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Molecular Pharmacology: *Perspective*

# Casting a Wider Net:

# Whole Cell Assays to Capture Varied and Biased Signaling

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**Running Title**: Cellular Assays Detect Pluridimensional and Biased Efficacy

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**Abbreviations**: cyclic AMP- cyclic adenosine monophosphate: MAPK- mitogen-activated protein kinase : ERK1/2-Extracellular signal-regulated protein kinases 1 and 2;DAMGO-[D-Ala2, N-MePhe4, Gly-ol]-enkephalin)

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Abstract:

The observation of complex receptor behaviors has shown how ligands can have multiple efficacies and also can differentially stimulate certain cellular signaling pathways over others (i.e. biased signaling). Conventional pharmacological assays (usually proximal to the receptor) will detect ligands that produce the signal defined by the assay (i.e. $Ca^{2+}$, cyclic AMP etc.) but otherwise may miss biased ligands which produce little activation of pathways not measured by the assay. Theoretically this is less of a hazard for generic whole cell assays which may be sensitive to multiple signaling inputs. Whole cell assays have the advantage of detecting effects induced by a variety of receptor interactions with cytosolic proteins including those which may be previously unknown. These ideas are discussed within the context of the highthroughput flow cytometry measurement of receptor internalization described by Wu *et al* (this issue). The internalization of receptors can be a useful therapeutic modality and the paper by Wu *et al* illustrates how this new assay, targeted to downstream cellular effects, can uncover unique ligand efficacies linked to receptor internalization.

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## Introduction

The two characteristic properties of all drugs are ***affinity*** for a biological target (this gets them to the target) and ***efficacy*** to change the behavior of that target (what they do when they get there). The concept of drug efficacy has changed dramatically over the past 15-20 years and a major reason for this revolution is the increasing availability of multiple receptor and cellular assays that allow observation of receptor behavior through numerous vantage points. The pharmacologists' window into drug efficacy is the assay; the type of assay employed actually defines the efficacy that is observed. With this increase in the number of ways to monitor receptors has come an appreciation of the diverse behaviors these proteins have in their repertoire.

## How Assay Technology Changed Pharmacology

The new views of receptor behavior afforded by new assay technology have introduced two important ideas into pharmacology. The first is that drugs can have many and diverse efficacies; this behavior has been given the term ***pluridimensional efficacy*** (Galandrin and Bouvier, 2006). For example, the β-adrenoceptor ligand propranolol produces inverse agonism for cyclic AMP but positive agonism for ERK1/2 activation (Azzi et al, 2003; Baker et al., 2003). The cannabinoid ligand desacetyllevonantradole is a positive agonist for $G_{i1}$ and $G_{i2}$ but an inverse agonist for $G_{i3}$ (Mukhopadhyay and Howlett, 2005). A diverse range of positive and negative agonism has been found for β-adrenoceptor ligands tested in adenylate cyclase vs mitogen-activated protein kinase assays (Galandrin and Bouvier, 2006).

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The second receptor behavior relevant to drug discovery is the observation that ligands need not produce identical effects on receptors but rather can ***bias stimulus*** through receptors to different signaling pathways in the cell (for review see Kenakin, 2010a). This has clearly been shown for a number of seven transmembrane (7TM) receptor functions, notably G-protein signaling vs. β-arrestin recruitment to the receptor. For example, this has been reported for biased ligands such as [$Trp^{1}$]PTHrp-(1-36) (G protein-biased) vs. PTH-1A ([D-$Trp^{12}$,$Tyr^{34}$]PTH-(7-34) (β-arrestin-biased) for the PTH receptor (Gesty-Palmer et al., 2006), the Substance P analogue SpD ([D-$Arg^{1}$,D-$Phe^{5}$,D-$Trp^{7,9}$,$Leu^{11}$]Substance P ; G protein dependent response) vs. bombesin (G protein independent β-arrestin activity) ( Mackinnon et al., 2001) and SII ($Sar^{1}$, $Ile^{4}$, $Ile^{8}$-AngII) (biased toward producing activation of the β-arrestin pathway vs. angiotensin II- Wei et al., 2003; Ahn et al., 2003).

