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Original article

Troubleshooting and deconvoluting label-free cell phenotypic assays in drug discovery

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ABSTRACT

Introduction: Central to drug discovery and development is to comprehend the target(s), potency, efficacy and safety of drug molecules using pharmacological assays. Owing to their ability to provide a holistic view of drug actions in native cells, label-free biosensor-enabled cell phenotypic assays have been emerging as new generation phenotypic assays for drug discovery. Despite the benefits associated with wide pathway coverage, high sensitivity, high information content, non-invasiveness and real-time kinetics, label-free cell phenotypic assays are often viewed to be a blackbox in the era of target-centric drug discovery. **Methods:** This article first reviews the biochemical and biological complexity of drug-target interactions, and then discusses the key characteristics of label-free cell phenotypic assays and presents a five-step strategy to troubleshooting and deconvoluting the label-free cell phenotypic profiles of drugs. **Results:** Drug-target interactions are intrinsically complicated. Label-free cell phenotypic signatures of drugs mirror the innate complexity of drug-target interactions, and can be effectively deconvoluted using the five-step strategy. **Discussion:** The past decades have witnessed dramatic expansion of pharmacological assays ranging from molecular to phenotypic assays, which is coincident with the realization of the innate complexity of drug-target interactions. The clinical features of a drug are defined by how it operates at the system level and by its distinct polypharmacology, ontarget, phenotypic and network pharmacology. Approaches to examine the biochemical, cellular and molecular mechanisms of action of drugs are essential to increase the efficiency of drug discovery and development. Label-free cell phenotypic assays and the troubleshooting and deconvoluting approach presented here may hold great promise in drug discovery and development.

1. Introduction

Drug discovery strategies have undergone dramatic transformation in the past decades, at least partly enabled by advances in pharmacological assays. Historically, drug discovery has been heavily weighted toward low-throughput phenotypic assays using isolated animal tissues (Kenakin, 2009; Trzeciakowski, 1987). Drug pharmacology is inferred with a phenotypic response of these tissues, such as contractions of the guinea pig ileum, whose physiological origin may not necessarily be well-understood. Over the past two decades advances in molecular genetics, detection technique and automation have made molecular assays dominating the target-directed drug discovery (TDDD) (Swinney & Anthony, 2011). Nowadays, screening a large library of compounds is a common practice for fishing out lead molecules based on a specific molecular mechanism predetermined by the molecular assay used (Inglese et al., 2007). This strategy has benefits associated with operational and biological simplicity, but suffers fundamental weaknesses in that the specific molecular mechanism is often not directly related to the pathophysiology of diseases. A recent analysis of new molecular entities approved by the FDA between 1999 and 2008 indicated that for the first in class molecules, 37% resulted from projects that used phenotypic screening whereas target based screening identified 23% (Swinney & Anthony, 2011). In light of the high drug attrition rate of current TDDD (Booth & Zemmel, 2004; Munos, 2009; Paul et al., 2010) and the increasing focus on unexploited biological mechanisms for diseases that involve many genes (Pammolli, Magazzini, & Riccaboni, 2011), there are renewed interests in modern phenotypic assays for improving and expediting the drug discovery process (Lee, Uhlik, Moxham, Tomandl, & Sall, 2012).

In recent years, cell phenotypic assays enabled by label-free biosensors have been emerging as novel pharmacologic assays for cell biology and drug discovery (Fang, 2006, 2010a, 2011a; Rocheville & Jerman, 2009; Scott & Peters, 2010). Label-free cell phenotypic assays non-invasively track in real time the cell decision making process, leading to a whole cell phenotypic response (Fang, Ferrie, Fontaine, Mauro, & Balakrishnan, 2006; Fang, Ferrie, Fontaine, & Yuen, 2005; Fang, Li, & Peng, 2005). Furthermore, these assays have wide pathway coverage, high sensitivity and do not require cell engineering, so it is possible to provide a holistic view of drug-target interactions and their functional consequences in native cells, as well as allows to approach the

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development and implementation of different assays in a systematic and generic way (Fang, 2011a; Kenakin, 2010a; Scott & Peters, 2010). However, since label-free cell phenotypic assays mirror the innate complexity of drug pharmacology, these assays are often viewed to be difficult to be adopted in the drug discovery process. Given that the protocols about how to deal with technical pitfalls of label-free cell phenotypic assays have been reviewed (Fang, Ferrie, & Tran, 2009; Schröder et al., 2011), I here first review key aspects of drug-target interactions that affect the label-free profiles of drugs in living cells, and then present troubleshooting and deconvoluting approaches about how to relate label-free cell phenotypic signatures to specific biochemical, cellular and molecular mechanisms of action (MOAs) of drugs.

2. Complexity of drug-target interactions

Drug-target interactions are intrinsically complex (Fig. 1). Over the past two decades, the concept of designing highly selective ligands to avoid unwanted side effects has become predominant paradigm in drug discovery. However, a growing body of post-genomic biological data has been revealing a far more complex picture of drug action. The binding characteristics, in particular the kinetics, of a drug can influence its clinical features (Copeland, Pompliano, & Meek, 2006) as well as its label-free profiles in cells (Fang, 2012). A drug may selectively modulate some of the pleiotropic signaling pathways of a specific target, leading to distinct *ontarget pharmacology* (see Glossary) (Kenakin & Miller, 2010; Kinzer-Ursem & Linderman, 2007). The pathway-selective ontarget pharmacology is termed *ligand-directed functional selectivity* or biased agonism for G protein-coupled receptor (GPCR) ligands (Urban et al., 2007). Owing to distinct expression and organization patterns of targets and their associated signaling components, different cells may respond to a drug with distinct profiles, a phenomena termed *phenotypic pharmacology* (Nelson, 2008; Nelson & Challiss, 2007). A drug may also bind specifically to multiple targets, thus having *polypharmacology* (Davis et al., 2011; Knight, Lin, & Shokat, 2010). A drug may work individually, or together with another drug, to modulate multiple targets within a specific signaling network that is essential to their clinical efficacy, thus resulting in specific *network pharmacology* (Hopkins, 2008; Metz & Hajduk, 2010). Furthermore, the connectivity network often surmounts the action of the drug by any feedback mechanisms of regulation (Barabási & Oltvai, 2004). Metabolism and circulation may also impact the clinical characteristics of drugs (Gleeson, Hersey, Montanari, & Overington, 2011).

The ever improving resolution of pharmacological assays will certainly continue revealing biochemical, cellular and molecular details of drug-target interactions and their functional consequences. This is illustrated by aspirin. Salicylate, the active metabolite of aspirin and the active constituent of the extracts of willow bark, meadow sweet and wintergreen, has been long used for alleviating rheumatic pain, the discomfort associated with childbirth and eye inflammation without knowing its MOA (Yeomans, 2011). Aspirin, a drug originated from salicylic acid, has been used as an analgesic, anti-thrombotic, antipyretic, anti-inflammatory, anti-edema and cancer preventive agent. Aspirin also causes acute effects on gastric mucosa, ulcer complications and intestinal damage (Musumba, Pritchard, & Pirmohamed, 2009). The *molecular mode of action* (MMOA) of aspirin is still under debate today (Fuster & Sweeny, 2011). Beside its primary MMOA related to the selective acetylation of cyclooxygenase-1 (COX-1) (Vane, 1971), aspirin also causes the acetylation of several other cellular proteins including the tumor suppressor p53 (Marimuthu et al., 2011). Salicylate inhibits the NF-κB pathway (Kopp & Ghosh, 1994) and directly activates AMP-activated protein kinase (Hawley et al., 2012), while genstiate and 2,3,5-trihydroxybenzoic acid, the two aspirin catabolites, activate GPR35 (Deng & Fang, 2012a).

