Take it personal! Genetic differences in G protein-coupled receptors as studied with label-free technology

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CHAPTER 1

General Introduction

About this thesis

This thesis describes the study of the influences of genetic variation on a specific class of drug targets, the G protein-coupled receptors (GPCRs), using a combination of personal cellular models and novel label-free assay technology. The results obtained herein will likely assist in the translation of early *in vitro* experiments to more clinically relevant studies in the course of the drug discovery pipeline. Eventually, the findings in this thesis hopefully contribute to the development of clinically more effective drugs and advance the current 'one-size-fits-all' paradigm into the realm of precision medicine. In this first chapter, I introduce the concepts of precision medicine, importance of GPCRs as drug targets and prevalent sources of genetic variation. Moreover, I discuss the advantages and opportunities that arise from combining a novel label-free assay technology with personal cell lines. In the last section of this chapter, I specifically outline the objectives of this thesis.

Precision medicine

Historically, conventional disease treatments have been based on diagnosing a patient with a general disease state and providing a corresponding generalized drug treatment. However, while successful to a degree, such one-size-fits-all treatments may be ineffective or harbor dangers for the individual patient. Inter-individual variability in drug effectiveness poses a significant challenge for the conventional strategies. Even today's best sold, 'blockbuster' drugs, poster children of the current treatment paradigm, work in only 35% - 75% of patients due to influences of genetics, lifestyle and environmental differences [1, 2]. Hence, modern medicine is undergoing a paradigm shift towards a more personalized, patient-customized treatment model, for which a large part is based on a deeper understanding at a molecular level [3, 4]. For this emerging concept known as personalized or precision medicine, it is paramount to better understand the effects of a drug not only in the overall population, but in the individual patient as well [5]. Customization using a sub-population or patient's individual characteristics, e.g. genetic information, could decrease risks of ineffective treatment, dosing or side-effects [2, 6, 7]. Genetic testing is already available for approximately 2000 clinical conditions today, most of which are in oncology. Two successful examples are genetic tests for HER2-positive breast cancer which serve as a predictor of response to the drug Herceptin, and CYP450 polymorphisms which affect the action and metabolism of drugs such as selective serotonin-reuptake inhibitors [6-9]. Despite the promise shown by these examples, most drug targets and disease mechanisms are still in

dire need of further research to determine whether, and how, genetic variation affects both pathology and drug responses.

GPCRs and genetic differences

The majority of drug targets today are GPCRs, a specific class of membrane proteins. In fact, 30-40% of all current drugs work by influencing GPCR function [10, 11]. This is no surprise as approximately 800 GPCRs are encoded by the human genome. Over 300 of these are considered druggable, i.e. they constitute current or future drug targets [12]. Due to their ubiquity, GPCRs are involved in almost all aspects of human physiology from vision to immune response [13]. In general terms, the role of a GPCR is to translate an extracellular signal, which can range from photons to odorants, hormones or neurotransmitters, into a cellular response. Depending on the nature of ligand and receptor, the cellular effect can vary from changes in morphology to proliferation, differentiation and survival (**Figure 1**).

Due to their physiological importance, it is highly interesting to decipher the influence of genetic variation in GPCR-mediated drug responses in the context of personalized medicine [5, 14]. Several studies have already linked GPCR polymorphisms to diseases and drug response variation [14-18], including for instance serotonin [15], dopamine [14, 16, 19-21], adenosine [22-24], purinergic [25, 26] and cannabinoid [17, 18] receptors, and many other commonly targeted GPCRs [14].

Single Nucleotide Polymorphisms

One prevalent source of genetic differences which can lead to an alteration in the drug target are Single Nucleotide Polymorphisms (SNPs). SNPs make up 84 to 95% of the total human genetic variation and are defined as single-base variations with a presence in at least one percent of the population. Consequently, SNPs are quite common, with on average around one SNP per 300 bases [27]. These variations can cause a multitude of differences in the end-product of genes, depending on their location and nucleotide difference. For example, a SNP can cause a new start- or stopcodon to appear, cause the transcript to be removed or even change the encoded amino acid with a different one, i.e. a so called missense SNP. SNPs that somehow change the amino acid sequence of the resulting protein are known as non-synonymous SNPs. It is believed that such changes are the most prevalent source of differences in GPCR response to drugs (**Figure 2**).

