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RESEARCH ARTICLE

Label-free optical biosensor: A tool for G protein-coupled receptors pharmacology profiling and inverse agonists identification

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Abstract

Current GPCR cell-based assays often rely on the measurement of a loaded fluorescent dye, fluorescently tagged targets, or the expression of a reporter. These manipulations may alter the cellular physiology of the target GPCR, and the measurements may be subject to off-target interference of compounds. Label-free optical biosensor-based technologies that provide a noninvasive methodology to study GPCR activation and signaling have been developed. These technologies enable the evaluation of drug effects on various GPCRs that couple to different signal transduction pathways using only one assay platform. This technology is highly sensitive and detects inverse agonism, therefore providing a convenient tool to study the pharmacology of drugs. Furthermore, its real-time kinetic measurements give researchers additional information about the biological responses induced by the drug. This assay platform when applied in early drug discovery efforts can provide valuable information on the mechanism of action and pharmacology profiles of drug candidates.

Keywords: *Label-free; optical biosensor; GPCR; pharmacology; inverse agonist*

Introduction

G protein-coupled receptors (GPCRs) represent one of the largest classes of cell membrane proteins and are the targets of approximately 45% of marketed human medicines (1,2). The ligand-receptor-binding assay was the gold standard of GPCR drug discovery for many decades. A better understanding of GPCR-signaling cascades in the past two decades has allowed cell-based functional assays to be developed. Functional assays are more versatile because they provide additional information and enable the identification of agonists, antagonists, allosteric modulators, and inverse agonists. Furthermore, in the cellular environment, the concentrations of intracellular mediators such as receptors, related enzymes, co-regulators, and associated proteins, are in a more physiological state than in the biochemical assay.

High attrition rates particularly at the preclinical and clinical stages continue to plague the drug development process. While there may be numerous reasons for the high attrition rate, one possibility is that drug candidates are advanced through the early stages of the drug development process without full interrogation of possible off-target interactions. Generic technology that provides incisive and predictive information about the lead compounds is urgently needed in the early stages of the process. It can reduce attrition rates and save enormous late developmental costs of drug candidates. In addition, assays that can measure multiple signal transduction pathways without the need to involve forced or promiscuous coupling through molecular engineering of cells, as well as technologies feasible to the testing of primary cells or samples from patients, will be particularly valuable to drug development.

The effects of drugs can be measured at various levels: from fundamental in vitro binding of the drug with the target protein to an overall biological response in animals or human subjects. The latter, which involves complex

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and integrated biochemical and physiological processes, is what matters in the clinic. The overall clinical effect is the relevant endpoint for drug discovery, but studying drug effects in vitro can help researchers understand how a drug induces therapeutic responses and adverse effects. In addition, applying in vivo studies in the early drug discovery process would not be practical because these tests are usually complex, slow, and expensive. Therefore, the more physiologically relevant an in vitro assay performed in early drug discovery processes, the better off we are at defining the pharmacological taxonomy of drugs and their subsequent therapeutic effects. The development of new in vitro assay technologies and the advancement of existing technology platforms are the catalysts for our improved understanding of drug activity. In this report, I review the general attributes of cell-based label-free technologies implemented in the drug discovery process and then focus the discussion on label-free optical-based technologies used in GPCR pharmacology profiling.

Label-free technology platforms

Current GPCR in vitro assays rely on the measurement of a single event such as ligand-receptor binding, specific signal transduction activity including levels of second messengers [Ca^{2+} and cyclic AMP (cAMP)], translocation of a particular target tagged with a fluorescent label, or the expression of a reporter gene. These technologies require manipulations or molecular engineering of cells by overexpressing fluorescent-tagged target GPCRs or loading of fluorescent molecules into cells. These manipulations may alter the cellular physiology of target receptors and subject them to interference from fluorescent and color compounds. The inclusion of certain labels and reporters, particularly labels for live cells, has been shown to affect various aspects of cellular behavior. For example, it has been shown that the live cell fluorescent dye 2′,7 ′-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF-AM) and rhodamine 6G (R6G) can dose-dependently inhibit the migration of macrophage and mononuclear cells, respectively, urging caution when using these dyes for cell-based assays (3,4).

