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Developing A Comprehensive Functional Assay System for LPA1 Receptor Screening

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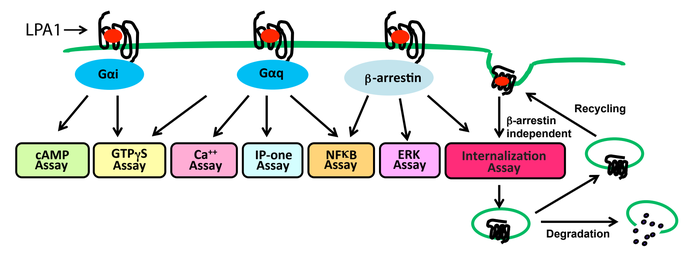
ABSTRACT

Lysophosphatidic acid (LPA) is a small, bioactive phospholipid that mediates multiple cellular responses, including proliferation, differentiation, motility, and survival in both normal physiology and disease. The effects of LPA require the action of heterotrimeric G proteins and act via membrane-bound G protein-coupled receptors. Currently, five LPA receptors and five S1P receptors have been widely studied. The receptors for LPA are expressed in diverse areas of the body such as the brain, circulation and digestive tract. The first LPA receptor, LPA1, when bound to the ligand, induces Gi-dependent cAMP suppression and Gq-dependent intracellular calcium mobilization. In general LPA1 is known to induce many responses including cell proliferation and survival, cell migration, neural progenitor, neuropathic pain, and cytoskeletal changes. LPA1 has also been linked to multiple disease processes, including cancer, fibrosis in kidney and lung, and male infertility. This therapeutic relevance of LPA1 has prompted considerable interest in development and validation of LPA1 receptor model systems to guide the identification of new LPA1 receptor agonists and antagonists. In this report, we present a comprehensive functional assay system encompassing *cAMP*, calcium, *ERK*, *internalization*, NFκB, *GTPγS and chemotaxis* assays using cells stably expressing LPA1 receptor or membranes. These optimized assays going through different signaling pathways are powerful tools in directing compound lead optimization holistically aiming toward discovering new therapeutic interventions with improved potency and efficacy while having reduced side-effects.

Introduction

G-protein coupled receptors (GPCRs) play critical roles in human physiology and are prime targets for drug discovery in the pharmaceutical industry. GPCRs are involved in many diseases, and are also the targets of approximately 30% of all medicinal drugs. These receptors are mainly coupled through $G\alpha_q$, $G\alpha_i$ or $G\alpha_s$ proteins. Advances in high-throughput detection technologies have resulted in an increased use of cell-based functional assays in early drug discovery, in particular for GPCRs. Multispan has optimized parallel functional assays such as *cAMP, calcium, IP-one, ERK, internalization and GTPγS* assays for wide range of GPCRs. In this report we present data for functional assays generated for LPA1 receptor. Our unique beta-arrestin dependent and independent internalization assay along with other functional assays can be used for high-throughput profiling and screening for pathway-specific compounds

Functional Assays Targeting Different GPCR Signaling Pathways



Materials and Methods

Stable Cell lines: LPA1-RH7777 (Multispan Inc., Cat # C1048-6)

Membrane: Membrane was prepared from stable cell line LPA1-RH7777 (Multispan Inc., Cat # MC1048-6), expressing full-length LPA1 receptor

Compound: Compound used as agonist was LPA (Cayman, 10010291)

Calcium Mobilization Assay: Cells cultured overnight in Poly-D-Lysine-coated plates were incubated for one hour with buffer containing calcium indicator dye FLIPR® Calcium 4 (Molecular Devices, R8142). Calcium flux was monitored upon addition of compounds using FlexStation III (Molecular Devices).

cAMP Assay: Cells treated with compounds were subjected to cAMP assay using cAMP Hi-Range Kit (Cisbio, 62AM6PEC).

Internalization Assay: Cells were treated with compounds, stained with anti-FLAG PE antibody, and receptor surface expression was measured by FACS (BD).

NFκB p65 ELISA Assay: Cells treated with compounds were subjected to NFκB p65 assay using phospho-NFkB p65 ELISA Kit (eBioscience, 85-86082-11).

Chemotaxis Assay: Cells treated with compounds were subjected to cell migration or invasion assay using BD HTS FluoroBlok insert plate, 351163).

GTPγS assay: Membranes obtained from stable cell lines expressing LPA1 receptor were subjected to GTPγS assay using SPA beads (Perkin Elmer, RPNQ0001).

Internalization Assay for LPA1 Receptor

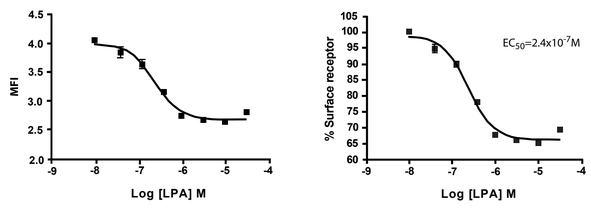


Figure 1. Dose-dependent internalization assay for LPA1 receptor using control agonist. The percent surface receptors were monitored by FACS analysis. Reduction of the surface receptor is 35% for LPA1 receptor after agonist treatment. The EC_{50} value obtained was comparable to the published data.