A common example is the association between SNPs on the chemokine 2 and 5

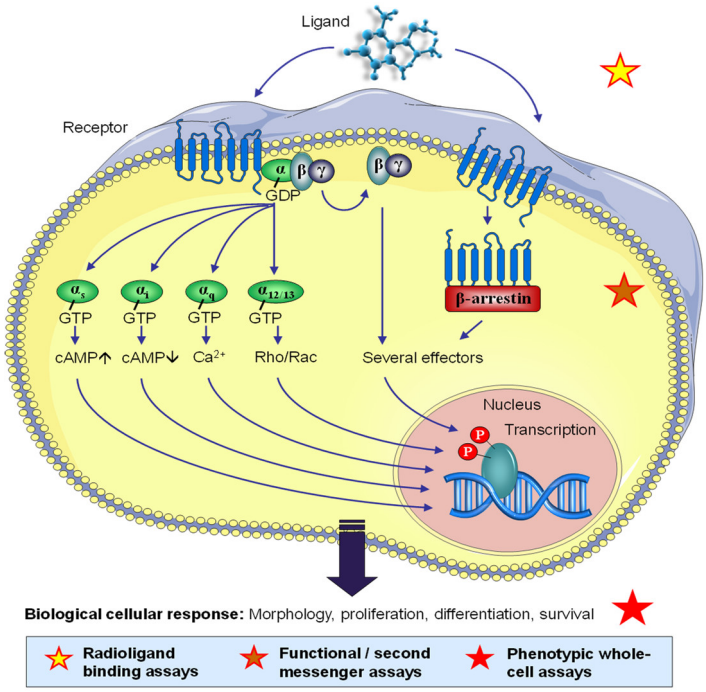

Figure 1: GPCR signaling and *in vitro* assays. When a ligand binds to and activates a GPCR, the receptor in turn activates the G protein. The trimeric G protein dissociates and can activate various secondary messenger pathways, leading via a cascade of reactions to an eventual cellular response. Traditional drug development programs are often target-focused, i.e. relying on *in vitro* assays which use reporter systems for the investigated target. Such reporter systems include for instance the use of radioactive labels or fluorescent dyes for ligand, target or effector labeling, or of more downstream reporter gene constructs. Such modifications, however, may influence target pharmacology. Label-free whole-cell assays are phenotypic assays that capture the biological cellular response in real-time, without focusing on merely one pathway and without requiring any such modifications, potentially providing a better physiological context. Image constructed using components from Servier Medical Art by Servier (*http://www.servier.com/Powerpoint-image-bank*).

receptors (CCR2 and CCR5) and the delayed or increased onset of AIDS after HIV infection [28]. In another instance, a SNP-caused tryptophan to arginine change in the β3-adrenergic receptor has been associated with obesity [29]. A set of four SNP locations on the dopamine D3 receptor have been associated with schizophrenia, where the susceptibility to the disease is most likely caused by the combined effect of these SNPs [30]. In the GRM1 glutamate receptor, the presence of SNPs in the splice region between two exons causes a new splice variant lacking one transmembrane domain, again associated with schizophrenia symptoms [31]. These examples emphasize that the possible influence of SNPs on GPCRs can be quite

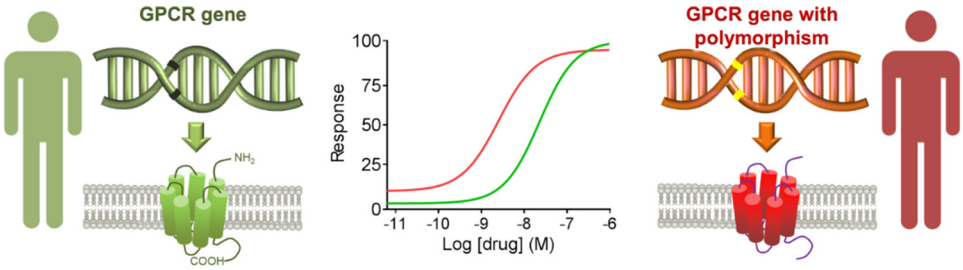

Figure 2: Effects on individuals of SNPs in GPCRs. GPCR polymorphisms can lead to differences in drug response between individuals, potentially changing drug effectiveness and risks of side-effects.

profound. However, the knowledge of polymorphism effects in GPCRs is still scant as of today. Hence we sought to find out more on the influence SNPs have on ligand-induced GPCR function in this thesis.

