



Unveiling drug selectivity via functional profiling and multi-prong approach to hit validation using ChemiScreen™ GPCR cell lines

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Abstract

FLIPR® and aequorin technologies have become the systems of choice for measuring the changes in intracellular calcium in a high throughput manner. They both provide rapid and sensitive read-out for many GPCR drug targets. Not all GPCRs, however, couple to G_q leading to calcium mobilization. We have developed a novel cell-based assay to make all GPCRs signal through calcium mobilization using cell lines with endogenous promiscuous G proteins and CRAC channel. It eliminates the need and problem associated with co-transfecting either promiscuous or chimeric G proteins. The functional readout of each GPCR expressed in this system has been validated for over 150 targets of different G protein coupling status, and compared to the binding assay and native readouts using small molecule agonists, antagonists, and allosteric regulators.

Case study and SAR cascade are presented using chemokine receptor CXCR2 as an example. To further validate the screening hits from a FLIPR assay we compared the result to a conventional radioligand filtration binding assay and many other different functional assays using ChemiScreen transfected cell line and native cell line expressing CXCR2. Here we evaluated the activities for known compounds to be selective for the CXCR2 receptor, SB 225002 and SB 265610. Also, we evaluated the activity of non-selective compounds for CXCR2 but are selective for other chemokine receptors, RS 102895 (CCR2b), RS 504393 (CCR2), and UCB 35625 (CCR1 and CCR3). Sample activity across different assay platforms and preserving sample rank order is presented to illustrate the versatility and multi-purpose applications for Millipore's GPCR product line.

Introduction

In the journey of bringing a small molecule from "bench to bedside", drug companies face many challenges associated with small molecule screening. Evaluating the activity of small molecules can be a daunting task, especially if the tools provided are not suitable for high throughput screening. Millipore provides an array of GPCR products to assist the investigator address these needs. From GPCR cell lines and membrane preparations to filtration devices and related accessories, Millipore offers a huge range of products to address your screening needs.

A case study of compound profiling is presented which demonstrates the cost effectiveness and advantages of using Millipore's GPCR ChemiScreen products. A SAR screen was conducted to determine if Millipore's ChemiScreen product lines can differentiate selective small molecules and inactive compounds at CXCR2, a G_i-coupled receptor that is an important target in disease states such as inflammation and rheumatoid arthritis. We used the CXCR2/Chem-1 cell line (Catalog #: HTS002C) and CXCR2 membrane preparation (Catalog #: HTS002M) to screen a panel of such compounds. In this study, we have implemented various functional assay platforms (fluorescent and aequorin calcium assay, GTPγS binding, and cAMP inhibition) to rank order compounds, demonstrating the versatility and multi-purpose applications of Millipore's product line. The results of these functional assays compare closely to the observations in traditional radioligand binding.

Methods

Fluorescent calcium flux assay: CXCR2-expressing Chem-1 cells were loaded with Fluo-8 (ABD Bioquest, Ltd.). Cells were pre-incubated with compound, and calcium flux in response to 2x EC50 recombinant human IL-8 was determined on a Molecular Devices FLIPR™^{TR} assay.

Aequorin assay: Chem-1 cells co-expressing CXCR2 and a mutant flash aequorin were loaded with 5μM coelenterazine. Luminescence in response to IL-8 was determined in quadruplicate on a Molecular Devices FLIPR™^{TR} assay.

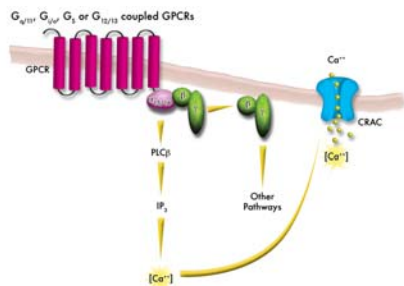
Radioligand binding assay: CXCR2 membrane preparation was incubated with [¹²⁵I]-IL-8 and compound. The reaction was filtered on a PEI-coated FC 96-well harvest plate (Millipore) and washed. The plate was dried and counted on a Trilux Microbeta counter.

GTPγS assay: CXCR2 membranes were incubated with [³⁵S]-GTPγS, compounds and unlabeled IL-8. The binding reaction is transferred to an FB filter plate (Millipore) and washed. The plate was dried and counted.

Cyclic AMP assay: CXCR2-expressing Chem-1 cells were transiently transfected with Adenylate Cyclase 6 and assayed for cAMP by the chemiluminescent cAMP HTS Immunoassay Kit (Millipore)

Millipore's ChemiScreen GPCR Technology

Millipore's GPCRProfiler panel contains 140+ GPCRs with calcium readout and more to come



1. Novel mammalian expression system to express more GPCR on cell surface.
2. Use endogenous promiscuous G proteins to direct all GPCRs to mobilize calcium.
3. Use endogenous CRAC (calcium release-activated calcium) channel to further amplify calcium signal for maximum FLIPR readout.
4. Validate the FLIPR assay for each GPCR with agonist, antagonists and allosteric modulators, with results consistent with a native assay.
5. Common readout for all GPCRs is ideal for compound comparison in selectivity profiling and HTSAR.

