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All Functional Assays in One Cell Line for Studying GPCR Signaling Bias

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ABSTRACT

G protein-coupled receptors (GPCRs) are the biggest therapeutic target class in drug discovery. Early models of the functional activity of GPCRs considered a classical two-state model: 'on' or 'off'. Based on this model, the properties of ligands were classified as agonists, antagonists, and inverse agonists. However, it is now clear that a given ligand is able to induce multiple signaling pathways, such as activation of G proteins and/or β-arrestin. Moreover, if more than one G-alpha protein subtype binds to its cognate receptor, each class of ligand could also affect the downstream signaling differently. These ligand properties are described as the functional selectivity or biased agonism and would, in principle, allow the selective activation of specific cell responses and physiological pathways. It is now well-accepted that many orthosteric ligands have the ability for bias signaling between different G proteins and/or between the G proteins and β-arrestins that are involved in receptor desensitization, internalization, and other second messenger signaling. Similar biases also apply to antagonist and modulators. In this report, we present our comprehensive recombinant assay system that allows us to examine different signaling pathways in the same cell line, obviating the misleading complications associated with using different cells for different assays. Specifically, data on optimized functional assays encompassing radioligand binding, GTPγS binding, cAMP, pERK, pGSK2, and internalization for β1-adrenergic, MOR, and KOR receptors will be shared.

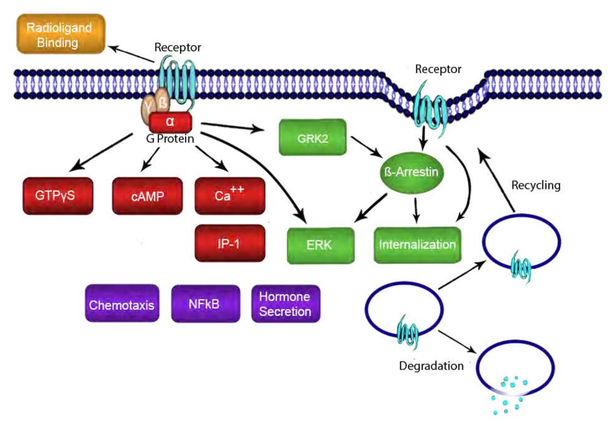

Figure 1. Illustration of GPCR signaling pathway mechanism and assay platform.

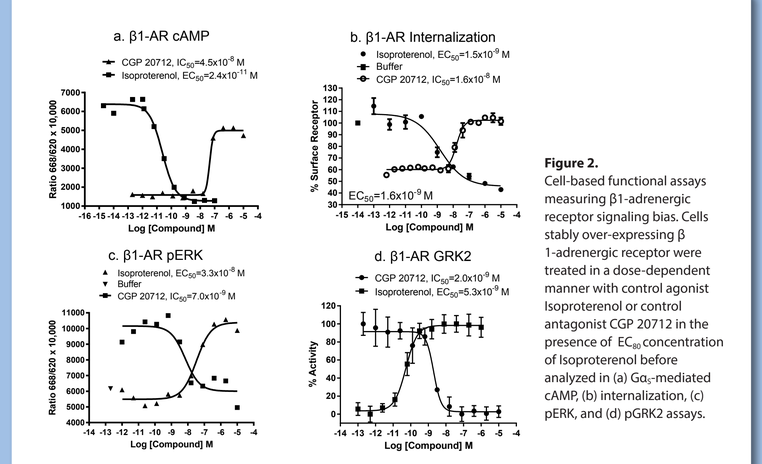

Figure 2.
Cell-based functional assays measuring β1-adrenergic receptor signaling bias. Cells stably over-expressing β 1-adrenergic receptor were treated in a dose-dependent manner with control agonist Isoproterenol or control antagonist CGP 20712 in the presence of EC_{80} concentration of Isoproterenol before analyzed in (a) $G\alpha_s$-mediated cAMP, (b) internalization, (c) pERK, and (d) pGRK2 assays.

Materials and Methods

Stable Cell lines: β1-adrenergic HEK293T (Multispan Inc., Cat# H1437), MOR CHO-K1 (Multispan Inc., Cat# C1350-1a), and KOR CHO-K1 (Multispan Inc., Cat # C1352-1a).

Membrane: Membranes were prepared from stable cell lines over-expressing full-length β1-adrenergic receptor (Multispan Inc., Cat# MH1437), MOR (Multispan Inc., Cat# MC1350-1a), and KOR (Multispan Inc., Cat # MC1352-1a).

Compounds: Compounds used as control agonists were Isoproterenol (Cayman, Cat# 5592), DAMGO (Phoenix, Cat# 024-10), and Dynorphin B (Tocris, Cat# 3196). Compound used as control antagonist was CGP 20172 (Tocris, Cat#1024).