2.1. Biochemical mechanism of action of drug-target interactions

Biochemical mechanism of action describes the kinetics-related mechanism of action of drugs (Swinney, 2004). Particularly, *drug residence time* (reciprocal of k_{off}) has been proposed to be important for the clinical features of drugs (Copland, 2010). Drug-target interaction is hallmarked by the binding of a drug to its target with specific affinity, kinetics, pathway, conformation and thermodynamics (Fang, 2012; Nunez, Venhorst, & Kruse, 2012), all of which can influence the label-free cellular profiles of drugs obtained. Instead of binding affinity, most cell-based assays measure the potency and efficacy of drugs. The potency describes the amount of a drug required to produce an effect of given intensity, and is proportional to binding

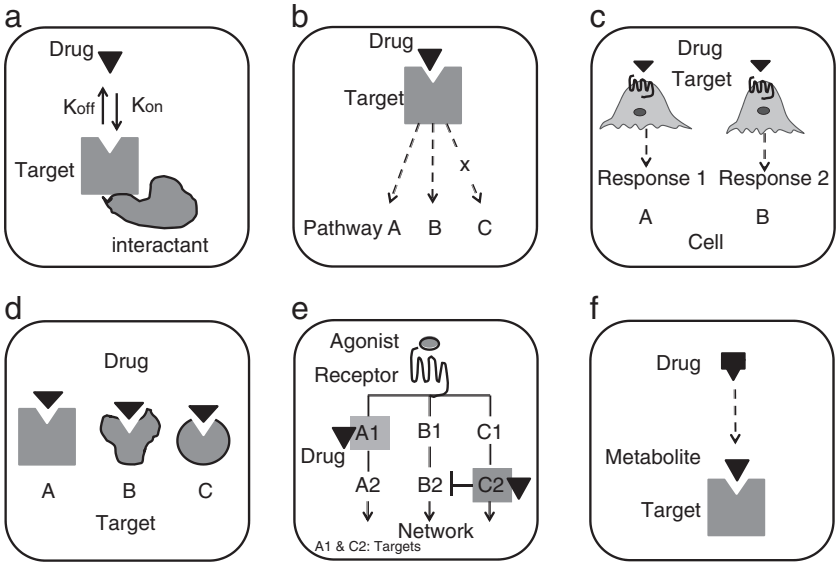

Fig. 1. The aspects of a drug-target interaction that influence label-free profiles. (a) The influence of the target-interacting protein (interactant) on the binding kinetics of a drug-target interaction. (b) Pathway-biased activity. (c) Cellular context dependent phenotypic pharmacology. (d) Polypharmacology. (e) Network pharmacology in which inhibiting multitargets (e.g., A1 and C2) in a network by a drug determines the activity of the drug. (f) Activity of drug metabolism-induced active metabolites.

affinity and efficacy. The efficacy is the ability of a drug to change the behavior of the target at the molecular, cellular, tissue or system level as a function of receptor occupancy.

As a traditional surrogate of *in vivo* efficacy, drug's binding affinity is often obtained in a closed system, in which both target and drug concentrations are constant over time (Fang, 2012). However, drug-target interactions *in vivo* are often far distant from equilibrium conditions due to dynamic flow and drug metabolism in the human body (Gleeson et al., 2011). Both rate constants of association (k_{on}) and dissociation (k_{off}) of a drug can influence the *in vivo* behaviors of drugs (Barnes, 2000; Copland, 2010; Disse, Speck, Rominger, Witek, & Hammer, 1999; Keighley, 2011) as well as the *in vitro* cellular profiles of drugs depending on how the drugs are tested (Ambrosio & Lohse, 2012; Charlton & Vauquelin, 2010). This could be more pronounced in real time kinetic profiling studies using label-free whole cell assays than the conventional endpoint molecular assays (Deng, Hu, & Fang, 2012; Deng, Wang, Su, & Fang, 2012; Goral, Jin, et al., 2011).

Different ligands may have distinct binding pathways to a specific receptor. Using unbiased molecular dynamics simulation Dror et al. (2011) found that drug molecules spontaneously associate with β_1- and β_2-adrenergic receptors to achieve final poses matching those determined crystallographically. Several beta blockers and a beta agonist all traverse the same well-defined, dominant pathway, but with different kinetics, as they bind to the receptors. For β_2 adrenergic receptor (β_2-AR) agonists, biophysical studies found that there are two distinct kinetic components, a slow and fast phase, when monitoring agonist-promoted changes in fluorescence of tetramethylrhodamine-labeled purified β_2-AR (Swaminath et al., 2005, 2004). The different conformational states of β_2-AR can affect function by altering the speed of G protein activation (Nikolaev, Hoffmann, Bunemann, Lohse, & Vilardaga, 2006). Furthermore, the cellular context can also influence the binding characteristics of drug-target interactions by presenting cellular context-specific conformations of the target via complexing with its interacting partners (Kenakin, 2010b; Nelson & Challiss, 2007). For instance, carbachol was shown to have a wide range of affinities binding to a single receptor subunit subject to different conformational constraints in different regions of rat brain (Birdsall, Hulme, & Burgen, 1980). In addition, a receptor may present more than one binding pocket for drug binding, leading to different types of ligands for the same receptor such as allosteric modulators and orthosteric binders. The pathway of distinct classes of ligands binding to the same receptor may be different (Conn, Christopoulus, & Lindsley, 2009). Thus, it is possible that distinct ligands targeting the same receptor could result in label-free cellular profiles with different kinetics in living cells.

2.2. Ligand-directed functional selectivity

Functional selectivity describes pathway-selective activity of ligands for a specific receptor. The concept of functional selectivity is well-known for GPCR ligands (Kenakin & Miller, 2010; Urban et al., 2007), but less characterized for ligands acting at other receptor classes (Choi et al., 2011). The pathway-selective ontarget pharmacology is rooted in the ability of a receptor to mediate pleiotropic signaling, and originated from the operational bias of the drug to select and stabilize a specific active conformation from dynamic conformational ensembles of the receptor (Boehr, Nussinov, & Wright, 2009; Park, 2012; Rosenbaum, Rasmussen, & Kobilka, 2009; Struts, Salgado, Martínez-Mayorga, & Brown, 2011).

Receptor pleotropic signaling is best illustrated by epidermal growth factor receptor (EGFR), one of the most well studied receptors (Sako, Minoghchi, & Yanagida, 2000; Wiley, Shvartsman, & Lauffenburger, 2003). EGFR activation is a complex process involving receptor-ligand binding, receptor dimerization, phosphorylation, trafficking, and cell signaling. Recently, Oda, Matsuoka, Funahashi, and Kitano (2005) had constructed a comprehensive map of molecular interactions for EGFR signaling which consists of a total of 219 reactions and 322 molecular species including 202 proteins. The map covers many known aspects of the pleotropic signaling of EGFR including receptor endocytosis, degradation and recycling, small GTPase signaling, MAPK cascade, PIP signaling, cell cycle, Ca^{2+} signaling and GPCR-mediated EGFR transactivation. GPCRs, a family of important cellular signal transduction proteins, are also well-known for their ability to mediate pleiotropic signaling through both G protein-dependent and independent mechanisms. The promiscuity of G proteins makes it possible for a single GPCR to couple to and signal via multiple G proteins (Hermans, 2003). Furthermore, a single GPCR can also mediate G protein-independent signaling such as β-arrestin-mediated signaling (DeWire, Ahn, Lefkowitz, & Shenoy, 2007; Rajagopal, Rajagopal, & Lefkowitz, 2010).

