentration-response with an EC₅₀ of about 35 nM, 10 μM ANI did not induce β2AR internalization in either of the cell lines (c,i): contrast to the β2AR agonist ISO (b,h) and the active compound LEVON (d,j). For ANI, the loss of surface fluorescence when cells were preincubated with the compound and then treated with fluorogen did not result from the expected internalization but some other mechanism. Hit molecules identified from the antagonist screen that were not known β2AR antagonists, including NAF (Fig. 6f,l), DOM, BOL, and PIZ (Supplementary Fig. 1), all prevented ISO-induced β2AR internalization in both cell lines, although significant capping appears to have occurred. Taken together with the β2AR-DHA competition binding assay, the validation approaches guide the identification of compounds that induce β2AR internalization via non-canonical pathways from compounds that non-specifically downgrade the expression of receptors.

Effect of Drugs on the Level of Surface and Total β2AR in Cells Over Time. Figure 7 shows the amount of surface and total AM2.2-β2AR as well as the total amount of β2AR-GFP over 6 hrs in the presence and absence of selected drugs. For convenience, the average median channel fluorescence at time 0 was normalized to 1. As shown in Figure 7a, total amount of β2AR-GFP in untreated cells is nearly stable over the course of the experiment. The level of β2AR-GFP appeared to elevate slightly for cells treated with both ISO and LEVON,
while the protein synthesis inhibitor cycloheximide (CYC) gradually reduced the total receptor expression in the cells, as expected. Six hrs after exposure to CYC, approximately 80% of the β2ARs remained. Interestingly, the results with ANI were completely different from the other drugs. The compound reduced the level of surface AM2.2-β2AR to the same level as ISO within 2 hrs after treatment and maintained the lower level. In contrast, the total receptor levels in both AAM2 and β2AR-GFP cells were decreased only for the first 2 to 3 hrs and returned to the same level as untreated cells thereafter. This behavior was observed in 5 separate experiments.

Similar experiments were also performed with β2AR functionally selective ligands including bucindolol (BUC), carvedilol (CAV), Propranolol (PRO), and atenolol (ATEN) and compared to the β2AR agonist ISO that activates both the adenylate cyclase (AC) and extracellular signal-regulated kinases1/2 (ERK1/2) pathways (Galandrin and Bouvier, 2006). We monitored the levels of total and surface receptor in the presence and absence of these drugs for up to six hours. This set of experiment defines the relationship between the activation of ligand induced GPCR downstream pathways and receptor endocytosis. As shown in Figure 8, none of the compounds tested reduce the level of surface receptor alone at up to 1 μM concentration within 5 hrs after the addition of the compound. On the other hand, BUC, CAV and propranolol PRO prevented ISO induced β2AR internalization at IC50s of 1.8 nM, 1.5 nM and 0.7 nM, respectively. The IC50 values are close to literature values for the binding affinity for β2AR and these ligands (Hoffmann et al., 2004; Pauwels et al., 1988; Ponicke et al., 2002).
Discussion

Even though GPCRs are the molecular targets of nearly 30% of all FDA approved drugs, most members of the GPCR superfamily have not yet been explored as drug targets, nor have their contributions to off-target drug side effects been systematically investigated. Thus the search for new ligands of liganded and orphan GPCRs is of considerable importance. These can include classical receptor agonists and antagonists as well as non-canonically, functionally selective ligands, the discovery of which may be the starting point for the design of new therapeutic drugs (Shenoy et al., 2006), including those that differentially activate a subset of receptor functions or induce internalization without activation, thereby optimizing therapeutic benefits while minimizing side effects (Kenakin, 2011; Mailman, 2007).

We have described a powerful platform for GPCR screening that combines FAP technology with high throughput flow cytometry that has been shown to detect and quantify the responses of β2AR, and we believe that this platform provides a unique opportunity to discover new drug leads regardless of their molecular properties.

Our protocol has been optimized for screens against the human β2AR with consistent results, and used to identify compounds that induced β2AR internalization as well as those that antagonized β2AR internalization. The molecules identified from the pilot screens include all the active β2AR ligands in the test library when using a cut-off filter that exceeds 3 times the standard deviation of the control signal. Bambuterol, the inactive pro-drug of the β2AR agonist terbutaline that requires a hydrolytic and/or oxidative reaction to produce the active compound was not identified as expected (Tunek and Svensson, 1988). At least 20 weak regulators of β2ARs with EC$_{50}$/IC$_{50}$ values greater than 1 µM were also identified. The potency of compounds measured by the receptor internalization assays, the soluble receptor competitive binding assay, and reported ligand-receptor
binding, are for the most part similar (Table 1), suggesting that this approach correctly identifies orthosteric ligands of the receptor.

