EuroscreenFast

Assay development & High-Throughput Screening of the pH-activated GPR65 & GPR68 receptors

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Introduction

G-protein-coupled receptors (GPCRs) are seven-transmembrane domain receptors allowing cells to sense their environment and receive external stimuli. These stimuli are very broad in nature and can include photons, biogenic amines, lipids, peptides, chemokines, hormones... Given this diversity of ligands, GPCR are participating in many different pathophysiological pathways such as pain, diabetes, inflammation, mental disorders, hormonal dysregulation, drug abuse, auto-immune diseases or cancer. This feature, and the fact they are easily druggable, has made them the subject of hundreds of successful drug discovery programs for several decades (1). Over the past 20 years, GPR4 (2), GPR65 (3), GPR68 (2) and GPR132 (4) have been identified as pH-activated receptors allowing cells to react to changes in extracellular pH. Recently, GPR4, GPR65 and GPR68 have been implicated in cancer as acidosis is a feature of the tumor microenvironment (5). EuroscreenFast developed recombinant GPR4, GPR65 and GPR68 cell lines and assays, and as a proof of concept used these to perform a high-throughput screening against a library of 260k compounds for antagonist activity on the recombinant GPR65 and GPR68. From this screen, 960 compounds showing a preferred activity towards either of the two receptors were selected for confirmation, at 1 and 10 µM, in duplicate. Subsequently, 180 and 50 compounds were confirmed for activity on GPR65 and GPR68 respectively, for full dose-response activity, with IC50 values ranging from 1 to 30 µM.

Materials and Methods

Cell lines

The coding regions for the GPR65 receptor (NP_003599.2) and the GPR68 receptor (NP_003476.3) were amplified by PCR from genomic DNA and then cloned in pEFIN5, a proprietary bicistronic expression vector developed at EuroscreenFast, in which the transcription of both the receptor and the gene of selection (neomycin) are under the control of a strong promoter of transcription through an IRES (internal ribosome entry site) sequence (6). The GPR65 and GPR68 pEFIN5 expression vectors were transfected in 1321N1 astrocytoma cells using Lipofectamine 2000 (Life Technologies). After selection with antibiotics, the pool of antibiotic-resistant cells was further characterized for receptor activity.

cAMP HTRF assay for Gs coupled receptors

1321N1 cells expressing recombinant human GPR65 (FAST-0927C) or GPR68 (FAST-0928C) were grown in media without antibiotics and detached by gentle flushing with PBS-EDTA before being recovered by centrifugation.

For both agonist & antagonist assay modes (384-well, suspension), cells are resuspended in assay buffer (KRH: 5 mM KCl, 1.25 mM MgSO4, 124 mM NaCl, 25 mM HEPES, 13.3 mM Glucose, 1.25 mM KH2PO4, 1.45 mM CaCl2, 0.5 g/l BSA, supplemented with 1mM IBMX) prepared at a basal activity pH of 7.6. Cells are then mixed with the test compounds diluted at increasing concentrations. In agonist mode, test compounds are prepared in pH 7.6 assay buffer. In antagonist mode, test compound samples are diluted with a pH 4.4 buffer to give a final assay pH of 6.8.

Plates are incubated 30 min at room temperature and then of each of the detection reagents of the cAMP Gs dynamic kit (Revvity) diluted in cell lysis buffer are added to each well. The plate is further incubated for 1 hour at room temperature before being read on an EnVision® Xcite (Revvity) according to the manufacturer specifications.

For High-Throughput Screening, compounds were prepared using a Fluent® Liquid Handler (Tecan), cells were dispensed with a Multidrop Reagent Dispenser (ThermoFisher scientific) and detection reagents were dispensed with a Minitrak (Perkin Elmer/Packard).