Lymphoblastoid Cell Lines

Most evidence supporting the influence of GPCR polymorphism effects are based on statistic association with occurrence of a disease, or by functional characterization in artificial, heterologous cell lines [14, 16, 17]. Both methods lack the final, well-defined physiological link that would allow us to understand more precisely how a polymorphism changes GPCR effects in an individual patient [32, 33]. Such understanding could be provided by directly measuring drug responses in patient material or cells as a model system.

An upcoming phenomenon in the past two decades are biobanks, which collect and store biological material to support modern medical research such as -omics approaches and personalized medicine. For this purpose, biobanks provide biomaterial resources including tissues, cells, blood, and serum from patients with specific diseases, specific populations or individuals with specific traits [34-36]. One type of cells used in many biobanks as a preferred choice for storing genetic material are lymphoblastoid cell lines (LCLs), which are derived from a person's B-Lymphocytes [37, 38]. Renowned consortia with LCL libraries include the Centre d'Étude du Polymorphisme Humain, the International HapMap and 1000 genomes projects [39-43]. In most cases, however, LCLs are merely used as a source of DNA or RNA for genotyping, expression or methylation studies [16, 37].

In this thesis, we set out to show that LCLs can be used as a model system to directly study polymorphism effects on GPCR function on a cellular level.

Label-free technologies

Traditional GPCR assays are often label-based, which have definite disadvantages when venturing to remain as close to the physiological situation as possible. These assays rely on (chemical) engineering by, for instance, radioligand tagging or overexpression of the receptor (**Figure 1**). All of such alterations to the cell may influence its physiology leading to for instance identification of false-positive or false-negative hits [44]. Furthermore, such assays are mostly pathway-biased as they typically focus on only one cellular event in a specific signaling pathway [45]. Another drawback is that they often lack the sensitivity required for receptors endogenously expressed in cell lines, as this is much lower level than in specifically engineered cell lines. In short, such assays are not well-suited for investigating subtle polymorphism changes on endogenous receptors in their native environment.

However, new assays are emerging that enable measurements in endogenous cell lines and hereby provide greater, more relevant biological insight. By eliminating any need for labels, label-free cellular biosensors have the capability of assessing endogenous receptor function in their native physiological settings [46]. They are more sensitive, less invasive and monitor drug effects on a whole cell in real-time [33, 47, 48]. Hence, label-free assays are also more translational towards a correlation between *in vitro* and *in vivo* findings [49, 50]. Moreover, the sensitivity of these label-free assays allows monitoring of standard effects such as GPCR activation or inhibition as well as detection of smaller changes such as biased signaling [33, 51], which may also be affected by polymorphisms [5]. In short, label-free technologies offer unique advantages for precision medicine as they offer the ability to monitor small changes in GPCR signaling or drug responses in the native cellular context.

Objective and overview of this thesis

Aim and set-up

The aim of the study was to provide detailed insight in the influence of genetic variation on ligand-induced GPCR function within the general human population. Our selection process of SNP containing GPCRs to be investigated with label-free technology and LCLs is visualized in **Figure 3**. In this thesis we focused on SNPs that are likely to have a profound effect on GPCR signaling responses by changing the amino acid sequence, in particular the so-called missense SNPs. The biobank employed in this research, the Netherlands Twin Registry (NTR; *http://www.tweelingenregister.org/en/*) [39], offered genotyped LCLs of individuals with a family structure consisting of parents and twin siblings. We first established an overview of such SNPs on each druggable, non-olfactory GPCR gene within these NTR individuals, after