In recent years, label-free technologies and knowledge of their potential applications have advanced greatly so that they can now be grouped into two distinct categories: (1) cell-based assays that are almost entirely in 96- or 384-well microplate format and (2) binding analysis assays that, in most cases, are still done with single or multiple sensor-based technologies or by measuring the heat capacity of a sample at relatively low throughput. These binding assays have proven invaluable in the investigation of biomolecular interactions. More importantly, they can provide direct monitoring of analytes binding to target molecules without modifying the molecules of interest with labels or using an indirect reporter system. Readers interested in label-free binding assays should refer to recent reviews that discuss different label-free binding technologies (5,6).

One main advantage of label-free technologies is real-time kinetic measurement. (7–10). Real-time monitoring of cellular processes offers distinct and important advantages over traditional endpoint assays. First, comprehensive representation of the entire length of the assay is possible, allowing the researcher to make informed decisions regarding timing of manipulations or treatments. Second, the actual kinetic response of cells provides important information about biological status, such as cell growth, arrest, morphological changes, and apoptosis. Furthermore, these technologies are noninvasive, and cells in the well can be used in sequential experiments to generate additional information. It also provides a generic approach to interrogate different GPCR targets that results in faster turnaround at lower cost.

Cumulative evidence has shown that label-free technologies can contribute to measurement of endogenous receptor activation, be used as a universal receptor deorphanization platform, and be used in the study of the mechanism of action of ligands (11,12). These technologies can also be used for the study of primary cells, when cell numbers are often limited. One major attraction is the feasibility of studying and comparing results generated from native nonengineered cells expressing the GPCR of interest to those obtained from a recombinant cell line transfected with the GPCR using the same assay platform without perturbation by labels.

Two major types of label-free technology have been applied to multiwell cell-based assays (Table 1). They are based on the measurement of impedance (CellKey™, ECIS™, RT-CES®, and xCELLigence™) and the optical

Table 1. Selected plate-based label-free systems for cell-based assays.

System Name	Biosensor	Well-formats	Vendor	Website
BIND®	Optical waveguide grating	16-, 96-, 384-, 1536-well	SRU Biosystems	http://www.srubiosystems.com
CellKey™	Impedance-based	96-, 384-well	MDS Sciex	http://www.mdssciex.com
ECIS™	Impedance-based	16-, 96-well	Applied Biophysics	http://www.biophysics.com
Epic®	Optical waveguide grating	384-well	Corning	http://www.corning.com
RT-CES®	Impedance-based	16-, 96-well	Acea Biosciences	http://www.aceabio.com
xCELLigence™	Impedance-based	16-, 96-well	Roche Applied Science	http://www.roche-applied-science.com

refractive index (BIND® and Epic®). All the impedance systems use special cell culture plates that have electrodes on the bottom of the cell attachment surface. They are based on the concept that interaction of cells with electrodes creates a resistance to the flow of current and that alterations in either the number or the shape of those cells will change the measured impedance. When a voltage with different frequencies is applied, the systems measure resultant currents caused by changes in cell morphology and interaction with the electrode (13). The label-free optical platforms (BIND® and Epic®) measure subtle changes in cells that occur over a period of minutes or seconds. Similar to the impedance system, a special plate is required. However, instead of an electrode, the plate has a waveguide grating bottom surface onto which cells are plated. The system shines broadband light on the bottom surface of the plate, light travels through the waveguide, and finally a single wavelength of reflected light is measured from the bottom of the plate. The reflected wavelength is affected by the presence of cells and the resulting mass of material in close proximity to the bottom of the plate (14). Hence, if a molecule interacts with the cells and induces a change in cell morphology or stimulates the translocation of intracellular contents toward the cell membrane closer to the waveguide surface of the plate (dynamic mass redistribution), there will be a corresponding shift in wavelength. In theory, this technology can detect cellular processes including recruitment of microorganisms, viruses and proteins, endocytosis, receptor internalization, exocytosis, apoptosis, cellular communication, and ligand-driven activation of cellular receptors such as GPCRs.