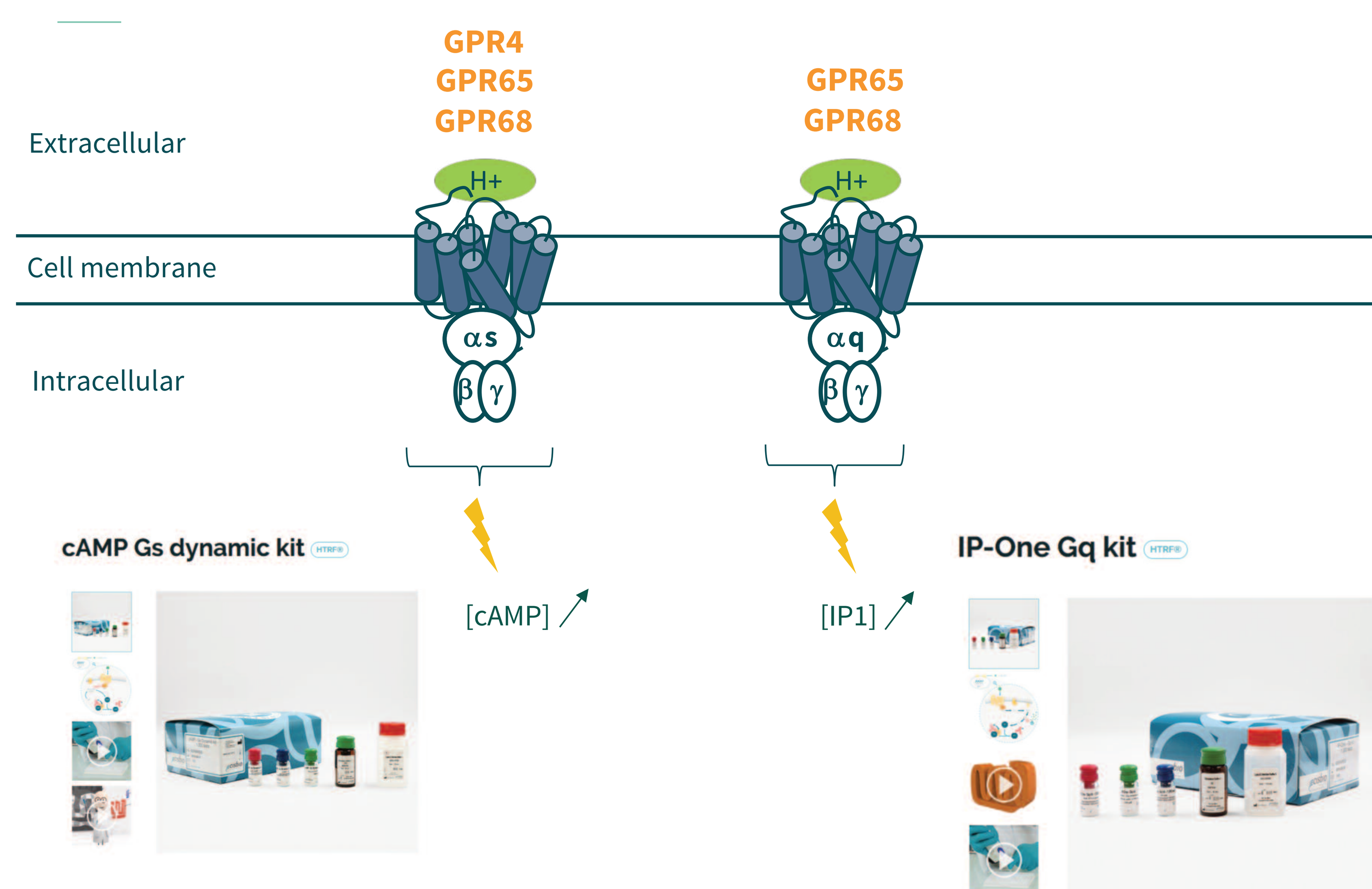

Figure 1. GPR4, GPR65 and GPR68 all signal in the cAMP pathway by coupling to the Gαs subunit, but GPR65 and GPR68 are also able to couple to the Gαq subunit and signal in the IP1/Calcium pathway.