Following the developed HTS protocols, compounds regulating surface β2AR internalization with potencies above 100 µM were validated by secondary assays. The bead-based β2AR-DHA competitive binding assay allowed us to distinguish the direct interaction between β2AR and its canonical ligands such as ISO from more complex GPCR signaling pathways. This HTS compatible assay was used as the first approach to confirm ligands competing for the same binding site as ALP. It is worthwhile noting that this experiment can be used to separate compounds that induce receptor internalization by non-canonical pathway but is insensitive to compounds that reduce surface receptor in an alternate or non-selective way such as protein synthesis inhibitors. Live cell microscopic analysis was used to record receptor trafficking in real time and to provide visual confirmation of the receptor location.

We have cross-validated both assays by testing the compounds identified by the high throughput concentration-response assays. Observations from these two assays demonstrated the potential for efficient removal of certain classes of false positives at this early stage of drug discovery, and validating changes in the cellular distribution of the receptor. During this investigation of hit molecules from the original PCL screen, we found several new ligands for β2AR, including LEVON, an αAR agonist that behaved in a very similar manner as the β2AR agonist ISO (Fig. 7 and Table 2).

In addition, the identification of unexpected compounds indicates our approach does not rely on the binding of compound to any specific binding site of the receptor, nor does it require receptor internalization via any specific pathway, thus it opens the door to the search for non-canonical modulators of the receptor. For instance, the “false agonist” ANI decreased the amount of surface displayed FAP-β2AR without inducing receptor internalization, which indicates that the “receptor internalization assay” can identify compounds that reduce the
number of surface receptors regardless of the mechanism of action. Originally believed as a protein synthesis inhibitor, ANI appeared to function differently from the protein synthesis inhibitor CYC. Unlike CYC that consistently down regulated the total level of β2AR-GFP and AM2.2-β2AR over time, ANI gradually reduced the total level of β2AR during the first couple of hours after exposure, which recovered unexpectedly to basal levels thereafter. The level of surface receptor remained low during the same period of time. Taken together with results from the DHA bead binding assay, these observations suggests that ANI is neither a canonical ligand of β2AR, nor is it a protein synthesis inhibitor. The identification of this compound proves that the platform described is capable of searching for compound inducing surface protein endocytosis via any mechanism, suggesting its promising contribution to the search for non-canonical receptor regulators, which would be of pharmaceutical significance due to their potential for reducing side effects.

The FAP technology makes it possible to understand the connection between GPCR downstream pathways and receptor internalization. On ligand activation, β2AR couples to Gαs or Gαi protein, and subsequently regulates both AC and mitogen-activated protein kinase (MAPK) pathways. Although most ligands have a balanced efficacy toward the two pathways, many are reported to have different efficacies (Azzi et al., 2003; Evans et al., 2010; Kenakin, 2002; Seifert and Dove, 2009; Vilardaga et al., 2009). Table 2 compares the efficacy pattern of selected PCL compounds on AC and ERK1/2 (a subfamily of MAPK) pathways as well as total and surface receptors, and the ability to induce surface receptor internalization.

Known β2AR ligands in Table 2 belong to four categories: 1) agonist of both AC and ERK1/2 pathways (ISO); 2) agonist of the ERK1/2 pathway and inverse agonist of the AC pathway (PRO); 3) agonists of ERK1/2 pathway and neutral ligands of the AC pathway (BUC and CAV); and 4) inverse agonist for both AC and ERK1/2 pathways (ATE) (Galandrin and Bouvier, 2006). Results shown that ISO and the hit molecule LEVON are the only compounds that resulted in an elevated level of total receptors along with a decreased level of
surface receptors arising from ligand-induced receptor internalization. These results indicate that the mechanism of action of LEVON is similar to that of ISO, as an orthosteric full agonist of β2AR. Although it is believed that vesicle mediated endocytosis is required for the activation of GPCR related MAPK pathway (Daaka et al., 1998), none of the other agonists of the ERK1/2 pathway has any effect on the level of total or surface receptors, nor do they induce receptor internalization during the first 6 hrs after drug treatment. All three compounds reported as β2AR antagonists and ERK1/2 agonists in literature (PRO, BUC, and CAR) effectively blocked ISO-induced internalization with nanomolar IC50.