Label-free optical biosensor for GPCR pharmacology profiling

Classical drugs targeting GPCRs generally fall into two categories: (1) agonists, which are drugs mimicking the actions of endogenous transmitters or hormones to stimulate GPCRs, or (2) antagonists, which have no intrinsic activity of their own but block activation of the GPCRs by agonists. This drug classification seems too simplistic for the new scientific evidence supporting multiple receptor states (15). While full and partial agonists have been known for many years, inverse agonists and allosteric modulators have only been identified in the last decade. Similar to partial agonists, inverse agonists are able to block the effects of full agonists at GPCRs. However, the unique property of these ligands is that while binding to the receptors, they induce an opposite signaling effect to that induced by agonists. The inherent activity of an inverse agonist is dependent on the receptor showing a spontaneous elevated basal response (constitutive activity). Thus, agonism can be better explained as the conformational selection of an active state of the receptor, inverse agonism as the selective enrichment of an inactive state, whereas neutral antagonists are drugs without the capacity for conformational selection of specific receptor states (16). For this reason, inverse agonists may be useful in pathological conditions where GPCRs undergo constitutive activity in vivo either due to mutation or because the receptors become overexpressed in the pathological condition.

It is now well known that GPCRs are able to exist in a spontaneously active state that leads to G protein activation (17). In experimental or pathological conditions, receptor overexpression may produce significant levels of spontaneously activated receptors and exceed the threshold for detectable constitutive activity. One simple molecular mechanism for inverse agonism is selective affinity for the inactive state of the receptor. It is important to note that inverse agonists behave as simple competitive antagonists in nonconstitutively active and ligand-activated receptors. Determining whether a drug produces full or partial inverse agonism is a great deal more complicated than characterizing agonists and depends on the dynamic range of the measuring systems in which inverse agonism can be detected.

Functional assays that measure signal transduction activity downstream from the receptors are typically more sensitive than ligand-receptor binding assays (18,19). Sensitivity, in this case, refers to the response (efficacy) and not the concentrations at which responses are detected (potency). In tightly coupled functional assays, the effects of even a low concentration of activated receptors would result in measurable levels of constitutive activity. In general, all ligands with affinity for receptors would be expected to produce either agonism or inverse agonism. If not, it would require that the ligand recognizes at least two conformational species of the receptor as being identical (20). In any case, failure to observe changes in receptor conformation cannot be taken as evidence that changes do not occur with ligand binding, only that the detection system is inadequate. Specifically, a fluorescent probe covalently bound to cysteine 265 of the β_2-adrenoceptor indicates a range of molecular microenvironments for the fluorescent probe bound to the protein (21). These changes in the protein environment of the probe indicate that the receptor adopts a Gaussian distribution of states reflecting the distribution of protein conformations. A recent survey of 380 known antagonists indicates that 322 (85%) are inverse agonists and 58 (15%) are neutral antagonists, suggesting neutral antagonists are the minority category of ligands (17).

The label-free optical waveguide grating biosensor in microplate format is still a nascent platform. However, its universal principle of detection and amenability to high throughput has stimulated interest in its applicability as

a generic methodology to facilitate a broad range of drug discovery assays (7,22,23). The dynamic mass redistribution (DMR) response, measured by optical biosensor, is a novel quantitative and global representation signature for all signaling. It is an integrated cellular response that involves G protein signaling and cellular events, including cytoskeleton rearrangement, and/or movement of signaling cascade proteins. Unlike other conventional assay platforms, which are generally limited to measurements of a single signaling event such as direct visualization of the translocation of fluorescently tagged targets in cells, this technology uses the integrated signature of DMR to study cell signaling and network interactions. Assay development using the optical biosensor for GPCR targets is fairly similar to other cellular assay formats but involves a noninvasive label-free optical readout. In addition, the same assay format and measurement are used no matter which GPCR target and signal transduction mechanism (Gs, Gi, or Gq) is involved in the study. This simplifies assay development, data analysis, and comparison of results between GPCR targets that couple to different signal transduction pathways.