It is difficult to separate the concepts of ***pluridimensional efficacy*** and ***biased signaling*** since both often involve the stabilization of ligand-specific receptor conformations (*vide infra*). In fact, the discovery of different efficacies for known molecules is directly linked to the testing of those molecules in different assays. This idea leads to the general concept that the discovery of new efficacies and the reduction of the likelihood of missing efficacy through biased signaling may be linked to the capability of the assay to detect signaling sequelae that result from varied receptor conformational active states. The number of unique types of molecules detected in different assays can easily exceed the number of assays used for screening. For example,

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it would be predicted that the testing of molecules in two assays such as a signaling assay and receptor internalization assay can detect up to seven different types of molecular efficacy (Kenakin, 2005). It is not the number of assays *per se* that is relevant but rather the range of detectors of molecular events that matters. From this point of view, cells furnish a complete set of interrogators of receptor conformation in the form of cytosolic signaling proteins (Magalhaes et al., 2012)- see Fig 1. This, in turn, optimizes the probability of detecting new and useful efficacies for molecules.

These ideas suggest that if a specific assay is chosen for detection of ligands, it will see a subset of molecules which happen to have the particular efficacy monitored by the assay or ligands biased towards that particular signaling pathway. The corollary to this idea is that a whole cell assay that monitors the outcome of multiple signaling pathways may have an advantage of detecting a range of efficacies with less influence of bias. This idea has been effectively discussed in terms of using the phosphorylation of P42/44 MAP kinase (ERK1/2) as a means of detecting the receptor activation of a range of G proteins in cells (Osmond et al, 2005). Specifically, a single assay of ERK1/2 activity in COS cells has been shown to detect receptor activation of $G_{\alpha s}$, $G_{\alpha q}$ and $G_{\beta\gamma}$ activation (Osmond et al, 2005). The measurement of cell surface receptors through flurogen activating protein tagged β-adrenoceptors described by Wu et al (this issue) offers another approach to the use of whole cell assays for detection of ligands with diverse and previously unknown efficacies.

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Receptor systems are dynamic in that receptors are synthesized, transported to the cell surface, have a lifetime at the cell surface where they transmit information from the extracellular space to the cytosol and then become internalized within the cytosol either to be degraded or recycled to the surface again. Before dedicated assays to view these processes became available, the desensitization and internalization of receptors was often deduced from the disappearance of agonist response. Under these circumstances, internalization necessarily was tied to the level of agonist response and the two processes tacitly assumed to be directly linked. However, the ability to track receptor internalization as a response separate from physiological pathway stimulation showed that some antagonist ligands produce no observable physiological activation but nevertheless do actively internalize 7TM receptors (Gray and Roth, 2001; Roettger et al, 1997; Willins et al., 1998). For example, the chemokine RANTES(9-68) produces no observable CCR5 receptor activation (as seen by an absence of calcium mobilization or chemotaxis; Gong et al, 1996) yet actively internalizes CCR5 receptors (Amara et al 1997).

There are numerous therapeutic indications for drugs that reduce or eliminate endogenous receptor signaling. Reduction in signaling can be produced by antagonist binding to the endogenous agonist binding site (orthosteric inhibition), or allosteric binding to reduce endogenous agonist binding and/or function. Another approach is to eliminate the receptor from the cell membrane through internalization, i.e. ligand-induced internalization can be a useful therapeutic modality. There are certain cases

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where receptor internalization actually may be a more effective means of antagonism. Blockade of receptor signaling at the cell surface requires an appropriate target occupancy, i.e. if the antagonist diffuses away from the receptor, then endogenous agonism can resume therefore antagonist offset kinetics is a complicating factor in the *in vivo* use of antagonists (Copeland et al, 2006; Vauquelin and Charlton; 2010). In contrast, internalization introduces a kinetic buffer into the removal of signaling in that the resumption of signaling then depends either on the rate of receptor recycling or synthesis. Specifically, it would be predicted that the re-emergence or synthesis of new receptors on the cell surface would be a slower process than the simple dissociation of an antagonist on the cell surface.

Direct antagonist-induced internalization of receptors provides an absolute cessation of extracellular ligand-receptor interaction which can be useful. For example, the ability of 5-HT(2A) receptor antagonists to down-regulate cortical 5-HT(2A) receptors may play a role in the therapeutic activity of some atypical antipsychotic drugs (i.e. clozapine, olanzapine-Yadav et al, 2011). Similarly, antagonist-induced down-regulation of 5-HT2A receptors may also play a role in protection against human polyomavirus (JCV) virus infections (Elphick et al, 2004; O'Connor and Roth, 2005). Another example of therapeutically relevant receptor internalization is found in the treatment of AIDS (Mack et al, 1998). Specifically, HIV-1 utilizes the CCR5 receptor to infect cells and absence of this receptor from the cell surface through a genetic mutation that prevents expression (Δ32 allele) leads to subsequent immunity from HIV-1

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infection (Dean et al, 1996; Huang et al, 1996). This suggests a viable option for prevention of HIV-1 infection, namely the direct internalization of CCR5 as discussed previously for RANTES(9-68).