The platform can be readily extended to other GPCRs, other families of surface receptors such as receptor tyrosine kinases, or any signaling receptors involving an endocytosis or exocytosis process (Sorkin and von Zastrow, 2009). The reported agonist assay has already been adapted to screen the NIH Molecular Libraries Small Molecule Repository (MLSMR) containing 340,000 compounds; data from this screen have been reported in PubChem (Pubchem AID 504448, 504454, 504459) and will be discussed elsewhere. The combination of FAP with HTFC clearly can take advantage of the sensitivity of fluorogens and FAP binding and the quantitative aspect of flow cytometry assays with high throughput capability, which allowed us to complete the MLSMR library screen in eight days. Theoretically the FAP assay can also be performed in a well-based tissue culture plate. However, one has to consider the relative speed, cost and quantitative aspects for the high-resolution screen or HCS. Because flow cytometry is more widespread (tens of thousands of units world-wide) than high content analysis (hundred of units), and the entry cost for a sample handling front-end is relatively low, we suggest that flow cytometry could be a popular counterpart or alternative to HCS.

It is now apparent that membrane trafficking is a highly complex yet essential signaling process where: 1) multiple receptors in a single cell can signal in response to a single stimulus; and 2) a single receptor can trigger multiple downstream effectors via different mechanisms depending on the ligand. The discovery of
biased agonists, native ligands that induce receptor ubiquitination and down-regulation, and compounds inducing differential sorting pathways all indicate the potential value of GPCR trafficking assays as tools in the field of drug discovery (Jean-Alphonse and Hanyaloglu, 2011). The development of the FAP-based high-throughput flow cytometry platform provides a unique opportunity to researchers in this area. This platform also has the potential to further increase the efficiency of screens through multiplexing. By fusing different FAP tags that bind to fluorogens emitting at different wavelengths, one can detect signal from different receptors expressed in the same cell simultaneously with no further increase in unwanted background fluorescence. This might be an attractive approach to study the behavior of a receptor/co-receptor pair upon ligand activation, such as the receptor/co-receptor pair CD4/CXCR4 for HIV entry, $\alpha_v\beta_3$/decay accelerating factor for sin nombre virus entry, or GPCR-induced epidermal growth factor receptor activation that has been connected to many different types of cancer. Variations on fluorogens and FAPs that make for pulse-chase experiments are also expected to be available soon.
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Author Contributions

Participated in research design: Wu, Waggoner, Jarvik and Sklar.

Conducted experiments: Wu, Tapia, Simons, and Foutz

Contributed new reagents or analytical tools: Fisher, Strouse and Jarvik

Performed data analysis: Wu

Wrote or contributed to the writing of the manuscript: Wu, Simons, Jarvik and Sklar
References


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Figure Legends

Figure 1. Internalization of FAP-tagged receptors followed by confocal microscopy. Image of live AAM2 cells (a) treated with 150 nM TO1-2p, image obtained 1-60 min after treatment; (b) stimulated with 20 µM ISO for 60 min before addition of 150 nM TO1-2p, image obtained 1-15 min after the final addition; and (c) addition of 20 µM ISO and 150 nM TO1-2p, image obtained 20-60 min after the simultaneous additions. Diagrams describe the experimental scheme to acquire each confocal image, and the presumed states of the receptor with FAP tag, fluorogen, and agonist.

Figure 2. Flow cytometric characterization of AAM2 cells with TO1-2p, agonist and antagonist. Kinetic (a) and equilibrium (b) binding of TO1-2p to AAM2 cells. (a) The solid black line, dashed line and dotted line represent the binding between TO1-2p to resting AAM2 cells, ISO stimulated AAM2 cells and wild type U937 cells, respectively. The binding between TO1-2p and AM2.2 has a fast half time of less than 5 sec. (b) TO1-2p is shown to bind with high affinity, displaying a Kd of ~2 nM. (c) The β2AR agonist ISO induced receptor internalization with an EC50 of ~800 nM. (d) The time course of surface β2AR internalization induced by ISO in the absence and presence of the β2AR antagonist ICI 118,551 (ICI) is plotted. Open triangles, open circles and open diamonds represent the signal from AAM2 cells treated with 0.2, 1, or 20 µM ISO, respectively; solid diamonds represent signal from resting AAM2 cells, and crosses represent signal from AAM2 cells treated with 10 µM ICI plus 20 µM ISO.