Dynamic conformational ensembles are increasingly recognized to be common for receptors (Boehr, Nussinov, & Wright, 2009; Kobilka & Deupi, 2007). GPCRs are traditionally viewed to be an "on–off" switch; that is, agonist binding switches the receptor from an inactive state to an active state (Kenakin, 2003, 2008; Neubig, Spedding, Kenakin, & Christopoulos, 2003). However, numerous biochemical, biophysical and modeling studies have shown that GPCRs can adopt multiple active conformational states with distinct functional effects (Deupi & Kobilka, 2010; Dror et al., 2011; Hubbell, Altenbach, Hubbell, & Khorana, 2003; Kahsai et al., 2011; Liu et al., 2012; Saulière et al., 2012). As a family of shapeshifting proteins GPCRs can exist as oligomers and form complexes with other GPCRs (Gurevich & Gurevich, 2008), interact with other signaling molecules such as G proteins and β-arrestin (Bockaert, Fagni, Dumuis, & Marin, 2004), and exist in specific membrane microdomains (Allen, Halverson-Tamboli, Rasenick, 2007), all of which can allosterically modulate the conformations of the receptor. It is interesting to note that the sampling of active state crystal structures for GPCRs is currently small (Onaran & Costa, 2012).

Many pathway profiling techniques have manifested that functional selectivity is common to many, if not all, GPCR ligands (Kenakin & Miller, 2010). Stereoisomers of the same ligand could promote functional selectivity (Seifert & Dove, 2009; Woo et al., 2009). Beside orthosteric ligands, drugs acting through allosteric binding sites could also promote biased activity (Leach, Sexton, & Christopoulos, 2007). For instance, neurokinin can trigger G_s and G_q signaling via NK1 receptor. The co-binding with neurokinin of an allosteric modulator LP1805 to the receptor led to enhanced G_q response, but antagonized the G_s activation (Maillet et al., 2007). Furthermore, functional selectivity is not limited to ligands with agonist activity. Biased activity of antagonists such as direct antagonist-induced internalization of receptors has been observed. The two 5-HT_{2A} antagonists, clozapine and olanzapine, were found to promote down-regulation of the 5-HT_{2A} receptor (Yadav, Kroeze, Farrell, & Roth, 2011). CCR5 receptor antagonist TAK652 was recently developed to be a biased antagonist that selectively blocks HIV-1 interaction with the CCR5 receptor, but still allows the chemokine CCL3L1 to internalize the receptor, thus having therapeutic potential for the treatment of HIV-1 infection (Muniz-Medina et al., 2009). CCR5 is a co-receptor for HIV entry, and high levels of CCL3L1 are protective against HIV progression through CCR5 internalization (Gonzalez et al., 2005). A small molecule antagonist, Gue1654, was recently found to selectively inhibit $G_{\beta\gamma}$ but not G_α signaling triggered upon activation of $G_{\alpha i\text{-}\beta\gamma}$ by the chemoattractant receptor 5-oxo-6E,8Z,11Z,14Z-eicostetraenoic acid receptor (Blattermann et al., 2012).

Functional selectivity can open up new avenues for the development of targeted and specific drugs (Kenakin, 2012; Mailman, 2007). Clinical data have shown that some but not all signaling produced by agonist drugs is beneficial for the treatment of diseases. For instance, as effective analgesics opioid drugs such as morphine can also produce side effects such as respiratory depression, possibly through the activation of β-arrestin 2 (Raehal, Walker, & Bohn, 2005). As effective anti-hyperlipidemia drug niacin is also known to induce flushing, a mechanism attributed to the activation of β-arrestin via GPR109 receptor (Walters et al., 2009). Screening and identifying pathway-selective

ligands has become a very active area in GPCR drug development, in particular, biased drugs that selectively inhibit disease causing GPCR mutations (Mendes, van der Spuy, Chapple, & Cheetham, 2005; Smit et al., 2007) or some active conformational states that are more prevalent in pathological conditions compared to those present under normal conditions (Park, 2012).

The functional selectivity is traditionally inferred with the differential pharmacological properties of a ligand obtained using assays measuring different biochemical pathways (Galandrin, Oligny-Longpre, & Bouvier, 2007). The pituitary adenylyl cyclase-activating polypeptide (PACAP) type-1 receptor is known to signal through both G_s and $G_{q/11}$ proteins (Wettschureck & Offermanns, 2005). A reversed order in relative potencies of the agonists PACAP-27 and PACAP-38 were observed when their potency to cause cyclic AMP production were compared with those to trigger inositol phosphate production, suggesting that the two agonists promote distinct conformational states, each having its own preferential coupling with and signaling through G_s vs $G_{q/11}$ proteins.

Since most molecular assays used to quantify the functional selectivity are performed using genetically manipulated cell lines, biased agonism was once thought to be an artifact of recombinant technology. Label-free cell phenotypic profiling using native cells further showed that biased agonism is a natural cellular control mechanism (Fang, 2011a; McLaughlin et al., 2005). For endogenous β_2-AR in A431 cells, label-free profiling has identified multiple distinct types of dynamic mass redistribution (DMR) signals upon stimulation with different agonists (Fang & Ferrie, 2008; Ferrie, Sun, & Fang, 2011; Goral, Jin, et al., 2011). Since ligands could have different efficacies and promote biased signaling, drug pharmacology often depends on the readout of the assay used. Since cells provide a complete set of cytosolic signaling proteins to interrogate receptor conformations, label-free whole-cell assays that monitor in real time the outcome of multiple signaling pathways are considered to be advantageous in detecting a range of efficacies and biased activity of drugs (Fang, 2010a, 2011a; Kenakin, 2009).

2.3. Cellular context-dependent phenotypic pharmacology

Cells fulfill tissue specific functions via dynamic coordination of their activities with environmental changes. One way to achieve tissue specificity is to express specific set of receptors and their interacting proteins (Lattin et al., 2007; Salazar, Chen, & Rockman, 2007), while another is to tightly regulate the temporal and spatial gradients of signaling molecules and networks in cells (Kholodenko, 2006). Each type of cells may have unique cellular context tailored to their needs. The cellular context can influence, sometime determine, the functional responses of receptors as well as drug actions, leading to cellular context-dependent phenotypic pharmacology. The phenotypic pharmacology is believed to be related to the intrinsic property of the tissue, which creates a "system bias" for all agonists (Kenakin, 2012). However, it could be difficult to separate the system biased phenotypic pharmacology from ligand-directed functional selectivity. For instance, melanocortin-receptor (MC4R) can preferentially couple to different G proteins in different cellular backgrounds. In HEK293 cells expressing the MC4 receptor, the receptor prefers G_s coupling, while in CHO-K1 cells expressing the receptor it favors G_q coupling (Peters & Scott, 2009). In the two cell backgrounds different MC4R agonists may lead to distinct phenotypic pharmacology, which is interconnected with ligand-directed functional selectivity. This also indicates that it is highly possible to manifest ligand biased activity by controlling the cellular context using whole-cell label-free assays. For instance, the cholera toxin-treated cells can be used to differentiate G_s-coupled receptor such as β_2-AR agonists that primarily signal through cAMP pathway from agonists that do not signal through cAMP pathway (Ferrie, Sun, & Fang, 2011).

The functional responses of drug molecules can be sensitive to many cellular factors, including receptor expression level, receptor conformations, and heterogeneity in expression and organization of cytosolic interactants (Kinzer-Ursem & Linderman, 2007; Watson et al., 2000). The extracellular matrix (ECM) onto which cells are harbored is also part of environmental cues for regulating cellular functions (Giancotti & Ruoslahti, 1999). The mechanical properties, compositions and organization of the ECM can influence receptor signaling, for example, through governing the differentiation states and lineages of stem cells (Engler, Sen, Sweeney, & Discher, 2006), or through directing the adhesion complexes and the actin-myosin cytoskeleton of cells (Discher, Janmey, & Wang, 2005). Profiling of endogenous P2Y receptors in HEK293 cultured on distinct ECM-coated surfaces has revealed that interactions with the ECM coatings can shape the signaling of endogenous P2Y receptors (Tran, Sun, & Fang, 2012). Compared to those on tissue culture treated surfaces, fibronectin coating increased the potency of all P2Y agonists examined, while gelatin had little impact.

