Identifying Nonselective Hits from a Homogeneous Calcium Assay Screen

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The authors used a homogeneous calcium dye kit with a cell line transfected using a recombinant protein construct to screen a 50,000 compound library for G-protein coupled receptor (GPCR) agonists. Only 1 of the 365 primary hits activated Gq-coupled GPCRs, as shown using IP-ONE HTRF®. Furthermore, an agonist screen against the entire compound library and same heterologous cell line using AequoScreen™ technology generated no false positives and identified the same positive hit. Next, a multiplex assay composed of both Fluo-3 and Fura-2–loaded cells identified 1 false positive and the same true-positive hit out of the 365 primary hits. Finally, rescreening the 365 primary hits against the parental cell line loaded using the homogeneous calcium dye kit confirmed the specificity of the same true-positive hit only. In summary, the results suggest that AequoScreen™ technology, IP-ONE HTRF®, and multiplex assays are unique, orthogonal technologies to identify nonspecific hits. (Journal of Biomolecular Screening 2007:1-3)

Key words: G-protein-coupled receptor, homogeneous calcium assay, aequorin, functional drug screening system, Fura-2, Fluo-3, IP-ONE HTRF®

INTRODUCTION

TYPICAL G-PROTEIN-COUPLED RECEPTOR (GPCR) agonist screening campaigns challenge a random compound library against cell lines heterologously expressing specific GPCR targets, followed by “cherry-picking” the primary hits. The selected primary hits are subsequently rescreened against the parental cell line to identify and eliminate compounds having off-receptor, endogenous activity (nonselective hits). Although homogeneous calcium dye kits for high-throughput screening (HTS) historically work well for both agonists1,3 and antagonists,4 we observed a high false-positive rate in a modest agonist campaign (50,000 compounds). We rescreened hundreds of primary hits against the parental cell line (same homogeneous calcium dye kit) and confirmed only 1 selective active hit. This low confirmation rate is too costly in terms of resources for rescreening5 plus a waste of precious, if not irreplaceable, compounds. Thus, we explored alternative assays that provide the same sensitivity but improved specificity.

In this report, we (1) screened a library for agonists using a homogeneous calcium dye kit, (2) screened the same library for agonists using AequoScreen™ technology, and (3) retested primary hits from a homogeneous calcium dye kit screen using 2 orthogonal assays and compared results.

MATERIALS AND METHODS

Cell line and cell culture

The GPR54 cell line of Chinese hamster ovary (CHO) cells stably transfected with the coding sequence of the human metastin GPR54 receptor (GPR54 cell line, GenBank: NM_032551 was a kind gift from Euroscreen s.a., Brussels, Belgium). The GPR54, cell line was cultured in Ham’s F-12 medium with 10% fetal bovine serum, G418, and Zeocin. The control CHO cell line (parental cell line) was stably transfected with Aequorin and maintained in the same growth media.

Homogeneous calcium dye assay

Assays were executed according to the standard protocol provided by BD Biosciences (Franklin Lakes, NJ). Briefly, 24 h prior to plate seeding, cell culture medium was replaced with assay medium. (Assay medium is identical to growth medium minus antibiotics G418 and Zeocin.) Cells were dissociated from cell culture flasks using Hank’s buffer with 5 mM EDTA and resuspended in assay medium to a final density of 0.25M cells/mL. Cells, 50 µL, were seeded into 384-well plates (Corning, NY, cat. 3709) and incubated at 37 °C, 5% CO₂ for 18 to 24 h. Calcium dye (BD™ PBX Calcium Assay Kit) was incubated with cells (without removal of culture media) for 30 min at 37 °C. Cells were challenged against the compound library using the Hamamatsu Corp., Bridgewater, NJ.

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Received Aug 1, 2006, and in revised form Oct 4, 2006. Accepted for publication Nov 21, 2006.

Journal of Biomolecular Screening 12(X); 2007 DOI:10.1177/1087057106298538

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FDSS6000 (Bridgewater, NJ), with excitation 480 nm and emission 540 nm. Maximum net increase in fluorescence following calcium mobilization was recorded; signals from wells challenged using 10 µM ligand were positive controls, and those from wells with buffer only were negative controls.

**AequoScreen™ assay**

The GPR54 cell line was cultured and dissociated from cell flasks as described above. After centrifugation, cells were resuspended in DMEM/F-12 medium with 0.1% bovine serum albumin (BSA) to 5M cells/mL. Coelenterazine h was added to the cells at a final concentration of 5 µM and incubated at room temperature for 18 to 24 h. Next, cells were diluted to a final concentration of 0.5M cell/mL using DMEM/F-12/0.1% BSA. The cells were loaded into the cell vat on the FDSS6000. Cells were added to 384-well plates preloaded with 10-µM compounds, and photons generated following calcium mobilization were collected and integrated using a 2-D photon counting sensor for 1 min.

**IP-ONE HTRF® assay**

The cell-based IP-ONE HTRF® assay from Cisbio (San Diego, CA) was performed according to the manufacturer’s protocol. Briefly, the GPR54 cell line was incubated overnight in a 384-well plate at a density of 12,500 cells/well. Next, medium was removed, and 20 µL of 10-µM compounds made in stimulation buffer was added to appropriate wells. Cells were incubated at 37 °C, 5% CO2 for 60 min before adding 10 µL d2 conjugate and 10 µL K conjugate. Cells were incubated at room temperature for 60 min before being read using a plate reader (Molecular Devices, Sunnyvale, CA), with time-resolved fluorescence at 620 nm and 660 nm (ex 337 nm). The assay measures IP-1 accumulation; IP-1 is the stable breakdown product of labile IP-3, generated following GPCR activation.