Figure 3. Screens of a Prestwick Chemical Library plate for FAP-β2AR ligands: internalization (for agonists) and prevention of ISO-induced internalization (for antagonists). Screen shots of time vs. fluorescent signal of a complete 384 well plate targeting β2AR agonists (a) and antagonists (c). Pseudo color in (a) and (c) represents density of the cells that emit the same level of fluorescent signal at any given time point, where blue represents the highest cell density and red the lowest. Insert shows magnified data from the first 26 wells of the plate plotted in (a), each cluster represents data from a single well, and the gap between clusters are air bubbles to
separate the samples. The bigger gap toward the end of the insert indicates the end of that row as the last two wells of each row were intentionally left free of samples. Analyzed data from row N for the two screens are plotted in (b) and (d), respectively. Points 1 and 2 in (b) represent the signal from positive control well N1 (12.5 µM ISO) and negative control well N2 (vehicle); point 3 represents a “hit” where cells were treated with 10µM β2AR full agonist Salmeterol (Advair™). In (d), points 1 and 1’ represent the signals from positive control wells N1/N23 (9 µM ICI and 1µM ISO/vehicle); point 2 represents the signal from negative control well N2 (1 µM ISO); point 3 represents a “hit” where cells were treated with 9 µM β2AR antagonist Timolol; and point 4 represents a near-hit from cells treated with 9 µM αAR selective antagonist Prazosin.

Figure 4. Concentration-response curves of FAP receptor internalization or stabilization, using selected "hit" compounds from the PCL agonist screen (a-c) and from the antagonist screen (d-f). (a) levonordefrin, an αAR agonist (EC$_{50}$ = 4.5×10$^{-5}$ M). (b) anisomycin (EC$_{50}$ = 3.5×10$^{-8}$ M). (c) pimozide (EC$_{50}$= 2×10$^{-5}$ M). (d) propafenone (IC$_{50}$ = 2.1 × 10$^{-7}$ M). (e) naftopidil (IC$_{50}$ = 1.2 × 10$^{-7}$ M). (f) pizotifen (IC$_{50}$ = 8.6 × 10$^{-6}$ M).

Figure 5. β2AR-GFP to DHA-bead competitive binding assay. (a) Scheme of the high throughput flow cytometry compatible β2AR-DHA competitive binding assay. Soluble β2AR-GFP can bind to dihydroalprenolol derivatized beads at high affinity (left receptor), while ligands of β2AR (agonists or antagonists) can block the binding between the receptor and the beads (right receptor), leading to the decrease of fluorescent signal from the bead. (b) concentration-response curves of selected agonists measured by the β2AR-DHA competitive binding assay. The Y axis is normalized bead fluorescence intensity, in arbitrary units. (c) concentration-response curves of selected antagonists measured by the β2AR-DHA competitive binding assay. ALP served as the positive control of the experiment. All experiments were done in duplicate at least 3 times. EC$_{50}$ values for all β2AR ligands and several active compounds can be found in Table 1. Abbreviations used: BAM, bambuterol; (+) ISO, (+) isoproterenol; SALB, salbutamol; CLEN, Clenbuterol; SALM, salmeterol; ACE, acebutolol; BET, betaxolol; PRON, pronethalol; (+) LEV, (+) levobunolol; PENB, penbutolol.
Figure 6. Confocal microscopy of AAM2 cells and β2AR-GFP cells in parallel demonstrate the extent of receptor internalization. Images of AAM2 cells (a-f) and β2AR-GFP cells (g-i). For AAM2 cells, saturating TO1-2p was added to the cells together with the compounds to track the translocation of FAP-β2AR. Untreated cells show receptor largely on the plasma membrane, little receptor internalization, for both cell types (a, g). Cells treated for an hour with 10 µM β2AR agonist ISO show more receptor internalized than on the surface, or high receptor internalization, for both cell types (b, h). Test cells treated with 10 µM Anisomycin (c, i) show little internalization, classifying these compounds as non-agonists of receptor internalization. Cells treated with the β2AR antagonist ALP plus 1 µM ISO show little internalization (e, k). Test cells treated with 10 µM naftopidil plus 1 µM ISO (f, l) also show little internalization, classifying this compound as an antagonist of receptor internalization.

Figure 7. Time course of cell surface and total β2AR. Cell total or surface receptors were measured at 0, 30, 60, 90, 120, 180, 240, 300 and 360 min after drug treatment. (a) Untreated β2AR-GFP cells and cells treated with 10 µM ISO, 10 µM ICI together with 1 µM ISO, 10 µM ANI, 10 µM CYC, or 10 µM LEVON. (b) AAM2 cells pre-incubated with 1 µM TO1-2p prior to drug treatment and analysis. (c) Untreated AAM2 cells and cells treated with 10 µM ISO, 10 µM ICI together with 1µM ISO, 1µM ANI, 10µM CYC, or 10µM LEVON. An aliquot of cells was removed from the stock and cooled on ice for 15 min. 1 µM TO1-2p was added to the cells and incubated for 15 min before the measurement by flow cytometer.