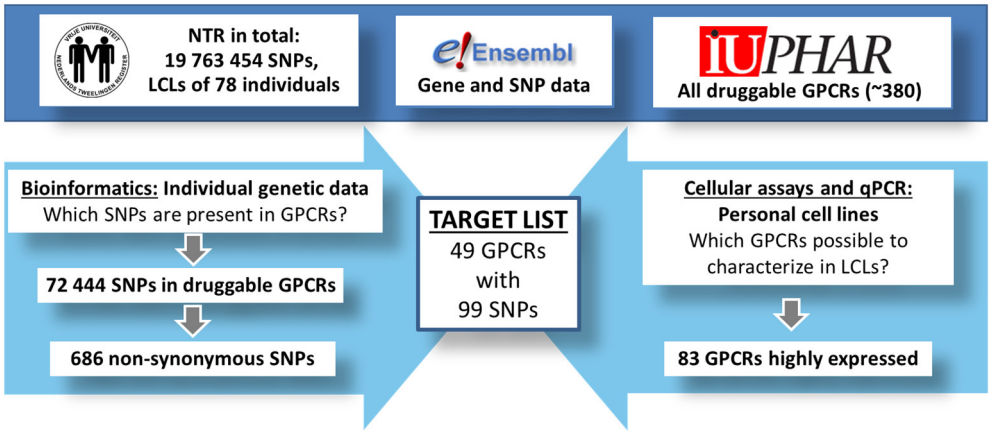

Figure 3: Flowchart of target selection. The selection was aimed at identifying all druggable GPCRs containing non-synonymous SNPs that were well enough expressed to allow functional characterization in LCLs from the NTR. For the bioinformatics, the selection was limited to non-synonymous SNPs in druggable GPCRs. The genotype data of the cell lines was provided by the NTR, as part of the Genomes of the Netherlands (GoNL) consortium [39]. A list of all druggable non-olfactory GPCRs was downloaded from the IUPHAR database. SNPs within each gene were extracted from the NTR data using PLINK, an open-source whole genome association analysis toolset, and annotated with their SNP-consequence types (gene data, SNP location and consequences were extracted from Ensembl). Cellular assays and qPCR were used to determine which GPCRs were expressed above a threshold that allowed functional responses to be measured using the label-free technology.

which we pursued several interesting cases in GPCRs commonly used in drug research.

Three separate cases of common polymorphisms that affect GPCR signaling and cellular effects were discovered, each revealing different properties including the sensitivity of partial versus full agonists, different chemical scaffolds and intron versus missense SNPs. These examples should provide the reader with insights that will hopefully lead to the development of clinically more effective drugs and drug treatment paradigms in the long term.

Outline of this thesis

The concept of using patient-derived cell lines as model systems is introduced and discussed in **Chapter 2**. This chapter furthermore highlights the advantages of label-free technology for assays on such cell lines.

Chapter 3 focuses on the optimization and application of an impedance-based label-free assay, the xCELLigence, to suspension cells such as LCLs to allow direct measurement of cellular effects of GPCR signaling.

Chapter 4 presents the case of the Adenosine A_{2A} receptor, in which an intron SNP is related to differential cellular effects of a partial agonist, but not full agonists or antagonists. **Chapter 5** summarizes the effects on a highly common non-synonymous polymorphism on the Cannabinoid Receptor 2, to which different chemical scaffolds show different sensitivity. **Chapter 6** presents the case of the Glucose-Dependent Insulinotropic Polypeptide (GIP) Receptor, in which a missense SNP that has often been associated with diseases changes the cellular effects of the endogenous ligand.

The research presented in these chapters highlights that coding and non-coding, common and less common genetic variations in GPCRs can affect endogenous signaling as well as drug effects.

An overall conclusion from the results described in this thesis and forthcoming opportunities for drug discovery and treatment are presented and discussed in **Chapter 7**.