A recent double-blinded study has demonstrated the application of an optical biosensor to study the mechanism of action of ligands, distinguishing inverse agonists from neutral antagonists and agonists (23). In this study, the Epic® system was used to successfully identify compounds that function as agonists, neutral antagonists, or inverse agonists from a panel of 12 compounds against two recombinant cell lines expressing the human dopamine D3 receptor (CHO-D3R) or human muscarinic acetylcholine M1 receptor (CHO-M1R). Antipsychotics, such as amisulpride and haloperidol, induced dose-dependent negative-DMR responses in CHO-D3R cells opposite to that induced by the natural ligand dopamine, indicating an inverse agonism at the D3R (Figs. 1 and 2). The positive-DMR response produced by subsequent addition of dopamine was also attenuated by both compounds in a dose-dependent manner, showing the compounds antagonized dopamine responses at the D3R. The effects of amisulpride were observed only on CHO-D3R cells and not on CHO-M1R cells, suggesting that amisulpride is a D3R-specifc inverse agonist (Fig. 1). In contrast, haloperidol stimulated a positive-DMR response at a lower potency on CHO-M1R cells, which also antagonized subsequent carbachol-induced responses dose dependently. This result shows that haloperidol, a D3R inverse agonist, also acts as a partial agonist at the M1R (Fig. 2). The observation is in accord with published data indicating many antipsychotic drugs are inverse agonists at the human D2R (24). The optical biosensor provides a sensitive label-free cell-based assay platform for the evaluation of drugs' mode of action. The ability to use

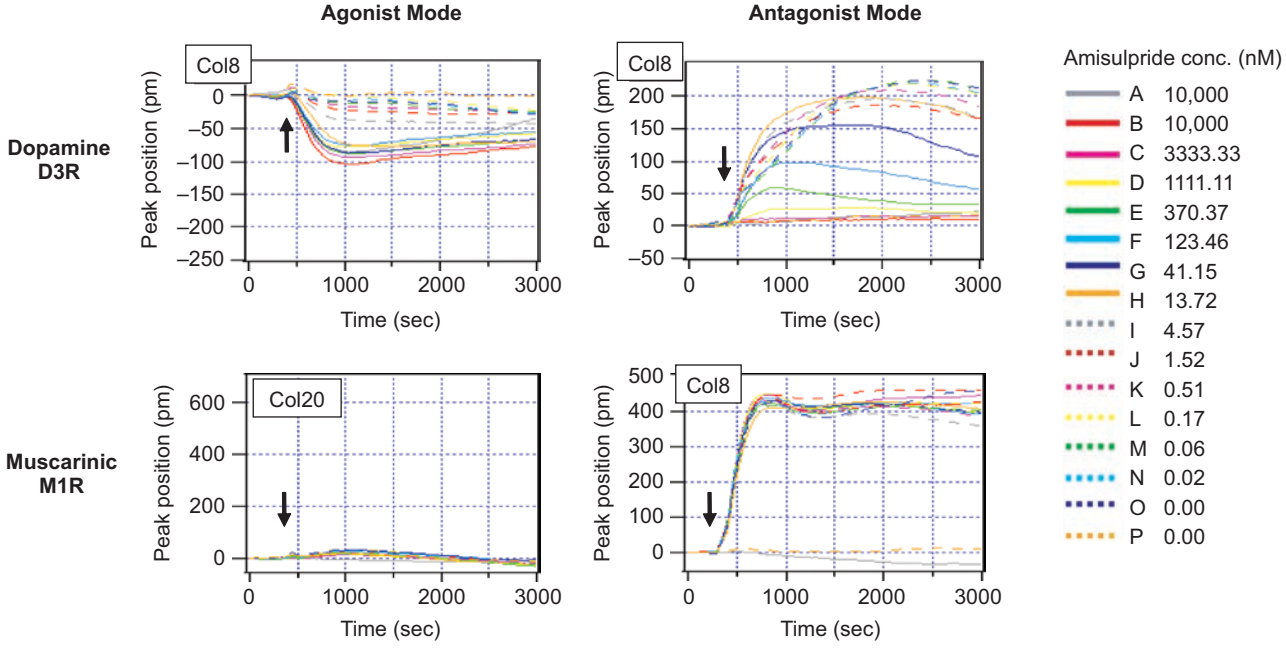

Figure 1. The dynamic mass redistribution (DMR) profile of amisulpride on CHO-D3R and CHO-M1R cells in agonist and antagonist modes. In the agonist mode, amisulpride in various concentrations (0.02–10,000 nM) was added to the wells after the plate had been equilibrated in the Epic® biosensor for 2 hr and a 5-min DMR baseline was taken. Amisulpride-induced DMR response was monitored for another 45 min. For the antagonist mode, amisulpride at various concentrations was incubated with the cells for 3 hr prior to taking a 5-min DMR baseline measurement. Dopamine (50 nM) or carbachol (500 nM) was added to the CHO-D3R cells or CHO-M1R cells, respectively, and the induced DMR response was monitored for another 45 min. Each curve represents the kinetic DMR response of the cells incubated with amisulpride at the concentration indicated. (Reproduced with permission from Ref. 23.)

a single assay platform to determine compound effects on various GPCRs coupled to different signal transduction pathways facilitates pharmacological profiling and selectivity determination of lead compounds in the early drug discovery process.