NFκB Assay for LPA1 Receptor

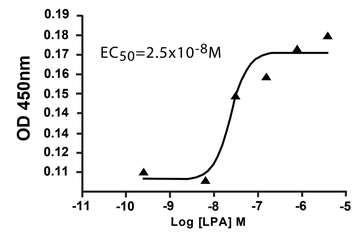


Figure 2. Dose-dependent NFκB p65 ELISA assay for LPA1 receptor expressed in RH7777 cells with control agonist. The EC_{50} value obtained was comparable with published data. Further assay development is in progress.

LPA1 Cell Migration Assay

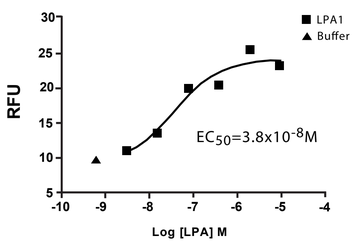


Figure 3. Dose-dependent cell migration assay for LPA1 receptor expressed in RH7777 cells with control agonist. The EC_{50} obtained with agonist LPA was comparable to published data. Additional assay development is in progress.

GTPγS Assay for LPA1 Receptor

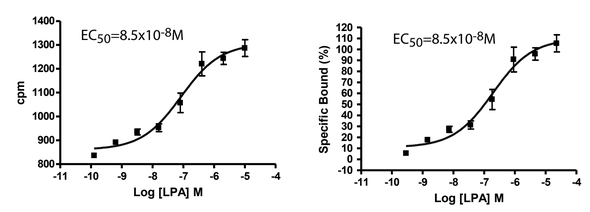


Figure 4. Dose-dependent GTPγS assay for LPA1 receptor expressed in RH7777 cells with control ago-nist using ^{35}S radioligand. Control agonist showed expected EC_{50} value for LPA1 receptor based on literature.

Ca++ Assay for LPA1 Receptor

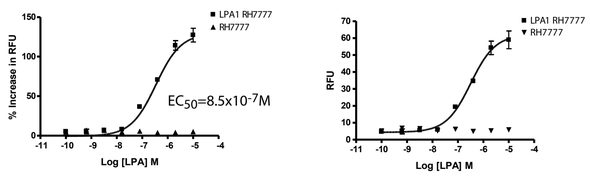


Figure 5. Dose-dependent intracellular Ca^{++} flux was monitored upon treatment with control agonist in stable expression LPA1 RH7777 cells. The EC50 obtained with agonist LPA was comparable to published data.

cAMP Assay for LPA1 Receptor

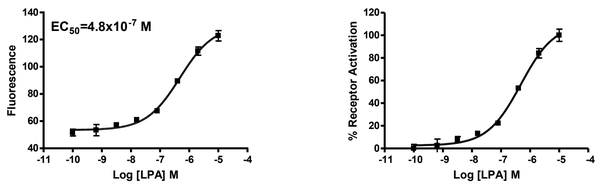


Figure 6. Dose-dependent inhibition of forskolin-stimulated cAMP level upon treatment with con-trol agonist, in RH7777 cells stably expressing the human LPA1 receptor, measured with cAMP HiRange kit (Cisbio 62AM6PEC). The EC50 obtained with agonist LPA was comparable to published data.

Parallel Functional Assays for LPA1 Receptor Developed by Multispan Inc.

Platform	Ca^{++}	IP-one	cAMP	Internalization	ERK	NFκB	GTPγS	Cell Migration
Validated LPA1 Assays	✔	▪	✔	✔	▪	✔	✔	✔

Reagents	LPA1 Cell Line	LPA1 Division Arrested cells	LPA1 Membrane
Catalogue Number	C1048-6	DC1048-6	MC1048-6

Conclusions

- LPA1 receptor has been validated in cell based functional assays including *internalization, NFκB, chemotaxis, cAMP, calcium and membrane GTPγS* assays using a known agonist. Together, these assays enable having a holistic view of lead compound activities and a plethora of options to screen for the most potent compounds regulating relevant receptor functions while avoiding others.
- Consistent EC_{50} values similar to published reports have been obtained, demonstrating excellent pharmacological performance of the LPA1 receptor.
- Our unique internalization assay directly quantifies the disappearance of the receptors from the cell surface capturing both beta-arrestin dependent and independent receptor desensitization, providing a highly desirable new way of screening compounds with high therapeutic efficacy and little side effects that are known to the existing therapies targeting this receptor family.
- Screening and profiling using the composite of functional assays measuring different signaling pathways triggered by the LPA1 receptor provide important information on compounds during screening and lead optimization in drug discovery.