Internalization Assay: Cells treated with compounds were subjected to internalization assay using anti-Flag specific antibody.

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

pERK Assay: Cells treated with compounds were subjected to pERK assay using Advanced pERK (Thr202/Tyr204) Kit (Cisbio, Cat# 64AERPEG).

pGRK2 ELISA Assay: Cells treated with compounds were subjected to pGRK2 assay using pS29 specific mAb.

β-Arrestin Signaling Assay: Cells transduced with a Bacmam viral construct expressing Transfluor® GFP-β-arrestin2 were washed and fixed prior to imaging and analysis on ImageXpress® Micro Confocal High-Content Imaging System with MetaXpress® software (Molecular Devices LLC).

Binding Assay: Membranes obtained from stable cell lines over-expressing β1-adrenergic receptor, MOR, or KOR were subjected to saturation or competition binding assay using SPA beads (Perkin Elmer, Cat # RPNQ0001).

GTPγS Assay: Membranes obtained from stable cell lines over-expressing β1-adrenergic receptor, MOR, or KOR were subjected to GTPγS assay using SPA beads (Perkin Elmer, Cat # RPNQ0001).

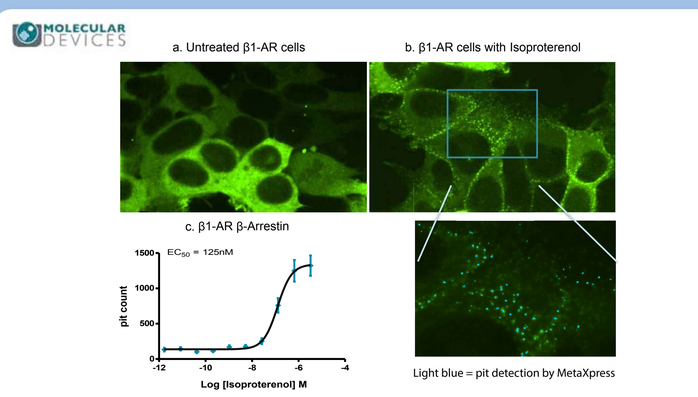

Figure 3. β-arrestin signaling assay in β1-AR stable cell line. Cells stably over-expressing β1-adrenergic receptor were transduced with a GFP-tagged transfluor biosensor, then treated in a dose-dependent manner with control agonist Isoproterenol before imaging and analysis of GFP-β-arrestin translocation on ImageXpress® Micro Confocal High-Content Imaging System. (a) Imaging of untreated β1-AR cells, (b) Imaging of β1-AR cells with Isoproterenol, (c) Dose-response curve of pit count analysis vs Isproterenol concentration in β1-AR β-arrestin assay.

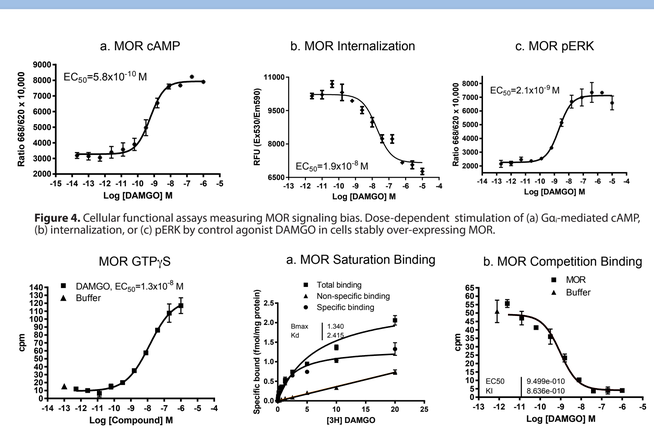

Figure 4. Cellular functional assays measuring MOR signaling bias. Dose-dependent stimulation of (a) $G\alpha_i$-mediated cAMP, (b) internalization, or (c) pERK by control agonist DAMGO in cells stably over-expressing MOR.

Figure 5. Dose-dependent stimulation of [35S] GTPγS binding by control agonist DAMGO in cells stably over-expressing MOR.

Figure 6. Dose-dependent binding of [3H] DAMGO radioligand to membranes from cells stably over-expressing MOR in (a) saturation binding and (b) competition binding.

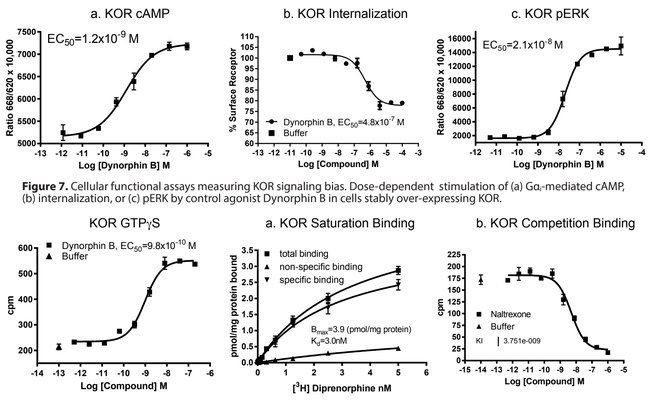

Figure 7. Cellular functional assays measuring KOR signaling bias. Dose-dependent stimulation of (a) $G\alpha_i$-mediated cAMP, (b) internalization, or (c) pERK by control agonist Dynorphin B in cells stably over-expressing KOR.