2.4. Polypharmacology

Polypharmacology describes the specific binding of one drug to multiple targets, often including targets other than the intended therapeutic targets (Hopkins, 2008; Hopkins, Mason, & Overington, 2006; Wermuth, 2004). Once thought to be an undesirable property under the influence of the philosophy of 'one drug for one target for one disease', polypharmacology has been increasingly recognized to be important for the *in vivo* efficacy and toxicity profiles of many drugs in recent years (Frantz, 2005; Pang, Kim, Jung, Cho, & Lee, 2012). Many literature and data mining studies based on reported affinity values of drugs binding to various targets have led to the realization that most, if not all, drugs show clinically relevant polypharmacology (Chen, Wild, & Guha, 2009; Hopkins, 2008; Hu & Bajorath, 2010; Lu, Pan, Hu, & Wang, 2012; Overington, Al-Lazikani, & Hopkins, 2006; Paolini, Shapland, van Hoorn, Mason, & Hopkins, 2006; Plake & Schroeder, 2011; Wishart et al., 2006; Yildirim, Goh, Cusick, Barabasi, & Vidal, 2007; Zhu et al., 2009).

Analyses of drug-target networks have led to two interesting findings. First, most drugs display polypharmacology, and the promiscuity of many drugs is important for their clinical success. A recent analysis showed that the drug-target network consists of many clusters of connected drug targets, and it has a giant component consisting of mostly receptors and transporters that are interconnected with the known drug-target interactions among about 50% of all drugs from the top 10 indication classes (Rask-Andersen, Almén, & Schiöth, 2011). The drugs in the giant component have higher polypharmacology (~3 targets per drug) than drugs outside the component (~1.5 targets per drug). In addition, the main innovations within the giant drug cluster occurred before the 'one drug, one target' focus of recent times, pointing to the importance of polypharmacology for the discovery of new drugs. Second, closely related members of the gene family that has generally similar function also show similar drug promiscuity. Antipsychotic drugs commonly affect the functioning of entire families of serotonin and dopamine receptors, and promiscuous antipsychotic drugs such as clozapine are known to be more effective to treat depression than targeted ones (Grunder, Hippius, & Carlsson, 2009; Pratt, Winchester, Dawson, & Morris, 2012; Roth, Sheffler, & Kroeze, 2004). It is worth noting that these drug-target network analyses are mostly limited to validated drug targets, thus underestimating the promiscuity of drugs.

Profiling of kinase inhibitor drugs at the human *kinome* level has pointed to genuine multitarget effects of these drugs (Anastassiadis, Deacon, Devarajan, Ma, & Peterson, 2011; Collins & Workman, 2006; Davis et al., 2011; Keith, Borisy, & Stockwell, 2005). For instance, imatinib was originally developed as a highly selective inhibitor of BCR-ABL kinase for treating chronic myeloid leukemia. It was later found that imatinib also has significant activity against several other clinically relevant kinases such as c-KIT and platelet-derived growth factor receptor (PDGFR), leading to expansion of its clinical utility for the treatment of other diseases such as myelodysplastic/myeloproliferative diseases associated with PDGFR gene re-arrangements, gastrointestinal stromal

tumor associated with c-KIT activity, and hypereosinophilic syndrome and/or chronic eosinophilic leukemia with the FIP1L1-PDGFRα fusion kinase (Scheinfeld, 2006). Sorafenib, the multikinase inhibitor drug, is known to inhibit RAF-kinase, vascular endothelial growth factor 2 (VEGFR2), VEGFR3, PDGFR, c-KIT and FLT3, thus affecting both tumor proliferation and tumor angiogenesis pathways (Wilhelm et al., 2008). Kinase inhibitors may also have polypharmacology activity beyond kinases. For instance, several tyrphostin analogs, the first generation of tyrosine kinase inhibitors, were found to be agonists for GPR35 (Deng, Hu, & Fang, 2011); sorafenib was also found to exhibit promiscuity by binding to 5HT2A, 5HT2B and 5HT2C with high to moderate binding affinity (Lin et al., 2012).

Identifying hidden MOAs of drugs is important to comprehend their clinical features as well as for drug repurposing. However, screening for multitarget compounds is a difficult task, partly due to varied affinities and activities of candidate compounds for distinct targets. Similarity analysis based on chemical, cellular, side-effect, and phenotypic features is one approach that has shown some successes for predicting novel drug-target associations. Analysis of the chemical similarity of drugs with probe molecules having known affinity to a specific target can predict previously unknown targets for some drugs, as well as adverse drug reactions (Keiser et al., 2007; Lounkine et al., 2012). Some of predictions have been validated experimentally, such as methadone for antagonizing muscarinic M3 receptor, emetine for antagonizing α2-adrenergic receptor, and loperamide for antagonizing neurokinin NK2 receptor (Keiser et al., 2007). Similarity analysis of known side-effects of drugs has also discovered novel drug-target associations (Campillos, Kuhn, Gavin, Jensen, & Bork, 2008). For instance, the proton pump inhibitor drug rabeprazole was found to share similar targets with the nervous system drugs such as pergolide, paroxetine, fluoxetine and zolmitriptan, all of which inhibit the dopamine receptor DRD3 and bind to the serotonin receptor HTR1D. Chemogenomics and large-scale ligand profiling have also provided evidence that many drugs act on multiple targets (Allen & Roth, 2011; Fliri, Loging, Thadeio, & Volkmann, 2005; Klabunde, 2007; Lamb et al., 2006; Zhao & Iyengar, 2012; Zhao & Li, 2010).

Polypharmacoly, known or unknown, can complicate label-free whole-cell profiling and analysis. The label-free whole-cell profiles of promiscuous drugs result from complex interactions of drug actions through multiple targets within the cells studied. This could be even more complicated, when considering the extent of redundancy in cells as showed by the limited clinical effect of altering the activity of many genes one at a time (Zambrowicz & Sands, 2003).

2.5. Network pharmacology

Network pharmacology describes the action of drugs in a disease module or a biological network. A disease module represents a group of *nodes* whose perturbation (mutations, deletions, copy number variations, or expression changes) can be linked to a particular disease phenotype (Barabási, Gulbahce, & Loscalzo, 2011). Over the past decades increasing evidence suggests that drug efficacy and toxicity should be understood by its action in biological networks (Pujol, Mosca, Farrés, & Aloy, 2010). In human cells, most cellular components are functionally interdependent via various interactions including molecular binding, biochemical reactions, expression regulation, and signaling protein assembly, all to which contribute to creation of the *interactome* (Russell & Aloy, 2008).