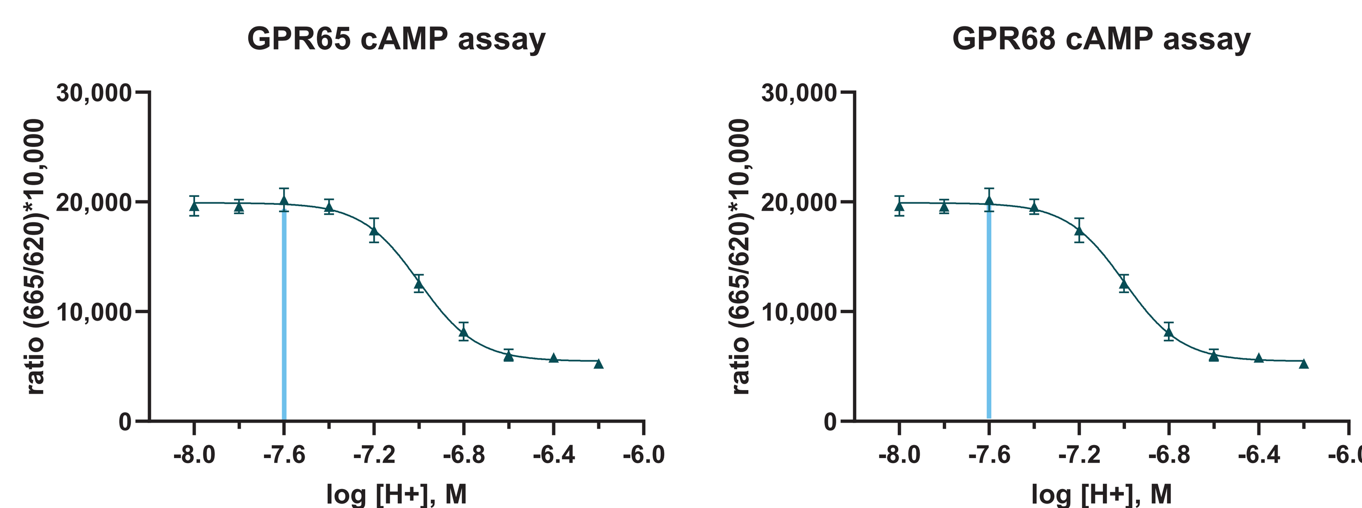

Figure 3. Results for the GPR65 and GPR68 receptors in a cAMP agonist mode assay. Basal pH (7.6) is marked.