Highthroughput screens designed to detect allosteric effects can yield molecules that otherwise would not be seen in conventional screening formats (Kenakin 2010b; Burford et al, 2011). The basic difference in these assays is the inclusion of low concentrations of endogenous agonists to determine the possible effects of co-binding synthetic ligands either to block, potentiate or change the quality of the endogenous signal. Cases where receptor internalization may be involved in allosteric function have been seen with antagonists that create bias in the endogenous system. For example,it has been shown that high gene copy numbers for CCL3L1 can lead to delays onset of AIDS in HIV-1 infected patients (Gonzalez et al,2005). Activation by chemokines leads to prevention of HIV-1 cell entry through internalization of receptor thus the implication for the CCL3L1 gene-copy data is that high levels of CCL3L1 protect against HIV progression through CCR5 internalization. In fact, chemokine-induced CCR5 internalization is a strategy utilized with the use of PSC-RANTES as a treatment against HIV-1 infection (Hartley et al, 2004). Therefore, a biased antagonist that blocks HIV-1 interaction with the CCR5 receptor, but allows chemokines, such as CCL3L1, to internalize receptors, theoretically offers a therapeutic advantage over non-biased antagonists. An example of this type of ligand is TAK 652, a biased antagonist that selectively blocks HIV-1 while preserving chemokine function (this antagonist is 11-12

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times more potent at blocking HIV-1 entry over blockade of chemokine-induced CCR5 internalization -Muniz-Medina et al, 2009).

The most simple mechanism to account for biased agonism and antagonism is ligand-induced stabilization of unique receptor conformational active states (Kenakin, 1995). Within this theoretical framework, ligands would stabilize conformations that are predisposed to cellular internalization; a possible mechanism for this could be ligand-directed control of receptor phosphorylation. This has been described as a phosphorylation-mediated 'barcoding' of receptors that is dependent on receptor conformation. The fact that receptors are barcoded through unique intracellular patterns of phosphorylation leads to long-term programing of the receptors behavior (Tobin, 2008; Tobin et al, 2008; Nobles et al, 2011; Butcher et al. 2011). An example of this effect is seen with the μ-opioid receptor agonists DAMGO and etonitazene that stimulate the phosphorylation of both Thr370 and Ser375; in contrast morphine leads to phosphorylation of only Thr370 (Doll et al, 2011). One of the most prominent outcomes of these effects is receptor internalization and subsequent cytosolic reactions (i.e. degradation or recycling to the cell surface).

In general, the more an assay depends on cell complexity the more it will differentiate subtle ligand effects. Downstream assays such as cell sorting are an effective way of detecting the overall outcome of ligand-directed conformational stabilization, i.e. texture in efficacy. An assay such as that reported by Wu *et al* is a way to observe the end-organ response of these interactions without the need to define the

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mechanism. The importance of these types of discovered textures in efficacy is underscored by the reports of previously unknown cytosolic protein interactants with 7TM receptors that may lead to alteration of receptor function and disposition (Magalhaes et al, 2012). For example, desipramine binds to $\alpha_2$-adrenoceptors but produces no overt $\alpha_2$-adrenoceptor response. However, while conventional signaling assays show no observable effect of despiramine, recently it has been shown that this ligand causes β-arrestin-3 recruitment to the receptor (with no $\alpha_2$-adrenoceptor signaling) and that this effect is linked to internalization $\alpha_2$-adrenoceptors. This efficacy may be associated with a useful clinical phenotypic profile in depressive disorders (Cottingham et al, 2011). As new assays become available it would seem reasonable to subject known drugs to new interrogations to possibly link interesting *in vivo* drug phenotypes with measurable in vitro activities (O'Connor and Roth, 2005).

Consideration of the multiple conformational nature of receptor systems through molecular dynamics (Fraunfelder et al, 1988; 1991; Woodward, 1993; Hilser and Freire, 1997; Hilser et al, 2006; Kenakin, 2002) predicts that signaling bias and texture in efficacy should be a common, not rare, effect. Specifically, uniformity in efficacy would require that ligands have identical affinities for numerous receptor conformations; this would be an unlikely thermodynamic prediction. Therefore, some kind of ligand bias would be predicted for most if not all synthetic ligands. The detection of such bias is directly related to the assays used to observe drug effect, a situation analogous to the discovery and progression of ideas around inverse agonism. When first reported by

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Costa and Herz (1989), inverse agonism was thought to be rare. The 'rarity' of inverse agonism appears to have been related to the lack of availability of constitutive receptor assay, since in the years subsequent to the discovery, the reports of increasingly common inverse agonism have paralleled the availability of constitutive receptor assays (Kenakin, 2004). It will be interesting to see if biased agonism and antagonism follows the same progression, i.e. as more experience is gained with highthroughput assays capable of detecting complex behaviors will bias signaling in ligands be seen to be a more common phenomenon?

