Comparison of Orthogonal Technologies to Confirm Agonist Hits Generated Using the FDSS6000

Introduction

In this report we identify a subset of primary hits from a No Wash, fluorescence based agonist campaign using a recombinant cell line. We cherry picked the hits for retesting against a panel of assays to confirm activity.

Materials and Methods

CHO cells stably transfected with the coding sequence of a human receptor and Aequorin (Recombinant) or the parental cell line, stably transfected with Aequorin (Parental) were used in the study. The various assays, Aequorin (Euroscreen), IP-ONE (Cis-Bio), RT-CES (ACEA), Calcium No Wash (BD Biosciences), were used according to manufacturer's instructions.

Results

A primary screening campaign of 50,000 compounds against BD PBX Calcium Assay Kit loaded Recombinant cells yielded 365 hits, defined as signal greater than 50 % of the positive controls. By contrast, the same library and cell line, prepared for Aequorin assay and analyzed using the FDSS6000 yielded only 1 hit (Table 1).

Table 1: Fluorescence Based Assay Hits are Non-Specific, not Confirmed Using Orthogonal Technologies

Primary Hits			Primary Hit Retests		
Library	Fluorescence	Aequorin	Signal $_{Recomb}$ / Signal $_{Parent}$ >10	IP-One	RT-CES
50 000	365	1	1/365[a]	1/365[a]	1/85[b]

a. 365 Fluorescence-generated primary hits
b. Subset of 365 primary hits

Rescreening of the 365 hits against the Parental and Recombinant cell lines revealed only 1 hit with specific activity, that is activity 10 fold higher using the Recombinant cell line versus Parental (Table 1). Another assay, IP-ONE, used for measuring IP-1 accumulation following GPCR activation, confirmed one hit. Finally, a subset of the 365 hits was tested using RT-CES, technology used to measure cell growth, morphology, proliferation, adhesion, and morphology changes over several hours, in response to agonists. Interestingly, all retests confirmed the one and only hit discovered using the Aequorin assay.

To test the possibility that the false positive compounds interacted with the fluorescence dye directly, we utilized the dual excitation capability of the FDSS6000. Cells were rendered nonadherent, preloaded with either Fluo-3 AM or Fura-2 AM dyes, washed, and combined into one well (Multiplex). We hypothesized that a compound that affected one fluorescence dye signal might not affect the other dye signal. Thus, if we use the criteria of selecting compounds that were positive using both dyes, we could reduce the false positive rate significantly. As shown in Figure 1, only 2/365 compounds were double positive. Interestingly, 17 compounds were single positive with Fluo-3 AM loaded cells only, suggesting autofluorescence at excitation 488 nm. The remainder were double negative, suggesting that the false positive compounds were not directly interacting with the fluorescent dyes.

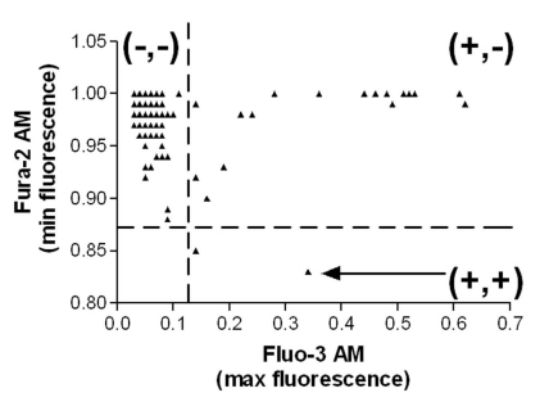

Fig. 1: Correlation of agonist hits using GPR54 cell line loaded using either Fura-2 AM (Y-axis) or Fluo-3 AM (X-axis) (Multiplex). Results are expressed as the minimal fluorescence at ex380 em540 for Fura-2 AM versus maximal fluorescence at ex480 em540 (Fluo-3 AM). Results fall into three groups: (-,-), double negative; (+,-), positive in Fluo-3 AM loaded cells, 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.

Summary

In summary, we suggest screening for GPCR agonists with No Wash dye kits in cell based assays may generate a significant number of nonspecific positive hits. When compared against the parental cell line response all nonspecific hits were identified using both Aequorin and IP-One technologies and nearly completely identified using multiplex technology, washed cells. In conclusion, follow-up assays for No Wash dye agonist campaigns should include orthogonal technology / assays to identify nonspecific hits.

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