Figure 8. Dose-dependent stimulation of [35S] GTPγS binding by control agonist Dynorphin B in cells stably over-expressing KOR.

Figure 9. Dose-dependent binding of [3H] Diprenorphine or [3H] Naltrexone radioligand to membranes from cells stably over-expressing KOR in (a) saturation binding and (b) competition binding.

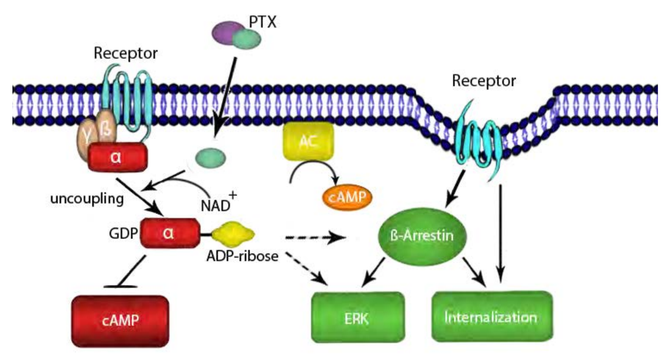

Figure 10. Pertussis toxin (PTX) impairs G protein heterotrimer interaction with receptors, thereby blocking $G\alpha_i/G\alpha_o$ receptor coupling.

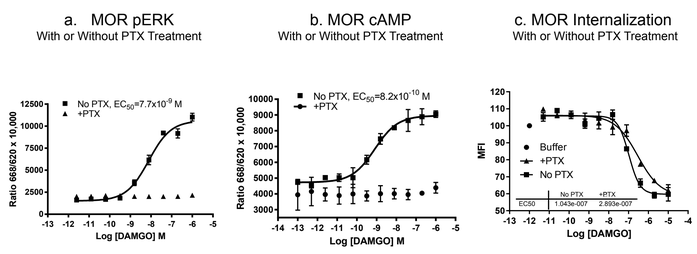

Figure 11. Pertussis toxin (PTX) completely blocked Gαi-mediated MOR pERK and cAMP signaling, whereas PTX had no effect on MOR internalization, suggesting it is going through β-arrestin instead of G protein pathway. Cells stably over-expressing MOR were treated with PTX or vehicle overnight prior to dose-dependent stimulation with control agonist DAMGO and analyzed in (a) pERK, (b) $G\alpha_i$ -mediated cAMP, or (c) internalization assays.

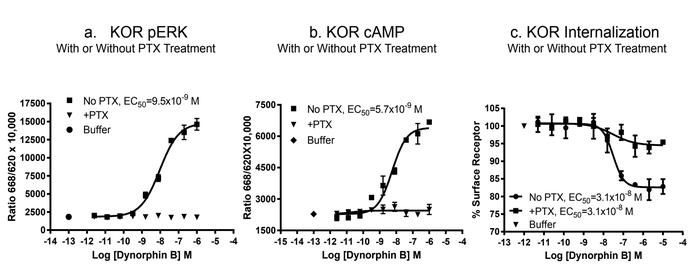

Figure 12. Pertussis toxin (PTX) completely blocked $G\alpha_i$-mediated KOR pERK and cAMP signaling, whereas only partially blocked KOR internalization, suggesting it is going through both β-arrestin and G protein pathway. Cells stably over-expressing KOR were treated with PTX or vehicle overnight prior to dose-dependent stimulation with control agonist Dynorphin B and analysis in (a) pERK, (b) $G\alpha_i$ -mediated cAMP, or (c) internalization assays.

Conclusions

- It is imperative to compare different GPCR signal pathways in one cell line. Consistent IC_{50}/EC_{50} values with literature were reported on optimized *internalization, pGRK2, cAMP, and pERK* functional assays for β1-AR, MOR, and KOR.
- Our Pertussis toxin (PTX) treatment studies illustrated that PTX blocked $G\alpha_i$-mediated pathways in both MOR and KOR pERK and cAMP assays, whereas PTX had no effect on MOR internalization and only partially blocked KOR internalization, which were consistent with literature.
- Together these assays can provide a holistic view of the activities of compound leads and a plethora of options to select the most potent compounds regulating relevant receptor functions while excluding others during lead optimization in drug discovery.