Analyses of biological networks have led several interesting observations. First, biological networks are well organized and consist of many nodes (e.g., proteins, metabolites, diseases) connected by links or *edges* (e.g., protein-protein interactions, metabolic reactions, or genes). The most noticeable is the emergence of a set of highly connected *hub proteins* that hold the whole network together, and the hub proteins tend to be encoded by essential genes (Jeong, Mason, Barabási, & Oltvai, 2001). The deletion of genes encoding the hubs leads to greater phenotypic outcomes than the deletion of genes encoding less connected proteins (Yu et al., 2008). Second, disease-causing genes occupy only about 10% of human genes (Amberger, Bocchini, Scott, & Hamosh, 2009). It is estimated that of the approximately 25,000 genes, only about 1700 have been associated with specific diseases, about 400 of which are essential disease genes. Non-essential disease genes tend to segregate at the network periphery whereas essential genes are associated with the functional center of the interactome (Barabási, Gulbahce, & Loscalzo, 2011). Proteins involved in the same disease have an increased tendency to interact with each other (Goh et al., 2007). Third, owing to the presence of compensatory signaling routes that bypass the inhibition of individual proteins, biological networks display robust phenotypes which are remarkably resilient to perturbation via inhibition or even deletion of single targets (Barabási & Oltvai, 2004; Hellerstein, 2008).

Both polypharmacology and network pharmacology have been proposed to offer new means to address the issue of high attrition rates arising from toxicity and lack of efficacy, and have emerged as new paradigm in drug discovery, in particular, for complex diseases such as cancers and cardiovascular diseases (Bell, 2010; Frantz, 2005; Pang et al., 2012). Network biology theory predicts that compared to exquisitely selective compounds for a single target, a promiscuous drug or a drug combination that simultaneously modulates multiple proteins in one process or parallel processes can be more efficacious for the treatment of complex disease (Boran & Iyengar, 2010; Kamb, Wee, & Lengauer, 2007; Keith et al., 2005; Schadt, Friend, & Shaywitz, 2009). Recently, Dar, Das, Shokat, and Cagan (2012) used a *Drosophila* model of multiple endocrine neoplasia type 2 (MEN2), a disease caused by activating mutations in RET kinase, to screen a library of small molecules that inhibit RET and other kinases. They found that inhibiting Raf and Src increased efficacy, whereas inhibiting mTOR led to toxicity. Based on this finding, they rationally designed an inhibitor targeting Src, Raf and S5K kinases, all within the network of RET signaling. Results showed that this inhibitor can rescue about 80% of the MEN2 flies, an effectiveness in a MEN2 mouse model better than vandetanib, a kinase inhibitor approved to treat thyroid cancer resulting from RET mutations that cause MEN2.

Network biology has significant implications in how to relate label-free cellular phenotypic signatures to the MOA of drugs. Label-free receptor assays probe drug actions with wide pathway coverage. Owing to the often unknown promiscuity of drugs and probe molecules, the label-free whole cell profiles may not be explainable by specific target-binding data. Different cellular backgrounds often present very different biological networks, thus making it difficult to directly compare label-free profiles of a drug-receptor interaction from one to another cell. Pathway deconvolution using probe molecules has been used together with label-free biosensors to understand drug actions in living cells (Fang, 2010b; Fang, Ferrie, Fontaine, et al., 2005; Fang, Li, et al., 2005; Ferrie, Sun, & Fang, 2011; Morse, Tran, Levension, & Fang, 2011; Schröder et al., 2010). The probe molecules such as G protein toxins and pathway modulators are often used to alter the activity of a specific target in the cells, and their effects on the drug-induced biosensor signature are used to determine the cellular mechanisms of drug action. However, the same probe molecule may not result in the same effect in different types of cells, since different cells often have different expression patterns of signaling proteins, and signaling protein(s) neighboring to the probe targeted proteins may compensate for the loss of function of the probe inhibited protein(s) in a tissue-specific manner. Furthermore, the probe molecules could alter the activity of other network neighbors, thus complicating the data interpretation.

3. Label-free cell phenotypic assays

Pharmacological assays, the essential component in drug discovery and development, have undergone dramatic improvements and expansion in the past decades. Historically, phenotypic screening using

whole animal organisms has been quite successful in discovery of new medicines, even in the era of TDDD (Swinney & Anthony, 2011). Measuring a phenotype of animal tissues such as contraction of guinea pig muscles or the beating of guinea pig right atrium tissues was essential to early drug discovery. Whole animals such as mice have gained popularity over time as the model system to investigate drugs. These model systems take advantages of the fully assembled biological systems for interrogating test drugs (Pratt et al., 2012; Zambrowicz & Sands, 2003). However, these phenotypic assays are hardly applicable to large-scale compound screening and medicinal chemistry optimization due to biological complexity and operational costs. Often, serendipity is part of organism-based drug discovery, in which serendipitous phenotype was observed in animals after exposure to naturally occurring small molecules (MacRae & Peterson, 2003).

Over the past three decades, molecular assays have become dominating in target-centric drug discovery process, partly owing to their operational and biological simplicity and party due to the desire to search highly selective drugs for a specific target. These assays measure specific signaling molecules to infer the actions of drugs. The operational simplicity makes these assays amenable to high throughput screening, while the biological simplicity makes them possible to guide medicinal chemistry optimization. However, since drugs are optimized towards a specific MMOA using artificial systems, the activity of drugs obtained hardly translates into *in vivo* profiles (Swinney & Anthony, 2011).

In light of the recent trend of increasing attrition rate in clinical trials (Scannell, 2012), the potential of most drugs to have polypharmacology and the complexity of disease networks, there are renewed interests in phenotypic assays for drug testing. Newer generation of phenotypic assays employ operationally less demanding model systems such as fruit fly, and zebrafish (Zon & Peterson, 2005). These assays directly search for compounds that cause desirable physiological or cellular phenotypes in animal models of a disease. The detected change in phenotype might be linked to their therapeutic impacts for a disease state. However, owing to poor mechanistic understanding of biology, the animal tissue-based approaches have given in for various cell line-based phenotypic screens such as cellular death (Kepp, Galluzzi, Lipinski, Yuan, & Kro, 2011) or expression and location of several proteins (Young et al., 2008).

Label-free biosensors including electric and optical biosensors were long recognized to be a cell morphological sensor (Giaever & Keese, 1993; Ramsden, Li, Prenosil, & Heinzle, 1994), and were used to investigate some cellular processes including cell adhesion, proliferation and death in the past (Fang, 2011b). Only recently it started becoming clear that these biosensors can also be used to perform receptor assays in living cells (Fang, Li, et al., 2005; Fang et al., 2006; Verdonk et al., 2006). This was made possible by label-free profiling of ligands in living cells using newly developed high throughput biosensor systems tailored to microplate-based assays. Early pathway deconvolution studies had showed the ability of the biosensor to translate drug-target interactions into biosensor signatures that mirror the biological complexity of receptor signaling (Fang, Ferrie, Fontaine, et al., 2005; Fang, Li, et al., 2005). Numerous follow-up studies confirmed these observations both theoretically (Fang et al., 2006) and experimentally (Antony et al., 2009; Chen, Shahid, Garcia, Penn, & Xi, 2012; Fang & Ferrie, 2008; Henstridge et al., 2010; Schröder et al., 2010; Stallaert, Dorn, van der Westhuizen, Audet, & Bouvier, 2012; Tanaka et al., 2008; Tran & Fang, 2009; Verrier et al., 2011; Yu et al., 2006). These developments have turned label-free from a morphological biosensor into a systems biology biosensor (Fang, 2006), and have made label-free reemerging as novel phenotypic assays for drug discovery (Fang, 2011a).

3.1. Whole cell phenotype

Label-free biosensors enable profiling of drugs at the whole cell level. Electric biosensors measure drug-induced changes in impedance of cells in proximity to the gold electrode surface (Verdonk et al., 2006). Owing to the insulating properties of cell membranes, the cellular impedance is arising from cell membrane directed electric currents flowing between or beneath the cells, leading to extracellular and transcellular currents (Fang, 2010a). The cellular impedance consists of resistance (a real part) and capacitance reactance (an imaginary part), and is a whole cell phenotype associated with morphological changes and ionic redistribution.

