GPCR FUNCTIONAL HTS PLATFORM FOR BIASED SIGNALING PATHWAYS

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Introduction

For many years, it has been known that G-protein coupled receptors (GPCR) can act simultaneously through parallel pathways by coupling to G proteins from different families and/or to β-arrestins. These pathways can be activated with different efficacies by a single ligand termed "biased agonist". These biased agonists might hold interesting promises as novel therapeutics with reduced side-effects compared to their balanced counterparts (Gesty-Palmer et al, 2009; Violin et al, 2010). Current Drug Discovery programs tend to focus on a single readout in early stages and might thus miss chemical entities with biased agonism properties.

We have worked on two proof-of-concept receptors, GPR43 (FFA2) and $S1P_1$ (EDG1), to set up an HTS platform monitoring two separate signaling pathways in parallel. For GPR43, we have designed a 384-well multiplexed assay using both Aequorin and cAMP HTRF™ for the evaluation of G_q- and G_i-coupling in response to known agonists; and for $S1P_1$, we have validated a 384-well G_i-coupled cAMP HTRF™ inhibition assay on the Tango™ β-arrestin U2OS cell line, allowing to screen for compounds signaling through G-proteins or β-arrestin in a balanced or biased manner. The assays set-up as well as results of the screening campaigns will be highlighted and discussed.

Material & Methods

(A) CHO-K1 cells expressing recombinant human GPR43 receptor and mitochondrial apoaequorine were loaded with Coelenterazine h and were injected on the test compounds in a 384-well plate. The resulting emission of light was measured using a FDSS6000 luminometer (Hamamatsu).

After a 30 minutes incubation with the test compounds at room temperature, HTRF™ conjugates were added to the cells and the cAMP levels were measured according to the manufacturer's instructions (CisBio International) on a Rubystar plate reader (BMG Labtech). This allowed to monitor two separate signaling pathways for the same receptor, in the same wells

In these experiments, the different kinetics of signal transduction allowed to discriminate between the rapid response to variations in Ca^{2+} flux (G_q signaling – aequorin assay) and the slower response to cAMP modulation (G_i signaling – cAMP HTRF™ assay).

(B) Tango™ β-arrestin U2OS cell line expressing recombinant human $S1P_1$ receptor were used to validate a cAMP HTRF™ assay. This cell line was then used to screen in parallel for compounds modulating the β-arrestin and/or the cAMP pathways, at a single concentration. Afterwards, active compounds were selected for confirmation and EC_{50} determination in both read-outs.

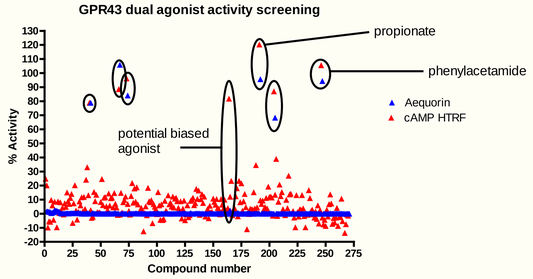

Figure 2. Screening of 270 compounds at 10 µM for agonist activity in recombinant human GPR43 Aequorin-cAMP HTRF™ multiplex assay. Reference compounds propionate and phenylacetamide were included as positive controls at 10 mM and 10 µM respectively. Five active compounds were selected for follow-up in dose-response testing, one of which displayed potential biased agonism properties.

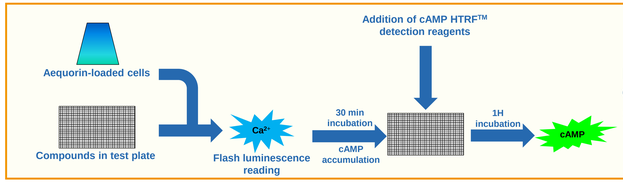

Figure 1. Schematic of the GPR43 multiplexing assay procedure

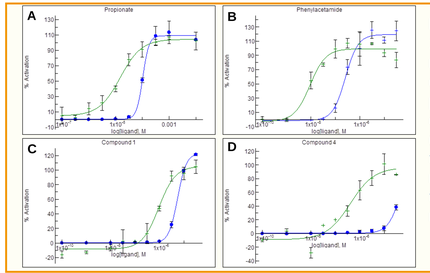

Figure 3. Dose-response testing following the screening at 1 concentration. Propionate and phenylacetamide reference compounds were tested along the five active compounds selected from the screening. Data are presented in % activation in Aequorin assay (blue) and in cAMP assay (green), for Propionate (A), Phenylacetamide (B), Compound1 (C), and Compound 4 (D). In all cases, the EC_{50} is left-shifted in the cAMP read-out but this shift is more pronounced for some of the compounds compared to the references. Individual values for all compounds in both read-outs are reported in Table 1.