US/European patents pending- M Hsu

Millipore provides a broad range of products and services to enable and accelerate drug discovery



- o HTS assays and profiling services for GPCRs
- o Cell lines expressing all classes of GPCRs for fluorescent and luminescent detection of calcium flux
- o GPCR membrane preparations for radioligand binding and GTPγS assays
- o cAMP assay kit for G_s and G_i-coupled receptors

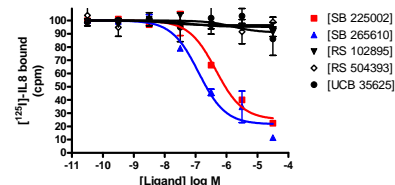
Results

Table 1. Summary of small molecule activity in different functional assays

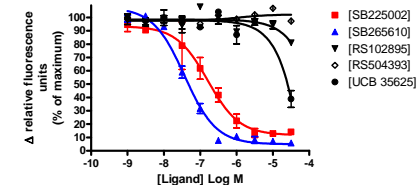
Functional Assay	SB 225002	SB 265610	RS 102895	RS 504393	UCB 35625
FLIPR (Ca ²⁺ flux)	162.7nM	37.7nM	No activity	No activity	> 10nM
GTPγS	1.2μM	342.6nM	No activity	No activity	No activity
cyclic AMP	185.2nM	3.0nM	No activity	No activity	No activity
Aequorin	74.6nM	11.7nM	No activity	-----	-----
* Radioligand Filtration Binding	200.4nM	55.4nM	No activity	No activity	No activity

Small molecule rank order: SB 265610 > SB 225002 > RS 102895 = RS 5044393 = UCB 35625

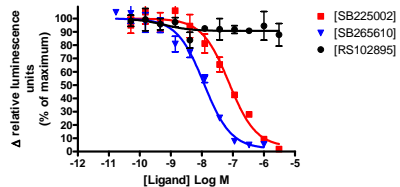
A. Radioligand Binding



B. Calcium flux (FLIPR assay)



C. Aequorin Assay



D. GTPγS Assay

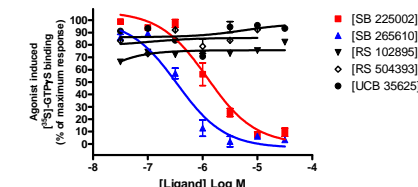


Figure 1. Comparing various functional assays versus radioligand binding. The data represented illustrates the various applications using Millipore ChemiScreen GPCR products as an ideal screening tool. When comparing radioligand binding values (A) to calcium flux assay results using the FLIPR™^{TR} (B), we were able to identify and differentiate between select inhibitors and non-specific compounds for the CXCR2 receptor. Moreover, small molecule rank order is preserved with both showing approximately a 4-fold difference in affinity between CXCR2 inhibitors SB265610 and SB225002. Also, aequorin (C) and GTPγS (D) assays were performed to further validate the flexibility and multi-functionality of Millipore's GPCR product line. Similarly, sample rank order is preserved between functional assay types.

Summary

- o We have demonstrated that using Millipore ChemiScreen GPCR products are effective tools in supporting many drug discovery SAR efforts.
- o In both radioligand binding and functional assays, we were able to successfully detect selective small molecules for CXCR2, as well as differentiate from inactive compounds for CXCR2.
- o Moreover, we were able to successfully rank order small molecule activity, which is an important aspect in the screening process.
- o In all assay formats performed, both SB 265610 and SB 225002 showed to be specific inhibitors for the CXCR2 receptor, which is consistent to what is reported in the literature.
- o SB 265610 has shown greater affinity for CXCR2 in all formats, yielding approximately a 5-fold difference in affinity when compared to SB 225002.

Related Products from Millipore

HTS002C	ChemiScreen CXCR2 Calcium-Optimized FLIPR Cell Line
HTS002M	ChemiScreen CXCR2 Membrane Preparation
ES106	Coelenterazine reagent, 500μg
17-416	Chemiluminescent cAMP HTS Immunoassay Kit
MAHFB1H60	MultiScreen® Harvest APFB Opaque 100μL well
MAHFC1H60	MultiScreen Harvest APFC Opaque 100μL well

References

- Auten *et al.*, J. Pharmacol Exp Ther. 2001 299: 90-95.
White JR *et al.*, J Biol Chem. 1998 273: 10095-10098.