Optical biosensors such as resonant waveguide grating (RWG) measure drug-induced dynamic mass redistribution (DMR) within the bottom portion of cells that is overlapped with the characteristic evanescent wave of the biosensor (Fang et al., 2006). The DMR is a whole cell phenotype associated with drug-induced protein trafficking, actin and cell adhesion remodeling and morphological changes (Chen et al., 2012; Fang, 2010a, 2011a; Fang et al., 2006).

3.2. Real time kinetics

Label-free biosensors can track receptor signaling and drug action in real time with high temporal resolutions. For optical biosensors, the DMR signal arising from a drug-target interaction is generally recorded as the shift in central resonant wavelength (in picometer, pm) or angle (in degree) of the biosensor as a function of time, depending on interrogation schemes (Fang, 2007; Fang, Ferrie, Fontaine, et al., 2005). For electric biosensors, the impedance signal of a drug-target interaction is generally recorded as a dimensionless parameter termed cell index as a function of time. The cell index is derived as a relative change in electrical impedance measured. Both assay duration and temporal resolutions can be adjusted, depending on experimental setup and the purpose of applications. For studying the life cycle of cells starting from cell adhesion, proliferation and death, the temporal resolution is generally within minutes and the assay duration is typically within days. For investigating receptor signaling, the temporal resolution is typically within seconds and the assay duration is often within hours.

3.3. Flexible assay formats

Label-free biosensors offer flexible assay formats to investigate different aspects of the cellular responses of drugs, owing to their non-invasiveness in measurements. Electric biosensors are considered to be minimally invasive due to the use of electric fields generated with sinusoidal voltages. Optical biosensors are truly non-invasive, since the illumination light has relatively long wavelength and is only used to incident the biosensor but not the cells (Fang, 2007).

The biosensor can be used to monitor the whole life cycle of cells on the biosensor surface, or determine the early waves of cell signaling processes (Fig. 2). The assays enabled by label-free generally include agonism assay and antagonism assay (Fang, Ferrie, & Tran, 2009). The one-step agonism assay is to directly monitor a drug-induced biosensor

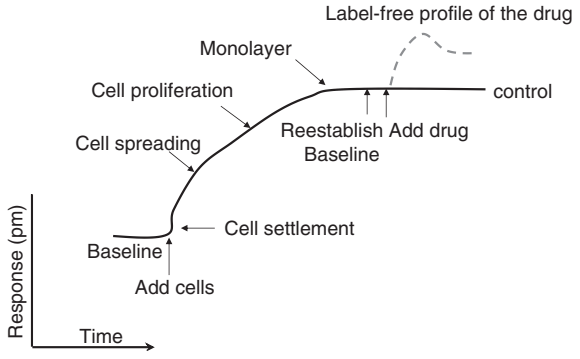

Fig. 2. The life cycle of cells on the biosensor surface and the timing for drug profiling.

response in real time, where the assay duration can be tens of minutes to hours and days. The antagonism assay is in general to measure the modulation of the receptor agonist-induced response by a drug. The antagonism assay can be performed in either co-stimulation or sequential manner. The co-stimulation assay is mostly to determine the competitive antagonism of the drug against the receptor agonist. The sequential two-step stimulation assay can be used to study competitive antagonism, allosteric modulation, pathway deconvolution, receptor desensitization and resensitization processes, and cell death prevention. Depending on applications, the two steps can be separated for varied time periods. In addition, the stimulation can take place at different time points during the life cycle of cells on the biosensor surface. For instance, the drugs can be added during the process of cell adhesion, cell proliferation, cell quiescence, or cell differentiation.

There are several key technical factors affecting assay development and quality. First, time resolution need to be tailored to the kinetics of biosensor responses arising from drug-target interactions, given that different drug-target interactions may result in different profiles with a wide range of kinetics. Second, cell culture need to be optimized, given that label-free profiles of many receptor-target interactions are high sensitivity to cellular backgrounds (e.g., passaged vs frozen cells, proliferative vs quiescent states, undifferentiated vs differentiated states). Third, cell assay environments may also need to be optimized. Assays under physiological conditions may be preferred for studying certain cellular processes, for instance, viral infection, receptor internalization and cell differentiation. On the other hand, most screening and profiling of drugs for many receptors can be performed under ambient conditions. Furthermore, since most label-free biosensors are sensitive to temperature fluctuation, it is important to minimize the temperature difference between drug and cell assay solutions, as well as temperature fluctuation throughout the assay. Lastly, appropriate controls (e.g., negative and positive controls) need to be performed in parallel to ensure the assay quality, given that there may be unexpected drifting of the biosensor baseline signal due to complicate environmental and cellular factors. In general, for robust receptor signaling studies, the drug responses are generally obtained after the cells form a monolayer, given that the cells in monolayer generally display a quite steady biosensor background, and most biosensors measure an averaged response from a population of cells (Fang, 2011a).

3.4. Wide pathway coverage

Label-free biosensors have high sensitivity with wide pathway coverage for several reasons. First, theoretical calculations suggest that the label-free cellular profile of a drug-target interaction is an integrated measure of cellular responses in living cells, in particular native cells (Fang et al., 2006). Second, numerous profiling studies have showed that label-free can be used to investigate the signaling of wide ranges of classes of targets, including receptor tyrosine kinases (Chen et al., 2012; Du et al., 2009; Fang, Ferrie, Fontaine, et al., 2005; Ona & Shibata, 2010; Verdonk et al., 2006), GPCRs (Fang, 2010a; Fang, Li, et al., 2005; Fang et al., 2007; Pai et al., 2012; Peters et al., 2010; Schröder et al., 2010; Yu et al., 2006), kinases (Verrier et al., 2011), ion channels (Denelavas et al., 2011; Fleming & Kaczmarek, 2009; Pänke, Weigel, Schmidt, Steude, & Robitzki, 2011), actin and microtubules (Fang, Ferrie, & Li, 2005; Ke et al., 2010), enzymes (Tran & Fang, 2009), immunoreceptors (Suzuki et al., 2008; Tanaka et al., 2008), Toll-like receptors (Hennessy, Sheedy, Santamaria, Barbacid, & O'Neill, 2011) and transporters (Wong, Gao, Ward, Henley, & Lee, 2012). Considering the signaling pathways involved in different classes of receptors, it is reasonable to conclude that label-free has wide pathway coverage. Finally, pathway deconvolution and functional selectivity studies have also demonstrated that label-free can differentiate pleotropic signaling pathways of receptors (Fang, 2010a, 2011a).

3.5. Multi-target profiling

Label-free is naturally a multi-target profiling technique, unlike conventional approaches that generally have limited capacity for multiplexing due to their pathway-biased measurements. The wide pathway coverage allows the biosensor to detect a wide range of agonists for different targets, both endogenous and overexpressed ones in a typical agonist screen. Screening the Library of Pharmacologically Active Compounds (LOPAC) from Sigma using quiescent A431 cells had identified agonists for the G_q-coupled histamine H_1 receptor, and the G_s-coupled adenosine receptors and β_2-AR (Tran & Fang, 2008). Profiling a GPCR agonist library had identified a great number of receptors that are functional in A431 cells (Fang, 2010b), and some receptors that undergo alterations during the differentiation of ReN cells, a neural progenitor stem cell line (Pai et al., 2012).

The naturally occurring cross-talks in cell signaling and redundancy in cell decision making process also make the biosensor a multiplexing technique for antagonist screening. In a typical antagonist screen the active hits identified for the targeted receptor also include some other classes of active compounds, beside ligands for the receptor. These "false positive" hits can not only be agonists for other endogenous receptors whose activation leads to heterologous desensitization of the target receptor, but also pathway modulators that alter the signaling of the target receptor. Screening antagonists for both histamine H_1 receptor and β_2-AR in the LOPAC had identified that beside known ligands for both receptors and known pathway modulators for GPCR signaling, the adenosine receptor agonists were also detected to be active for β_2-AR, while the protein kinase C activator phorbol 12-myristate 13-acetate was active for the H_1 receptor, both due to heterologous desensitization (Tran & Fang, 2008).