The effects of specific ligands on native cells expressing endogenous GPCRs have been studied by using an optical biosensor (11). Remarkably, distinct temporal fingerprints are observed that seem to be characteristic of the receptor coupling signal transduction pathways triggered by each ligand. In many cases, these appear dependent upon the cellular background and mechanism of cell attachment. Although GPCRs usually exhibit a preference for one G protein-coupling type, it has been shown that receptors can activate more than one pathway, depending on the agonist molecule and the cell type (agonist trafficking or functional selectivity) (25). These phenomena can now be studied by using the optical biosensor without the need for complicated molecular modifications. The key is having a strategy and toolbox to deconvolute the combination of signatures, such as knocking down receptors or corresponding pathways with siRNA, specific antagonists, or signal transduction pathway blockers.

Taking advantage of the noninvasive label-free nature of this technology, multiple relevant cell lines can be measured at once to determine the pharmacological profile of a drug in development. Furthermore, primary cells, or theoretically any cell type that adhere to the biosensor surface, can be studied to determine drug efficacy. This would permit comparing drug effects on primary cells obtained from patients and normal subjects, as well as determining the pathological relevant of constitutively active GPCR and inverse agonism. The extreme sensitivity and distinct optical signatures of GPCR signal transduction pathways are the other benefits of this technology. GPCR signal transduction typically proceeds through a series of amplification steps. An agonist-mediated maximal response, a measure of agonist efficacy, is dependent on the cellular events measured. As a result, the farther away from the receptor activation step the cellular events measured, the higher the sensitivity is to agonist-stimulated responses (18,19). The optical biosensor measures a cellular DMR signal integrating cell signaling and network interaction, a functional system that can amplify and turn a few constitutively active receptors that might not be detected by binding or second messenger assays into a measurable response. Thus, this label-free optical biosensor is an ideal tool for measuring physiological responses of living cells, especially for detection of inverse agonism.

Physiological relevance of inverse agonism

A significant advance in GPCR research of the past decade has been the expansion of the GPCR subtypes