**Multiplex assay**

The GPR54 cell line was dissociated from cell culture flasks using Hank’s buffer with 5 mM EDTA and resuspended in Hybridoma media (Sigma, St. Louis, MO) and 2.5 mM Probenecid (pH 7.5). Cells were divided into 2 groups, with 1 group loaded using 2.5 µM Fura-2 AM/0.05% w/v pluronic acid final and the other group loaded using 0.5 µM Fluo-3 AM/0.05% pluronic acid final. Following a 1-h incubation, RT cells were washed extensively and combined. To each well was added 45 µL containing 70,000 Fura-2-loaded cells and 25,000 Fluo-3-loaded cells. Cells were challenged against the compound library using the Hamamatsu FDSS6000, with excitation 480 nm, emission 540 nm (interrogating Fura-2 AM-loaded cells) and excitation 380 nm, emission 540 nm (interrogating Fura-2 AM-loaded cells) in a 1.2-sec cycle time. Maximum net increase in fluorescence following calcium mobilization (Fluo-3 AM loaded cells) and maximum net decrease in fluorescence following calcium mobilization (Fura-2 AM-loaded cells) were recorded; signals from wells challenged using 10 µM ligand were positive controls and those with buffer only were negative controls.

**RESULTS AND DISCUSSION**

A primary screening campaign of 50,000 compounds against the GPR54 cell line using a fluorescence-based homogeneous calcium dye assay, no washing, yielded 365 hits, defined as a calcium mobilization signal greater than 50% of the positive controls. By contrast, the same library and cell line, screened using AequoScreen™ technology, yielded only 1 hit (data not shown). Rescreening of the 365 hits against both the parental and GPR54 cell lines, homogeneous calcium dye, revealed only 1 hit with specific activity, reported as a GPR54 cell line response greater than the parental cell line response (Fig. 1A).

Another assay, IP-ONE HTRF® used for measuring IP-1 accumulation following GPCR activation, confirmed the same selective hit (Fig. 1B).

To test the possibility that the false-positive hit rate may be dye related, we developed a dual dye multiplex assay. We hypothesized that a compound affecting a given fluorescence dye signal may not affect the other dye signal. Thus, if we used the criteria of selecting compounds that were double positive, we could reduce false-positive hits significantly. As shown in Figure 2, 2 of 365 compounds were double positive (note: Fura-2 AM-loaded cells reported only 1 false-positive hit). Interestingly, 17 compounds were false positive using Fluo-3 AM-loaded cells, suggesting autofluorescence at excitation 480 nm. The remainder were double negative.

Not washing away culture media before adding homogeneous calcium dye may have biased the assay toward false-positive generation, through an unclear mechanism. Culture media containing 10% fetal bovine serum (FBS) plus metabolic byproducts from cells grown overnight may well influence assay performance. Although advertised as a "no-wash" product, cell washing1,4,7-9 or media aspiration10 steps are often part of the manufacturer’s suggested protocols. However, using homogeneous calcium dye assay kits with washing or aspiration steps decreases their value by (1) including potential cell perturbation—causing calcium mobilization artifacts through washing (a similar mechanism demonstrated using centrifugation and resuspension1), (2) not increasing screening throughput due to plate washing or media aspiration time and labor, and (3) in some kits, adding a proprietary quencher, a potential assay variable.

By contrast, 3 other assays reported herein, each requiring cell washing, generated nearly identical hit selectivity (no false-positive hits for IP-ONE HTRF® and AequoScreen™ technology). 1 false-positive hit (multiplex assay), and complete concordance apropos of hit sensitivity (1 selective hit common to all 3 assays).

In conclusion, AequoScreen™ technology, IP-ONE HTRF®, and multiplex assays are unique, orthogonal technologies to identify nonspecific hits.
ACKNOWLEDGMENTS

We thank Euroscreen for providing the GPR54 cell line, Shouming Du for his technical support, and Mary Jo Wildey for her support of the project.

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FIG. 1. Orthogonal assays confirm the same selective active hit. (A) GPR54 cell line, FL versus parental cell line, FL. (B) GPR54 cell line, IP-ONE versus parental cell line, FL. Arrows identify the same selective active hit in both panels. FL = homogeneous calcium dye assay. IP-ONE = IP-ONE HTRF® assay.

FIG. 2. Correlation of agonist hits using the GPR54 cell line loaded using either Fura-2 AM (y-axis) or Fluo-3 AM (x-axis). Results are expressed as the minimal fluorescence at excitation 380 nm, emission 540 nm for Fura-2 AM versus maximal fluorescence at excitation 480 nm, emission 540 nm (Fluo-3 AM). Results fall into 3 groups: (−,−), double negative; (+,−), positive in Fluo-3 AM-loaded cells and negative in Fura-2 AM-loaded cells; and (+,+), positive in both dye-loaded cells. The true-positive hit is marked with an arrow; a single false-positive hit is present.

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