4. Troubleshooting

Label-free biosensors imitate the biological complexity of drug-target interactions in living cells. The cells host complete sets of receptors and their signaling interrogators. Cell signaling is encoded by spatial and temporal gradients of signaling proteins, which act at the network and system level to govern cell decision process. Cell signaling can be viewed to propagate in waves. Receptor activation generally results in a series of network reactions including second messengers, protein trafficking and/or phosphorylation, *de novo* synthesis, exocytosis, gene expression alterations and ultimately cell fate determination. These signaling events take place at different time domains after receptor activation. On the other hand, a drug can bind to more than one target, each with its own binding characteristics and functional consequence in a cellular context dependent manner. As non-invasive whole cell sensing device, label-free simply tracks the complexity in cellular responses of drug-target interactions at the whole cell level. This section reviews approaches to deconvolute the complexity and to determine specific MOAs of drugs. To better illustrate a five-step strategy (Fig. 3), our discovery of a series of novel GPR35 agonists is used as the showcase. GPR35 is an orphan GPCR whose natural agonists are still under debate and biology is largely unknown today (Deng & Fang, 2012b; Deng, Hu, et al., 2012; MacKenzie, Lappin, Taylor, Nicklin, & Milligan, 2011). It is worth noting that such a five-step strategy is generically applicable to other types of cell-based and phenotypic based screening.

4.1. Pharmacological characterization

Shortly after the introduction of label-free cell phenotypic assay to the market, a question has been raised; that is, how to use such a label-free system to discover new drugs. To answer this we assembled an internally made chemical library consisting of 660 compounds and screened their agonist activity in native cell lines including human colon cancer cell line HT-29 (Deng, Hu, He, et al., 2011). These compounds are synthesized in house and originally designed for applications

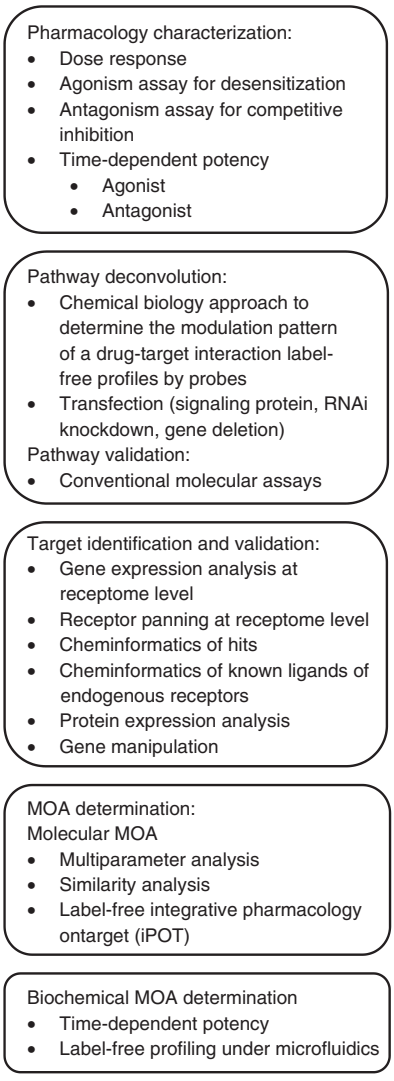

Fig. 3. Strategies to relate the label-free profiles to the mechanism of action of drugs.

in nonlinear optics and organic field-effect transistors, and have never been tested for their pharmacological activity in literature. Screening these compounds using DMR agonism assay had identified two chemical classes, 2-(4-methylfuran-2(5H)-ylidene)malononitrile and thieno [3,2-b]thiophene-2-carboxylic acid derivatives, that triggered robust DMR signals in HT-29. One of the hits is 6-bromo-3-methylthieno [3,2-b]thiophene-2-carboxylic acid (YE210). Pharmacological profiling suggests that all DMR-active compounds gave rise to dose-dependent responses, all of which fit well with one-phase sigmoidal non-linear regression, suggesting that these responses are due to the activation of an endogenous receptor in HT-29 cells. The DMR of all active compounds exhibited rapid onset kinetics and similar characteristics, suggesting that the signaling may start from a cell surface receptor.

4.2. Pathway deconvolution and validation

Since a label-free profile arising from a receptor-ligand interaction is a whole cell phenotypic response that tracks the native signaling pathways, the profile obtained can be directly used as the readout to deconvolute the signaling pathways when combined with chemical biology approach. Advances in chemical biology have discovered various activators, effectors, enzymes, and substrates for many receptor signaling pathways, and many probe molecules that selectively modify the activity of signaling proteins are widely available. Conventional assays can be used to further validate signaling pathways. Results showed that the YE210 response in HT29 was insensitive to the phospholipase C inhibitor U73122 and the adenylyl cyclase activator forskolin, but partially inhibited by Rho kinase inhibitor Y27632; furthermore, treating HT29 cells with YE210 did not result in any Ca^{2+} mobilization, neither cAMP production in native HT29 cells nor cAMP degradation in the forskolin stimulated cells (unpublished data). Western blotting showed that YE210 triggered a robust extracellular signal regulated kinase phosphorylation in HT29 cells (Deng, Hu, He, et al., 2011). These pathway deconvolution assays point to that YE210 may activate an endogenous $G_{12/13}$-coupled receptor.

4.3. Target identification and validation

In the era of genomics, target identification and validation can be achieved using multiple mechanisms. First, combining expression and functional data with analysis of known agonists for receptors at the *receptome* level can assist the identification of potential targets. Label-free panning endogenous receptors using known agonist library at the receptome level can be performed to predetermine how many receptors are functional in a specific cell line. DNA microarray-based gene expression analysis of common cell lines and tissues are available in several public databases such as BioGPS (Wu et al., 2009), and can be used for reference. Quantitative real time PCR at the GPCR receptome level is affordable nowadays and can be used to further validate the receptor expression. Structure activity relationship (SAR) analysis can be used to identify critical functional groups to activate the receptor, so it is possible to narrow down possible receptor families. This can be done in parallel with the analysis of the phylogenetic trees of human GPCR receptome (Fredriksson, Lagerström, Lundin, & Schiöth, 2003; Gloriam, Fredriksson, & Schiöth, 2007), the known G protein coupling preference and known ligands for receptors. Finally, Western blotting and immunostaining studies can be used to ascertain the presence and location of the receptors. Using a custom-made PCR set for almost all druggable GPCRs in human genome we had identified a subset of GPCRs including GPR35 is endogenously expressed in HT29. Immunofluorescence imaging showed that GPR35 is expressed at the protein level and located at the cell plasma membrane. DMR assays pointed to the essential role of the carboxylic acid group of YE210 to activate the endogenous receptor. Together with receptor panning results and chemoinformatics analysis, we hypothesized that GPR35 is the possible target for YE210 and its analogs in HT29 cells.

Second, gene manipulation techniques (e.g., gene transfection, mutagenesis, RNA interference, and gene deletion) can be used to validate the target (Fang, 2010b). Counter profiling between an engineered cell line and its parental cell line, or different cell lines, is effective to confirm the target specificity. RNA interference is widely used to suppress specific proteins in cells so it is possible to quickly examine the possible target (Hannon, 2002). ~60% knockdown of GPR35 using an RNAi revealed relatively lower sensitivity of the YE210-induced DMR to the expression level of GPR35 than partial agonists, suggesting that YE210 is a full agonist of GPR35 (Deng, Hu, He, et al., 2011).