References

1. Carey, J. *Making personalized medicine pay*. BusinessWeek 2010 [cited 2015 24.06.2015]; Available from: *http://www.bloomberg.com/bw/magazine/content/10_05/b4165058407403.htm*.
2. van't Veer, L.J. and R. Bernards, *Enabling personalized cancer medicine through analysis of gene-expression patterns.* Nature, 2008. **452**(7187): p. 564-70.
3. Kojima, R., D. Aubel, and M. Fussenegger, *Novel theranostic agents for next-generation personalized medicine: small molecules, nanoparticles, and engineered mammalian cells.* Curr Opin Chem Biol, 2015. **28**: p. 29-38.
4. Lu, Y.F., et al., *Personalized medicine and human genetic diversity.* Cold Spring Harb Perspect Med, 2014. **4**(9).
5. Venkatakrishnan, A.J., et al., *Molecular signatures of G-protein-coupled receptors.* Nature, 2013. **494**(7436): p. 185-94.
6. Mirnezami, R., J. Nicholson, and A. Darzi, *Preparing for precision medicine.* The New England journal of medicine, 2012. **366**(6): p. 489-91.
7. Katsanis, S.H., G. Javitt, and K. Hudson, *Public health. A case study of personalized medicine.* Science, 2008. **320**(5872): p. 53-4.
8. Weng, L., et al., *Pharmacogenetics and pharmacogenomics: a bridge to individualized cancer therapy.* Pharmacogenomics, 2013. **14**(3): p. 315-24.
9. Probst-Schendzielorz, K., R. Viviani, and J.C. Stingl, *Effect of Cytochrome P450 polymorphism on the action and metabolism of selective serotonin reuptake inhibitors.* Expert Opin Drug Metab Toxicol, 2015. **11**(8): p. 1219-32.
10. Overington, J.P., B. Al-Lazikani, and A.L. Hopkins, *How many drug targets are there?* Nat Rev Drug Discov, 2006. **5**(12): p. 993-6.
11. Rask-Andersen, M., M.S. Almen, and H.B. Schioth, *Trends in the exploitation of novel drug targets.* Nat Rev Drug Discov, 2011. **10**(8): p. 579-90.
12. Russ, A.P. and S. Lampel, *The druggable genome: an update.* Drug Discov Today, 2005. **10**(23-24): p. 1607-10.
13. Lagerstrom, M.C. and H.B. Schioth, *Structural diversity of G protein-coupled receptors and significance for drug discovery.* Nat Rev Drug Discov, 2008. **7**(4): p. 339-57.
14. Sadee, W., et al., *Genetic variations in human G protein-coupled receptors: implications for drug therapy.* AAPS pharmSci, 2001. **3**(3): p. 54-80.
15. Davies, M.A., et al., *Pharmacologic analysis of non-synonymous coding h5-HT2A SNPs reveals alterations in atypical antipsychotic and agonist efficacies.* Pharmacogenomics J, 2006. **6**(1): p. 42-51.
16. Docherty, S.J., et al., *A genetic association study of DNA methylation levels in the DRD4 gene region finds associations with nearby SNPs.* Behav Brain Funct, 2012. **8**: p. 31-44.
17. Ishiguro, H., et al., *Brain cannabinoid CB2 receptor in schizophrenia.* Biol Psychiatry, 2010. **67**(10): p. 974-82.
18. Tong, D., et al., *Association of single-nucleotide polymorphisms in the cannabinoid receptor 2 gene with schizophrenia in the Han Chinese population.* J Mol Neurosci, 2013. **51**(2): p. 454-60.

19. Al-Fulaij, M.A., et al., *Pharmacological analysis of human D1 AND D2 dopamine receptor missense variants.* Journal of molecular neuroscience : MN, 2008. **34**(3): p. 211-23.
20. Ishiguro, H., et al., *Mutation and association analysis of the 5' region of the dopamine D3 receptor gene in schizophrenia patients: identification of the Ala38Thr polymorphism and suggested association between DRD3 haplotypes and schizophrenia.* Molecular psychiatry, 2000. **5**(4): p. 433-8.
21. Lundstrom, K. and M.P. Turpin, *Proposed schizophrenia-related gene polymorphism: expression of the Ser9Gly mutant human dopamine D3 receptor with the Semliki Forest virus system.* Biochemical and biophysical research communications, 1996. **225**(3): p. 1068-72.
22. Bodenmann, S., et al., *Polymorphisms of ADORA2A modulate psychomotor vigilance and the effects of caffeine on neurobehavioural performance and sleep EEG after sleep deprivation.* British journal of pharmacology, 2012. **165**(6): p. 1904-13.
23. Childs, E., et al., *Association between ADORA2A and DRD2 polymorphisms and caffeine-induced anxiety.* Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 2008. **33**(12): p. 2791-800.
24. Freitag, C.M., et al., *Adenosine A(2A) receptor gene (ADORA2A) variants may increase autistic symptoms and anxiety in autism spectrum disorder.* European child & adolescent psychiatry, 2010. **19**(1): p. 67-74.
25. Buscher, R., et al., *P2Y2 receptor polymorphisms and haplotypes in cystic fibrosis and their impact on Ca2+ influx.* Pharmacogenetics and genomics, 2006. **16**(3): p. 199-205.
26. Wesselius, A., et al., *Association of P2Y(2) receptor SNPs with bone mineral density and osteoporosis risk in a cohort of Dutch fracture patients.* Purinergic signalling, 2013. **9**(1): p. 41-9.
27. Kruglyak, L. and D.A. Nickerson, *Variation is the spice of life.* Nature Genetics, 2001. **27**(3): p. 234-236.
28. Fellay, J., et al., *Common genetic variation and the control of HIV-1 in humans.* PLoS Genet, 2009. **5**(12): p. e1000791.
29. Mitchell, B.D., et al., *A paired sibling analysis of the beta-3 adrenergic receptor and obesity in Mexican Americans.* J Clin Invest, 1998. **101**(3): p. 584-7.
30. Sivagnanasundaram, S., et al., *A cluster of single nucleotide polymorphisms in the 5'-leader of the human dopamine D3 receptor gene (DRD3) and its relationship to schizophrenia.* Neurosci Lett, 2000. **279**(1): p. 13-6.
31. Frank, R.A., et al., *Clustered coding variants in the glutamate receptor complexes of individuals with schizophrenia and bipolar disorder.* PLoS One, 2011. **6**(4): p. e19011.
32. Eglen, R. and T. Reisine, *Primary cells and stem cells in drug discovery: emerging tools for high-throughput screening.* Assay Drug Dev Technol, 2011. **9**(2): p. 108-24.
33. Yu, N., et al., *Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: an approach to study G protein-coupled receptors.* Anal Chem, 2006. **78**(1): p. 35-43.
34. Artene, S.A., et al., *Biobanking in a constantly developing medical world.* ScientificWorldJournal, 2013. **2013**: p. 343275.