Figure 8. (a) Time course comparison of cell surface AM2.2-β2AR in the presence of 1% DMSO, 1 µM ISO, 1 µM BUC, 1 nM BUC, 1 µM CAV, 1 nM CAV, 1 µM (s)PRO, or 1 nM (s) PRO. Cell surface receptors were measured at 0, 30, 60, 90, 120, 180, 240 and 300 min after drug treatment. 10 µM ISO induces receptor internalization progressively, and DMSO has no effect on the expression or surface receptor. All other compounds tested do not induce receptor internalization up to 5 hrs after treatment. (b) Concentration-response curves of FAP receptor stabilization with BUC, CAV, and (s) PRO in the presence of 1 µM ISO. The presence
of up to 1 μM BUC, CAV, or (S) PRO alone do not induce any surface receptor internalization, and all three compounds effectively prevent ISO induced receptor internalization with nM efficacy ($\text{IC}_{50, \text{BUC}} = 1.8 \text{ nM}$; $\text{IC}_{50, \text{CAV}} = 1.5 \text{ nM}$; $\text{IC}_{50, (S)\text{PRO}} = 0.7 \text{ nM}$).
Table 1. Comparison of β2AR ligands in Prestwick Library. The table includes known agonists, known antagonists, and unusual compounds, in that order. Function of compound in the literature (Lit. Function) as an agonist or antagonist; affinity measured by radioligand competitive binding assay (Lit.), FAP-β2AR internalization assay (R Int), and soluble β2AR-GFP to DHA bead competitive binding assay (DHA).

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<td>R Int</td>
<td></td>
<td></td>
<td>Hit, Ago</td>
<td>HIT, Anta</td>
<td>HIT, Ago</td>
<td>HIT, Anta</td>
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<td>4.1±0.1</td>
<td>4.1±0.2</td>
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- Log IC50/EC50
Table 2. Comparison of efficacy pattern of selected β2AR ligands and PCL hits towards AC and ERK1/2 pathway, as well as their influence toward the amount of total and surface receptors, and ability to induce surface receptor internalization. INV: inverse agonist; NEUT: considered as neutral antagonist; AGO: partial or full agonist.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pathway (Galandrin and Bouvier, 2006)</th>
<th>Total Receptor</th>
<th>Surface Receptor</th>
<th>Internalization</th>
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<tbody>
<tr>
<td></td>
<td>AC</td>
<td>ERK1/2</td>
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<tr>
<td>(s)-ATE</td>
<td>INV</td>
<td>INV</td>
<td>No change</td>
<td>No change</td>
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<tr>
<td>BUC</td>
<td>NEUT</td>
<td>AGO</td>
<td>No change</td>
<td>No change</td>
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<tr>
<td>CAV</td>
<td>NEUT</td>
<td>AGO</td>
<td>No change</td>
<td>No change</td>
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<tr>
<td>PRO</td>
<td>INV</td>
<td>AGO</td>
<td>No change</td>
<td>No change</td>
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<tr>
<td>ISO</td>
<td>AGO</td>
<td>AGO</td>
<td>Increase</td>
<td>Decrease</td>
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<td>Decrease</td>
<td>Decrease</td>
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<tr>
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<td>n/a</td>
<td>Decrease -&gt;</td>
<td>Increase</td>
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<td>n/a</td>
<td>n/a</td>
<td>Increase</td>
<td>Decrease</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Measured IC<sub>S0</sub> = 2.0± 0.3nM  (inhibition of ISO induced internalization)

<sup>b</sup>: Measured IC<sub>S0</sub> ≈ 1.5 nM  (inhibition of ISO induced internalization)
Figure 1

(a) [Image of green fluorescence in cells]

(b) [Blank image]

(c) [Image of green fluorescence in cells]

Legend:

- Time 0
- Fluorogen
- Detection
- Agonist
- Fluorogen + Agonist
Figure 3
Figure 5

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Figure 6
Figure 7
Figure 8

(a) Norm Intensity vs. Time (min) for various treatments:
- DMSO
- ISO
- 1uM BUC
- 1nM BUC
- 1uM CAV
- 1nM CAV
- 1uM PRO
- 1nM PRO

(b) Norm Intensity vs. Log[Comp(M)] for various treatments:
- BUC
- BUC+ISO
- CAV
- CAV+ISO
- PRO
- PRO+ISO