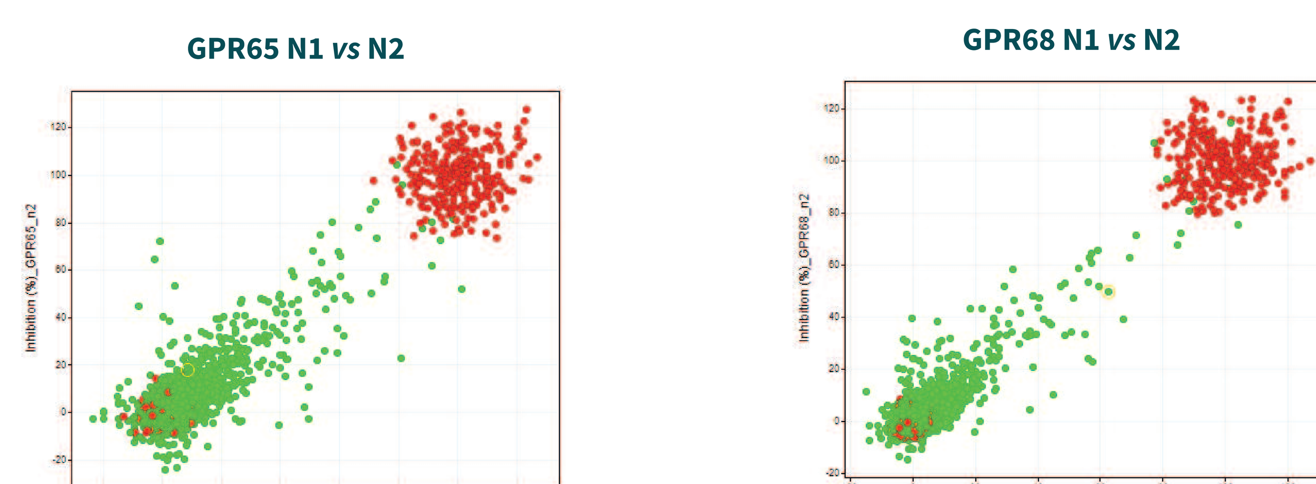

Figure 4. Pilot Screen. 3200 compounds (ten 384-well plates) were screened using the validated GPR65 and GPR68 HTS cAMP antagonist mode assays, at a single dose and in N=2 to assess for assay reproducibility. N1 vs N2 data for each receptor, red dots are control wells and green dots are test compound wells.

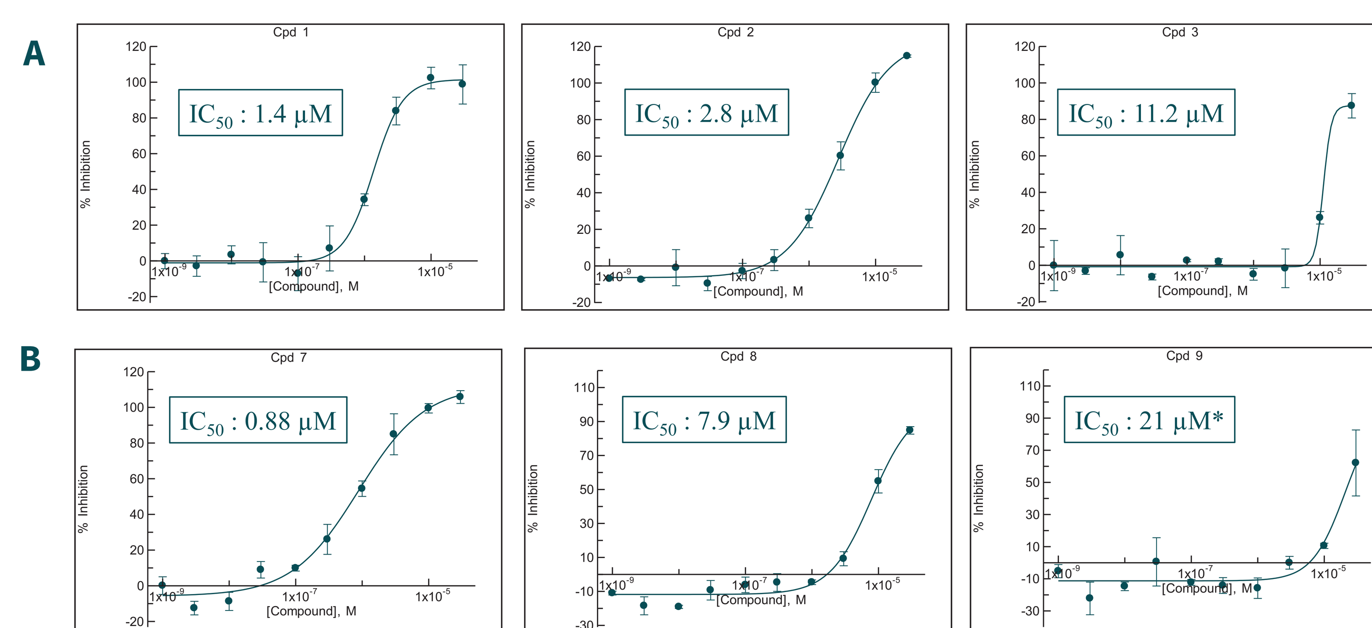

Figure 6. Out of the 960 selected primary hits, 230 were selected for full dose-response testing. (A) Selection of active compounds in a GPR65 cAMP HTRF antagonist assay. (B) Selection of active compounds in a GPR68 cAMP HTRF antagonist assay.