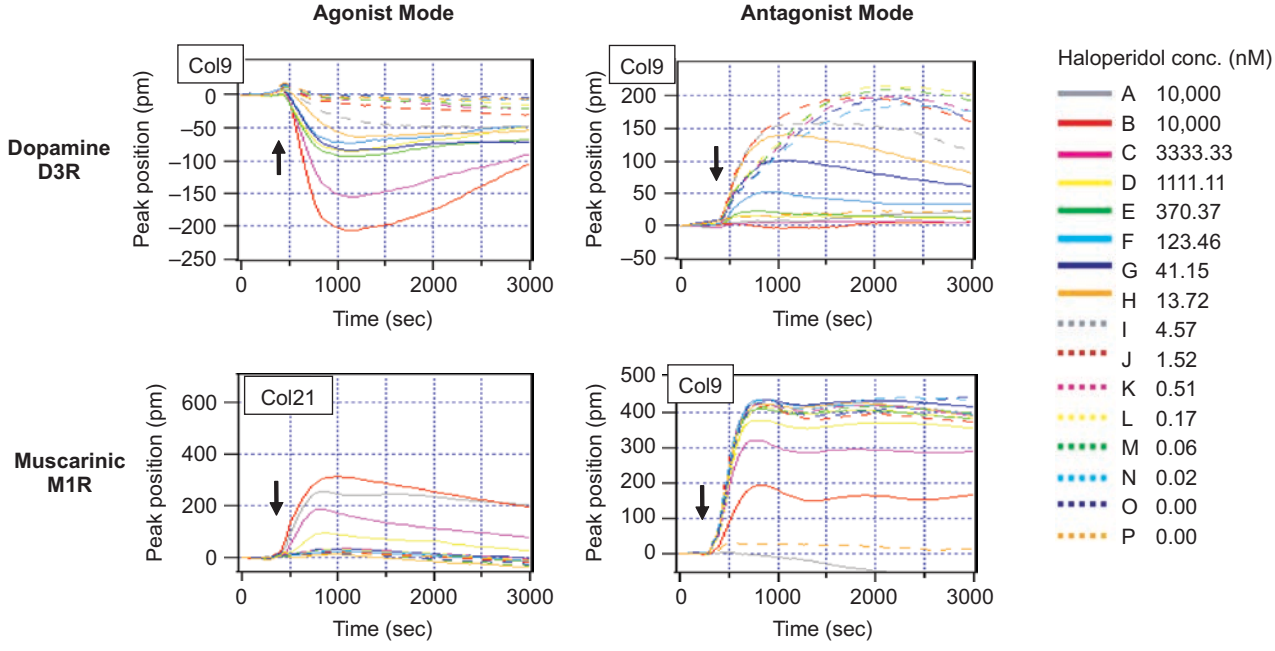

Figure 2. The dynamic mass redistribution (DMR) profile of haloperidol on CHO-D3R and CHO-M1R cells in agonist and antagonist modes. In the agonist mode, haloperidol in various concentrations (0.02–10,000 nM) was added to the wells after the plate had been equilibrated in the Epic® biosensor for 2 hr and a 5-min DMR baseline was taken. Haloperidol-induced DMR response was monitored for another 45 min. For the antagonist mode, haloperidol at various concentrations was incubated with the cells for 3 hr prior to taking a 5-min DMR baseline measurement. Dopamine (50 nM) or carbachol (500 nM) was added to the CHO-D3R cells or CHO-M1R cells, respectively, and the induced DMR response was monitored for another 45 min. Each curve represents the kinetic DMR response of the cells incubated with haloperidol at the concentration indicated. (Reproduced with permission from Ref. 23.)

for which constitutive activity and inverse agonism has been demonstrated. In 1995 there were five GPCRs for which spontaneous activity and inverse agonists had been identified: the δ-opioid peptide receptor, the β_2-adrenoceptor, the 5-hydroxytryptamine $5HT_{2C}$ receptor, the bradykinin B_2 receptor, and frog atria muscarinic acetylcholine receptor (26–30). In fact, there is now reason to believe that all GPCRs, including orphans, can exhibit a degree of spontaneous activity (17,31). In addition, there are other means such as mutations, single nucleotide polymorphisms (SNP), splice variants, and receptor autoantibodies that confer constitutive activity to a receptor. Typical examples are the thyroid-stimulating hormone receptor [~50 constitutively active mutant (CAM) receptors cause hyperthyroidism, the luteinizing hormone receptor (~14 CAM receptors cause male precocious puberty), the parathyroid hormone (PTH) receptor (3 CAM receptors cause dwarfism), and the Ca^{2+}-sensing receptor (~23 CAM receptors cause hypocalcemia and hypercalciuria) (32). In these cases, constitutive activity is causal to the disease. Inverse agonists might be beneficial where disease was precipitated by constitutive activity induced by mutations because they decrease the high basal activity, whereas neutral antagonists would have no effect.

The S49G mutation in the β_1-adrenoceptor, identified in patients with heart failure, appears to result in constitutive activity of the receptor (33). It displays increased agonist affinity and is more sensitive to the inverse agonist metoprolol compared with the wild-type receptor. In the $5HT_{2C}$ receptor, a C23S mutation occurs with an allele frequency of 0.13 in unrelated Caucasians. After expression in a recombinant cell line, the mutant appears to be even more constitutively active than the wild-type receptor (34). Activity in a recombinant system alone, however, does not always translate to in vivo response. Despite demonstrating constitutive activity of the $5HT_{1B}$ receptor in recombinant cell lines and having available both inverse agonists and neutral antagonists, these agents, in contrast to $5HT_{2C}$ receptor blockers, could not be differentiated in vivo (35). An awareness of how in vitro efficacy, using various recombinant assay systems, relates to the in vivo pharmacological properties is essential to the correct understanding of inverse agonist vs. neutral antagonist activity.