35. Ministers, C.o.E.a.C.o., *Recommendation Rec 4 of the Committee of Ministers to Member States on Research on Biological Materials of Human Origin*, S. European Commission, France, Editor 2006.
36. *https://ec.europa.eu/research/swafs/pdf/pub_archive/biobanks-for-europe_en.pdf*. 03.05.2016].
37. Sie, L., S. Loong, and E.K. Tan, *Utility of lymphoblastoid cell lines.* J Neurosci Res, 2009. **87**(9): p. 1953-9.
38. Sugimoto, M., et al., *Steps involved in immortalization and tumorigenesis in human B-lymphoblastoid cell lines transformed by Epstein-Barr virus.* Cancer research, 2004. **64**(10): p. 3361-4.
39. Willemsen, G., et al., *The Netherlands Twin Register biobank: a resource for genetic epidemiological studies.* Twin Res Hum Genet, 2010. **13**(3): p. 231-45.
40. Abecasis, G.R., et al., *A map of human genome variation from population-scale sequencing.* Nature, 2010. **467**(7319): p. 1061-73.
41. Welsh, M., et al., *Pharmacogenomic discovery using cell-based models.* Pharmacological reviews, 2009. **61**(4): p. 413-29.
42. Dausset, J., et al., *Centre d'etude du polymorphisme humain (CEPH): collaborative genetic mapping of the human genome.* Genomics, 1990. **6**(3): p. 575-7.
43. Wheeler, H.E. and M.E. Dolan, *Lymphoblastoid cell lines in pharmacogenomic discovery and clinical translation.* Pharmacogenomics, 2012. **13**(1): p. 55-70.
44. Lee, P.H., et al., *Evaluation of dynamic mass redistribution technology for pharmacological studies of recombinant and endogenously expressed g protein-coupled receptors.* Assay Drug Dev Technol, 2008. **6**(1): p. 83-94.
45. Tran, E. and F. Ye, *Duplexed label-free G protein--coupled receptor assays for high-throughput screening.* J Biomol Screen, 2008. **13**(10): p. 975-85.
46. Verdonk, E., et al., *Cellular dielectric spectroscopy: a label-free comprehensive platform for functional evaluation of endogenous receptors.* Assay Drug Dev Technol, 2006. **4**(5): p. 609-19.
47. Fang, Y., *Label-Free Receptor Assays.* Drug Discov Today Technol, 2011. **7**(1): p. e5-e11.
48. Rocheville, M., et al., *Mining the potential of label-free biosensors for seven-transmembrane receptor drug discovery.* Progress in molecular biology and translational science, 2013. **115**: p. 123-42.
49. Ferrie, A.M., H. Sun, and Y. Fang, *Label-free integrative pharmacology on-target of drugs at the beta(2)-adrenergic receptor.* Sci Rep, 2011. **1**: p. 33.
50. Morse, M., et al., *Ligand-directed functional selectivity at the mu opioid receptor revealed by label-free integrative pharmacology on-target.* PLoS One, 2011. **6**(10): p. e25643.
51. Stallaert, W., et al., *Impedance responses reveal beta(2)-adrenergic receptor signaling pluridimensionality and allow classification of ligands with distinct signaling profiles.* PLoS One, 2012. **7**(1): p. e29420.