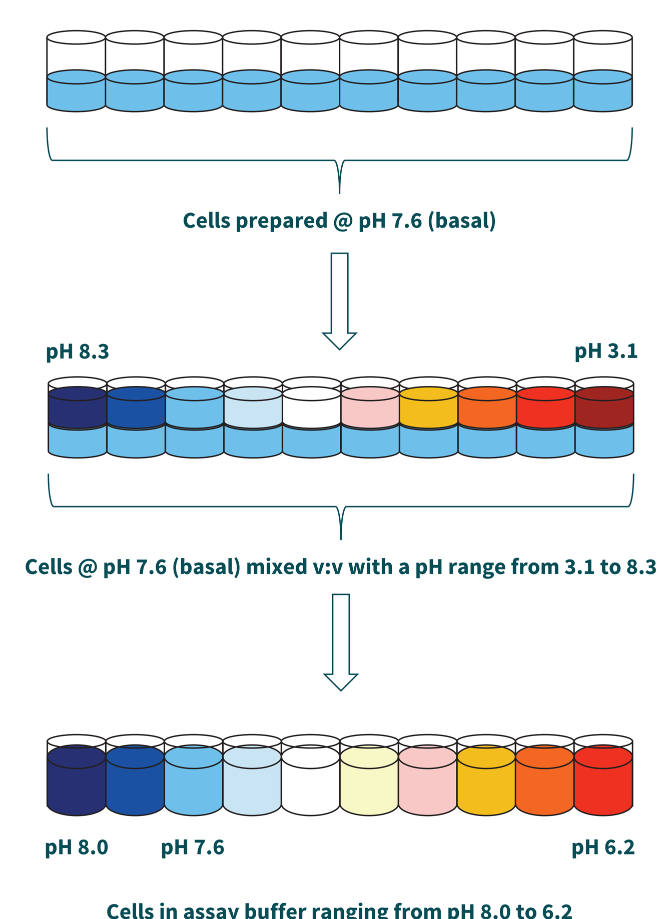

Figure 2. Assay design for 384-well assay format with cell suspension, for HTS compatibility. The GPR65 receptor in a cAMP HTRF agonist mode assay is exemplified but a similar design is used with the IPOne HTRF assay, and for GPR68 as well, using both readouts. Cells are prepared in assay buffer at a basal activity pH, dispensed in assay plate, then mixed v:v with a range of working pH prepared to achieve a known, final assay pH range.

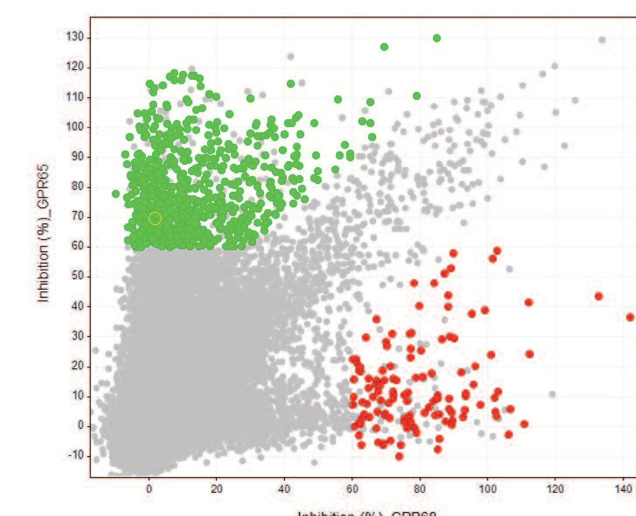

Figure 5. Complete HTS data of 260k compounds screened for antagonist activity at GPR65 and GPR68. A subset of 960 compounds was selected for follow-up testing based on >60% inhibition for a given target and >30% inhibition between the 2 targets. Green dots are GPR65 selective hits (780) and red dots are GPR68 selective hits (180).

References

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