Third, pharmacological tools (e.g., agonists, antagonists, allosteric modulators, and pathway modulators), in conjunction with multiple assay formats as described above, can also be used to ascertain the label-free whole cell profiles of drug-target interaction to a specific target. D-luciferin, one of the most widely used chemoluminescence substrates, was identified to be a partial GPR35 agonist using label-free cell phenotypic profiling, and further conformed pharmacologically using known GPR35 antagonists (Hu, Deng, & Fang, 2012).

4.4. Molecular mechanism of action determination

The cellular and molecular mechanism of action of different ligands for a specific receptor can be examined using multiple approaches. First, multi-dimensional analysis of label-free profiles can be used to relate a

kinetic parameter to a specific signaling event. For instance, different β_2-AR ligands triggered distinct types of DMR signals in A431 cells (Fang & Ferrie, 2008). Multiple kinetic parameters can be extracted and quantified the biased agonist activity of these ligands. Notably, ligands that cause receptor internalization were found to result in a slower positive-DMR (P-DMR) event than those do not. A P-DMR event is a DMR event with an increasing signal over time. Similarity analysis based on the multiple kinetic parameters can be used to further classify the MOA of drugs (Fang, 2010a). Similarity analysis has been widely used to determine the relationships among large biological data (Eisen, Spellman, Brown, & Botstein, 1998).

Second, similarity analysis of the entire kinetic responses can be performed to classify different ligands for a specific receptor into distinct clusters, each sharing a common cellular and molecular MOA (Fang, 2010b). Cellular impedance-based similarity analysis has led to identification of novel molecules that influence cell proliferation (Ke et al., 2010). Results showed that a set of compounds were co-clustered with paclitaxel and were confirmed to be relatively potent anti-mitotic agents. A drawback of such an approach is that it is effectively equivalent to traditional phenotypic screen such as cell death assays. Given the compensatory pathways in cell decision process, the compounds clustered together may not specific to a cellular or molecular MOA, particularly when a large compound library is screened.

Third, newly developed label-free integrative on-target pharmacology (iPOT) can be used to classify compounds in terms of their target selectivity and functional selectivity (Ferrie, Sun, & Fang, 2011; Morse et al., 2011). The iPOT approach leverages the sensitivity of drug-target interactions to cell background and pathway modulation, so it is possible to perform comparative pharmacology analysis. For adrenergic receptor drugs we had shown that the iPOT profiling of β_2-AR in native A431 led to a clustering pattern that seems to be correlated well with their *in vivo* indications of β-drugs (Ferrie, Sun, & Fang, 2011). This approach seems to be effective to integrate the system-based, ligand-directed, and kinetics-dependent biased activities of the drugs.

4.5. Biochemical mechanism of action determination

Label-free can manifest the biochemical MOA of drugs, beside their molecular MOAs. First, the time-dependent potency contains information related to the binding kinetics of agonists. This is because label-free is a real time kinetic profiling tool, and receptor activation occurs almost immediately after agonist binding, but agonists take time to reach equilibrium (Lohse et al., 2008). Second, the preincubation time-dependent potency of antagonists to block an agonist-induced label-free profile is also directly related to the binding kinetics of the antagonists. For instance, different muscarinic M_3 receptor antagonists have distinct kinetics binding to the receptor. The K_{on} value is 1.5×10^9, 4.83×10^8, 4.48×10^8, and 1.58×10^8 $M^{-1}.min^{-1}$, whereas the K_{off} value is 0.27, 0.07, 0.017 and 0.0015 min^{-1} for atropine, ipratopium, N-methyl scopolamine and tiotropium, respectively (Dowling & Charlton, 2006). For the DMR arising from the activation of endogenous M_3 receptor by acetylcholine, these antagonists gave rise to an incubation-time dependent potency to block the acetylcholine DMR (Deng, Wang, et al., 2012). As the preincubation time increases, antagonists with slower K_{on} and faster K_{off} tend to exhibit a decreased potency, but tiotropium that has the slowest K_{on} and K_{off} give rise to an increased potency. Furthermore, this study also showed that under co-stimulation condition tiotropium was found to be incapable of completely block the early onset signaling of the M_3 receptor, consistent with the model of "first come first serve" and the receptor signaling can propagate right after acetylcholine binding. In addition, DMR assays under microfluidic perfusion condition showed that the removal of free antagonists after initial binding had little impact the ability of pre-occupied tiotropium to block the acetylcholine response, suggesting that tiotropium is indeed a long-lasting antagonist with very slow rate constant of dissociation.

Third, label-free cell assays under microfluidics enable differentiation of agonist-occupancy dependent receptor signaling, and ligand-directed desensitization (Goral, Jin, et al., 2011; Goral, Wu, Sun, & Fang, 2011). This is possible since receptor signaling can take multiple waves through different pathways, and different signaling events have distinct requirement of ligand occupancy (Calebiro, Nikolaev, Persani, & Lohse, 2010). Using a microfluidic RWG biosensor system we found that in A431 cells the β_2-AR signaling can be initiated by its agonists with as short as 1 min pulse, and their DMR last for a long time (hours) (Goral, Jin, et al., 2011). The agonist pulse stimulated DMR patterns during receptor desensitization and resensitization processes was found to be ligand-dependent. Combining with chemical biology further elucidates different cellular mechanisms that govern the receptor desensitization and resensitization processes.

5. Conclusion

I have described strategies to determine the MOAs of drugs based on label-free cell phenotypic profiles. As a multi-target profiling technique that leverages the fully assembled biological systems, label-free cell phenotypic assays emulate the innate complexity of drug-target interactions and their functional consequences. Given that complexity is quite literally a fact of life, the label-free profiling is better positioned for multi-target based screens and offers far more useful information than conventional molecular assays. In addition, as a pathway profiling technique label-free also has advantages over other assays using a phenotype whose biological mechanism is poorly understood. Combining label-free with chemical biology, molecular genetics and chemoinformatics represents a rational strategy for relating the label-free whole cell profiles with the MOAs of drugs. Advances in label-free methodologies will also have great impacts on how to discover drugs with unique biochemical, cellular and molecular MOA profiles.

Abbreviations

β_2-AR	β_2-adrenergic receptor
TDDD	target-directed drug discovery
DMR	dynamic mass redistribution
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
GPCR	G protein coupled receptor
iPOT	label-free integrative pharmacology on-target
MC4R	melanocortin-4 receptor
MOA	mode of action
MMOA	molecular mode of action
PDGFR	platelet-derived growth factor receptor
RWG	resonant waveguide grating

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Glossary

Ontarget pharmacology: the functional activity of a drug after binding to a specific target
Ligand-directed functional selectivity: the ligand-dependent selectivity for a specific signal transduction pathway over another downstream the same receptor
Polypharmacology: the specific binding of a drug to more than one target
Phenotypic pharmacology: the cell specific action of a drug
Network pharmacology: the action of a drug in which the drug modulates more than one target in a signaling network
Biochemical mode of action: the interaction between a drug and its target that creates a binding kinetics-determined response
Molecular mode of action: the interaction between a drug and its target (or targets) that creates a specific response
Drug residence time: the amount of time that a drug spends in its target
Kinome: the whole set of protein kinases in the genome of an organism
Interactome: the whole set of molecular interactions in a cell
Node: a biological identity (e.g., gene, protein, protein complex, individual state of a protein) in a network
Edge: a link between two nodes in a network
Hub protein: a protein that is highly connected with other proteins having only a few interactions, but tends to avoid linking directly to another hub protein
Receptome: the whole set of receptors in the genome of an organism