NUCLEAR RECEPTOR PROFILING

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1. Overview

Nuclear Receptors (NRs) act as transcription factors that mediate the effects of certain hormones, drugs, and other xenobiotics by modulating the expression of specific genes involved in many cellular functions such as development, reproduction, and metabolism. Conserved functional domains of NRs, such as the DNA-binding domain (DBD), ligand-binding domain (LBD), N- and C-terminal transcriptional activation domains (AF-1 and AF-2, respectively), define this class of transcription factors[1]. Their disparate physiologic roles are determined by diversity in both ligand and DNA binding specificities, as well as in specific interactions with co-activator and co-repressor molecules that combinatorially mediate transcription.
A major focus in the current discovery of drugs targeting NRs is identifying drugs with reduced side effects by improving selectivity, not only from other receptors but also by selective modulation of the NR of interest. Cellular assays not only provide valuable information on functional activity, potency, and selectivity but also are ideally suited for differentiating full and partial agonists, inverse agonists, and antagonists.
In addition, there is increasing interest in understanding potential drug-drug and drug-nutrient interactions of potential new drugs[2]. This information can be provided by examining the activity of those receptors that modulate the expression of drug metabolism enzymes, in particular the constitutive androstane receptor (CAR), pregnane X receptor (PXR), and aryl hydrocarbon receptor (AhR). By examining a diverse panel of cell-based NR assays, in agonist, inverse agonist, and antagonist formats, one can gain the type of information that is essential in deciding which potential drugs to pursue in subsequent steps of the drug discovery process. In this article, seventeen compounds were examined across 25 NRs in agonist, inverse-agonist, and/or antagonist modes to serve as an example of the utility of profiling in making decisions regarding the liabilities of compounds of interest.

2. Types of NR activity

When discussing a drug that binds with specificity to a receptor there are several classifications that are dependent on the biological response and should be addressed in a drug profiling study (Figure 1).
An **agonist** is a ligand that increases the activity of a receptor above its basal, or constitutive level.
A **full agonist** will bind to the LBD of the receptor and initiates a conformational change that results in high affinity interaction with transcriptional coactivator molecules. A **partial agonist** will also bind to the receptor, perhaps with the same affinity as a full agonist, but the resultant conformational change provides a weaker association with coactivator molecules. An **inverse agonist** is an agent that binds to the receptor but induces a pharmacological response opposite to that of an agonist. A prerequisite for an inverse agonist response is that the receptor must have high constitutive or basal activity in the absence of an identifiable ligand. The conformational change of the NR that results from an inverse agonist either decreases its association with coactivator molecules or increases affinity for corepressor proteins.
There are several examples of inverse agonists of nuclear receptors, in particular of the estrogen receptor-related receptors (ERRα,β,γ)[3], retinoic acid-related orphan receptors (RORα,β,γ)[4] and constitutive androstane receptor-1 (CAR-1)[6].
A receptor **antagonist** is a type of ligand that blocks or ameliorates agonist-mediated responses.

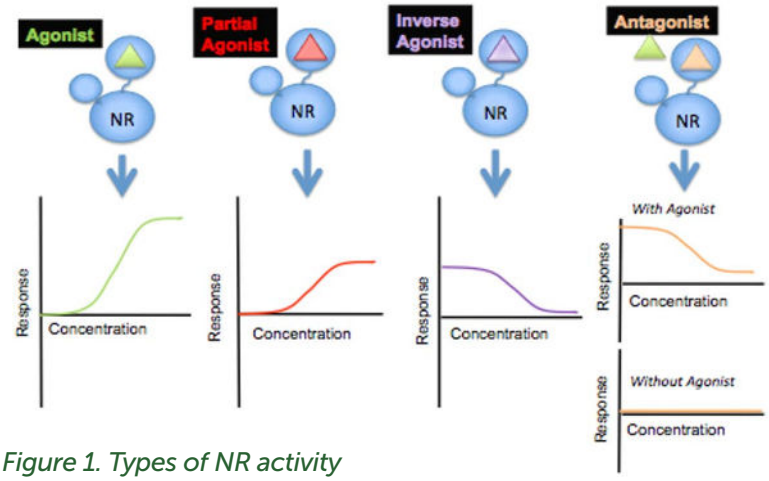


Figure 1. Types of NR activity

A differentiating factor between an inverse agonist and an antagonist is that the latter functions in the presence of an agonist. **Full and partial antagonists** depend on the conformational shape that results from association with the receptor. Full antagonists do not exhibit agonist properties whereas partial antagonist are also by definition partial agonists. It is important to note that while examining inverse agonists and antagonists toxicity is taken into account as a decrease in viability may present itself as either of these ligand-types. **Selective Receptor Modulators (SRMs)** are compounds that result in a conformational change between full agonists and antagonists and hence have varying degrees of coactivator/corepressor affinity[7]. This results in tissue-specific responses that are dependent on the cellular context, in particular the concentration of transcriptional cofactors. An important advantage to utilizing whole-cell based assays is that the whole spectrum of NR activity may be distinguished.