Therapeutic potential of inverse agonism

There are suggestions that a chronic elevated tone may be an important feature of some diseases. For example, in severe Jansen-type metaphyseal chondrodysplasia, ligand-independent constitutive activity of the parathyroid receptors may be the cause of the observed hypercalcemia and hypophosphatemia (36). Similarly, constitutive receptor activity may be relevant in other conditions such as retinitis pigmentosa, hyperthyroidism, or autoimmune diseases (37,38). In addition, an intriguing link between receptor overexpression, cell transformation, and cancer growth may involve GPCR constitutive activity. Some tumors have high expression levels of vasoactive intestinal polypeptide (VIP) and bombesin (39–45). Antagonists for VIP have been found to reduce tumor growth, likely by blockade of locally released VIP from the tumor (40,42,46). However, the extreme high levels of VIP receptors expressed in some tumors (i.e., 100,000-fold the natural level in pancreatic epithelioid carcinoma) should theoretically produce a constitutive cellular response that could lead to tumor growth (47). In cases where constitutive activity is pathologically relevant, inverse agonists will be uniquely valuable in the retardation of tumor growth.

A second explanation for the unique features of inverse agonists may not be due to short-term effects on elevated basal cellular responses but rather to the molecular properties of inverse agonists. For example, the selective affinity of the inverse agonists, cimetidine and metiamide, for histamine H_2 receptors has been linked with tolerance to H_2 receptor blockade (48–50). This tolerance is believed to occur through elevation of histamine receptor levels (48). In contrast, no tolerance or change in receptor levels was seen after long-term treatment with the neutral histamine H_2 receptor antagonist burimamide (51). The inhibition of spontaneous formation of receptor active states and subsequent phosphorylation and internalization of the receptor by inverse agonists could be a mechanism by which inverse agonists interfere with the natural cycle of receptor synthesis, membrane expression, and internalization. This could lead to an increased surface density of receptors in the presence of chronic inverse agonism (52). Receptor up-regulation is also observed with inverse agonism of β_2-adrenoceptors and α_1-adrenoceptors (53,54). However, the overall rate of receptor synthesis and degradation in the cell will be an important factor determining whether inverse agonists cause tolerance to antagonism through increases in receptor levels. This will depend on receptor type, cell type, and degree of negative efficacy. Depending on conditions, current evidence suggests that inverse agonists can produce useful effects by reducing pathologically induced constitutive activity or undesirable properties of developing tolerance to the drug (55). It has been postulated that the classification of "typical" and "atypical" antipsychotics may be related to the fact that these classes of ligand coincide with inverse agonism (atypical) and neutral antagonism (typical) activity for $5HT_{2C}$ receptors (56).

Several large-scale placebo-controlled clinical trials with carvedilol, metoprolol, and bisoprolol—β-blockers with β-adrenoceptor inverse agonist properties—have

demonstrated clinically relevant and statistically significant decreases in mortality and the number of patient hospitalized (57–61) When the heart failure model was applied to asthma, again only inverse agonists of the β_2-adrenoceptor improved the airway hyperresponsiveness to methacholine in a murine model of the disease (62). Thus, agonists enhance signaling acutely, but desensitization mechanisms hamper signaling with chronic use. In contrast, inverse agonists inhibit signaling initially but up-regulate and/or sensitize receptors with chronic use, resulting in enhanced signaling (63).

Conclusion

GPCRs can be spontaneously activated in the absence of ligand. This constitutive activity has also been firmly established in vivo, despite the often abundant presence of endogenous hormones and neurotransmitters. Furthermore, it appears that receptor splice variants, somatic mutations, and SNPs that affect the coding region of genes can display different levels of constitutive activity. This knowledge is only now emerging, and its consequences for drug therapy are not yet certain. Constitutive activity can also be imposed with tools from molecular biology. Thus, mutant receptors and fusion products between receptors and G proteins, among others, can be engineered to display increased levels of constitutive activity. Such constructs are useful for differentiating between neutral antagonists and inverse agonists, developing new drugs, and deorphanizing receptors.

The drug discovery paradigm is shifting from a focus on individual molecular targets to the exploitation of systemic biology and the understanding of drug pharmacology in a native context. Label-free optical biosensors constitute a valuable and seamless link between the direct screening of any target for small molecule binders and the biological complexity of native cells. This platform can be applied to early drug discovery efforts, including hit identification, hit validation, hit-to-lead optimization, and pharmacology profiling, providing valuable information on the mechanism of actions and pharmacology profiles of drug candidates.

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