Authorship Contribution

Wrote or contributed to the writing of the manuscript: Terry Kenakin

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Fig 1. Receptor signaling systems. Assays can be centered on direct coupling of the receptor to various G proteins or β-arrestin to reveal selected efficacies or to whole cell response to detect varied recepor behaviors in response to ligand binding.

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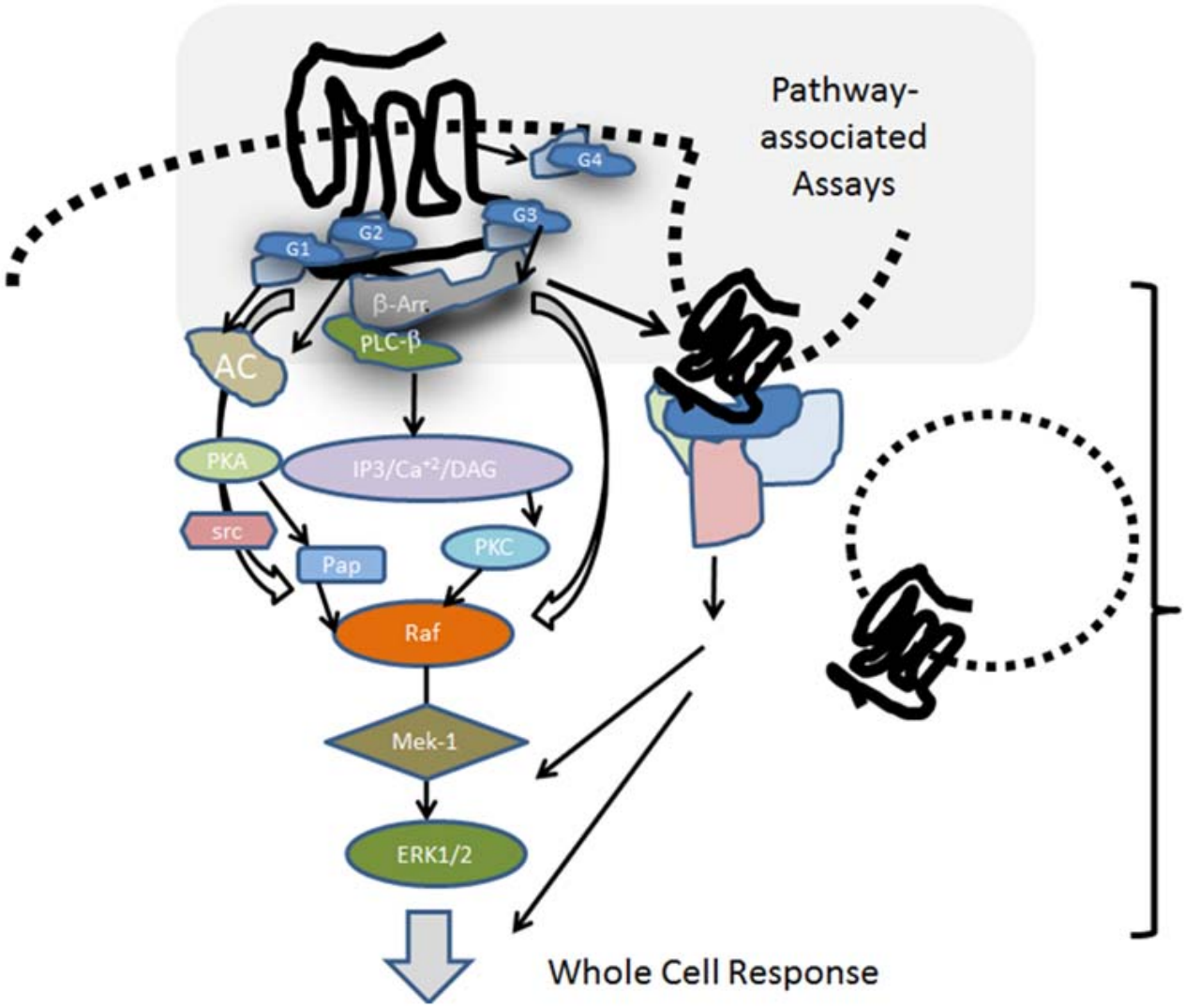


Figure 1