3. Specific Nuclear Receptor Assays

All assays were performed by INDIGO Biosciences, Inc. (State College, PA) contract screening laboratory. All receptors were examined using cell-based reporter assay systems (Figure 2) which feature engineered nuclear receptor-specific reporter cells prepared using their unique CryoMite™ process. Test compounds were screened for agonist, inverse agonist, or antagonist activities against human nuclear receptors expressed within healthy, dividing mammalian cells. The reporter systems utilize firefly luciferase reporter gene technology to provide optimal assay sensitivity and dynamic range when quantifying nuclear receptor activity. Following ligand-activation, the nuclear receptor complex acts to regulate expression of the luciferase reporter. Upon addition of detection reagent, the intensity of light emission from the luciferase reaction directly correlates to the activation status of the nuclear receptor.

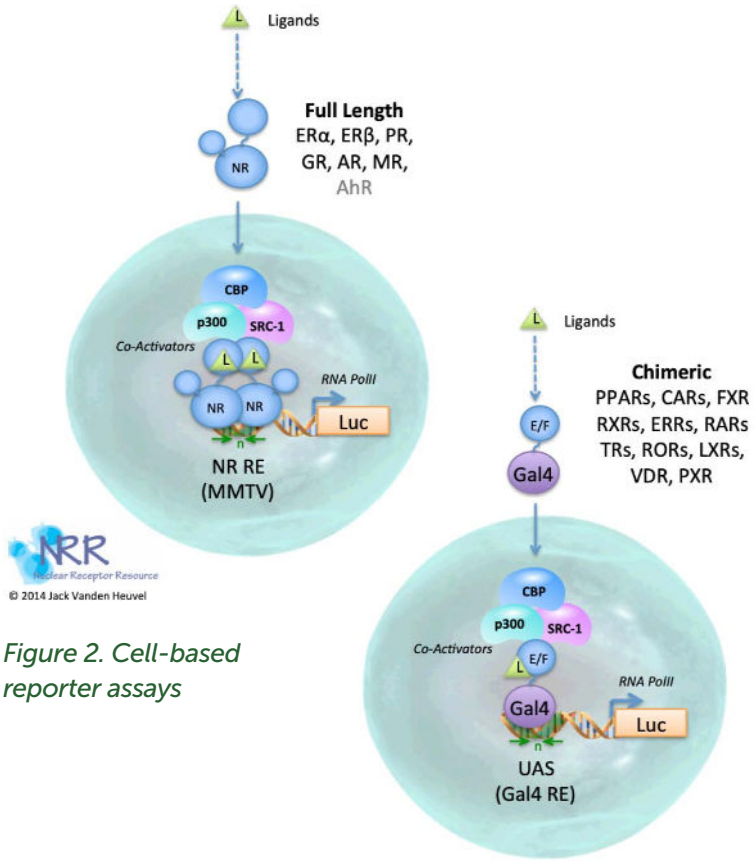


Figure 2. Cell-based reporter assays

When screening test compounds for antagonist activities it is particularly important to quantify changes in the relative number of live reporter cells at the assay endpoint. Test compounds that exert cytostatic, cytotoxic, or cytolytic activities invariably generate "false-positive" results in an antagonist and inverse agonist screens. In such cases, the observed drop in luciferase activity will be incorrectly attributed to inhibition of the nuclear receptor by the test compound. In reality, however, the treatment compound has pushed the reporter cells into division arrest, apoptosis, necrosis, or lysis. The Live Cell Multiplex (LCM) Assay provides an efficient fluorescence-based method of quantifying the relative number of live cells resident in treated wells of an assay plate. The fluorescence-based LCM Assay and the luminescence-based INDIGO NR Assay were performed sequentially using the same assay wells, thus eliminating false-positive inverse agonists and antagonists in the profile screening.

4. Functional Classification of Nuclear Receptors

As mentioned above, NRs act as sensors for various intracellular molecules including hormones and fatty acid metabolites. The effects of these ligands can be understood by the fact that numerous genes involved in the cellular processes, such as general homeostasis, growth, and defense against microbes, are under the control of NRs. The target gene and protein expression patterns of NRs and their physiological consequences create a network that can be monitored by multimodal approaches, such as systems biology[8]. By understanding in which diseases, pathways, and networks each NR participates, it is easier to anticipate the consequences of regulation of unintended targets by a compound of interest. Although the dominant paradigm in drug discovery is to design maximally selective ligands to act on individual NR targets, many effective drugs act via modulation of multiple proteins rather than single targets. Advances in systems biology are revealing a phenotypic robustness and a network structure that strongly suggests that exquisitely selective compounds, compared with multitarget drugs, may exhibit lower than desired clinical efficacy[9]. This new appreciation of the role of polypharmacology has significant implications for the two major sources of attrition in drug development, efficacy and toxicity[9]. Thus, defining the biological niche of each NR, as well as understanding overlapping pathways and functions, provides valuable context for evaluating a compound's liabilities and promise.