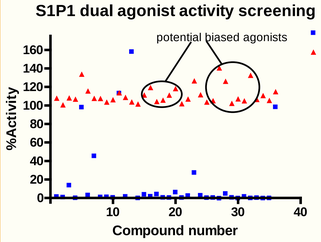

Figure 4. 2560 compounds were screened at 10µM on the Tango™ β-arrestin U2OS cell line expressing the recombinant human $S1P_1$ receptor. The compounds were tested in parallel in a G_i cAMP HTRF™ assay and in a Tango™ β-arrestin assay. Results were expressed as a %Activity relative to an EC_{100} of FTY720P. Nine active compounds were selected for follow-up in dose-response testing.

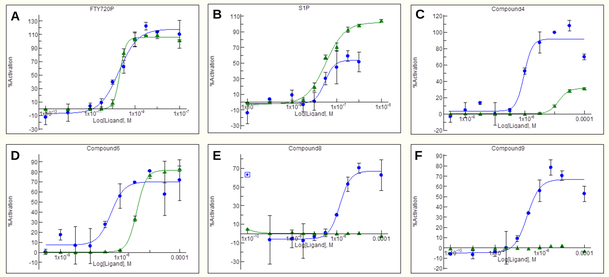

Figure 5. Dose-response testing following the screening at 1 concentration. FTY720P and S1P reference compounds were tested along the nine active compounds selected from the screening. Data are presented in % activation relative to FTY720P in cAMP assay (blue) and in β-arrestin assay (green), for FTY720P (A), S1P (B), Compound4 (C), Compound6 (D), Compound8 (E) and Compound9 (F). In this cases, EC_{50} can be equivalent or shifted and we also observe a difference in efficacy between the two signaling pathways. Individual values for all compounds in both read-outs are reported in Table 2.

Table 1. Measured EC_{50} values in both read-outs on GPR43

Compound ID	EC_{50} (nM)		% Activity at 10 µM or 10 mM*		EC_{50} Left-shift (Aeq/cAMP)
	Aeq	cAMP	Aeq	cAMP	
Propionate	103000	14900	103*	110*	6.9
Phenylacetamide	257	9.06	111	97	28
Compound1	5580	941	99	96	5.9
Compound2	5800	76.7	88	110	75
Compound3	7390	105	83	89	70
Compound4	42200	410	7.6	101	103
Compound5	359	10.8	120	102	33

Table 2. Measured EC_{50} and E_{max} values in both read-outs on $S1P_1$

Compound ID	EC_{50} (nM)		% Activity at 100 µM		EC_{50} Left-shift (βarr/cAMP)	E_{max} ratio (βarr/cAMP)
	βarr	cAMP	βarr	cAMP		
FTY720P	0.21	0.19	106	118	1.09	0.90
S1P	32	29	102	54	1.08	0.52
Compound1	4000	838	5	57	4.77	0.09
Compound2	2714	94	76	85	28.8	0.89
Compound3	18716	3244	36	127	5.77	0.29
Compound4	13626	940	32	92	14.5	0.34
Compound5	3720	4482	8	107	0.83	0.08
Compound6	3628	493	82	70	7.36	1.16
Compound7	3821	392	44	104	9.74	0.42
Compound8	Not active	1377	Not active	67	>100	0
Compound9	Not active	273	Not active	67	>300	0

Conclusions

- We have set up assay conditions where two read-outs can be screened sequentially in the same well : Aequorin Ca^{2+} flux assay (G_q signaling) and cAMP HTRF™ assay (G_i signaling)
- Tango™ β-arrestin recruitment and cAMP levels (G_i signaling) were screened in parallel on the same cell background expressing recombinant human $S1P_1$ receptor
- We have performed a small screening looking for compounds that would present biased agonism properties, using these two appraoches.
- In a follow-up experiment, candidate compounds were confirmed as positive hits.
- This methodology adds a new dimension to the hit-to-lead screening with an immediate confirmation in a secondary assay for balanced agonists and with the direct identification of new relevant entities presenting a biased efficacy for signaling to different G proteins.
- Interestingly, on the $S1P_1$ receptor, the screen identified compounds with biased efficacy as well as biased potency

References

(1) Gesty-Palmer *et al.*, Sci Transl Med. 2009; 1:1ra1

(2) Violin *et al.*, J Pharmacol Exp Ther. 2010; 335:572-579