Defining the system that each NR participates can be approached in several ways (Table 1) such as sequence similarity[10], potential disease implication,[1,11] or transcriptional networks[12]. The latter is particularly helpful since it encompasses NRs to which there are no known endogenous ligands (orphan receptors) or have few selective pharmacologic agents to evaluate biological consequences. In the present profiling experiment, the receptors are classified as follows:[12]

CNS, Circadian, and Basal Metabolism: LXRB, MR, RARB, RORA, RORB, RXRB, RXRG, THRA

Lipid Metabolism and Energy: ESSRA, GR, LXRA, PPARA, PPARD, PPARG, RXRA, THRB

Reproduction and Development: AR, ESR1, ESR2, PGR, RARA, RARG

Xenobiotic and Bile Acid Metabolism: AhR, CAR1, CAR2, CAR3, FXR, PXR, RORC, VDR

5. Results

Seventeen compounds were examined for potential agonistic effects at six doses in triplicate against twenty-five human nuclear receptors. The receptors examined were AhR, AR, CAR2, CAR3, ERα, ERβ, FXR, GR, LXRα, LXRβ, MR, PPARα, PPARδ, PPARγ, PR, PXR, RARα, RARβ, RARγ, RXRα, RXRβ, RXRγ, TRα, TRβ, and VDR. Similarly, three receptors were examined for inverse agonism including CAR1, RORα, and RORγ. In addition, twenty-four receptors were examined in presence of an ~EC_{50} concentration of reference agonist to determine potential antagonists (AhR, AR, CAR3, ERα, ERβ, FXR, GR, LXRα, LXRβ, MR, PPARα, PPARβ, PPARγ, PR, PXR, RARα, RARβ, RARγ, RXRα, RXRβ, RXRγ, TRα, TRβ, and VDR). Since inverse agonists and antagonists both exhibit decreased activity, there are examined concurrently in the summary figures.

With datasets of this size, the interpretation of the results can be a challenge. Some of the typical analysis and visualization approaches include: (1) Summary tables with fold induction or inhibition. This may or may not include results of statistical analysis such as significance versus vehicle control (i.e. Dunnett's t-test) or fold-change cut-off (i.e. 2-fold change). (2) Summary graphs grouped by receptor and mode. This should include curve-fitting by non-linear regression with calculation of potency (EC50 or IC50) and efficacy (peak activity, fold-change). (3) Dot-plots or similar means to quickly examine a single nuclear receptor's activity for a large number of compounds. (4) Grouping activity by compound. A useful analysis and visualization method is hierarchical clustering, which can assist in grouping compounds or receptors with similar activity.

Table 1. Nuclear receptors and their potential indications

NR	SYMBOL	ENTREZ GENE NAME	POTENTIAL INDICATIONS
NR0B1	DAX-1	Nuclear receptor subfamily 0, group B, member 1	Adrenal disease, fertility, gynecologic disorders
NR0B2	SHP-1	Nuclear receptor subfamily 0, group B, member 2	Obesity, dyslipidemia, diabetes, liver disease, cancer
NR1A1	THRA	Thyroid hormone receptor alpha	Obesity, dyslipidemia, hypothyroidism
NR1A2	THRB	Thyroid hormone receptor beta	Obesity, dyslipidemia, hypothyroidism
NR1B1	RARA	Retinoic acid receptor alpha	Cancer, psoriasis
NR1B2	RARB	Retinoic acid receptor beta	Cancer, psoriasis
NR1B3	RARG	Retinoic acid receptor gamma	Cancer, psoriasis
NR1C1	PPARA	Peroxisome proliferator-activated receptor alpha	Dyslipidemia, atherosclerosis, inflammation
NR1C2	PPARD	Peroxisome proliferator-activated receptor delta	Obesity, inflammation, cancer
NR1C3	PPARG	Peroxisome proliferator-activated receptor gamma	Diabetes, obesity, cancer, inflammation, osteoporosis
NR1D1	REVERBA	Nuclear receptor subfamily 1, group D, member 1	Circadian rhythm, atherosclerosis
NR1D2	REVERB	Nuclear receptor subfamily 1, group D, member 2	Circadian rhythm, atherosclerosis
NR1F1	RORA	RAR-related orphan receptor A	Atherosclerosis, dyslipidemia, inflammation, rheumatoid arthritis, osteoporosis, neurodegeneration
NR1F2	RORB	RAR-related orphan receptor B	
NR1F3	RORC	RAR-related orphan receptor C	Osteoporosis, immunosuppression
NR1H2	LXRB	Nuclear receptor subfamily 1, group H, member 2	Dyslipidemia, atherosclerosis, diabetes
NR1H3	LXRA	Nuclear receptor subfamily 1, group H, member 3	Dyslipidemia, atherosclerosis, diabetes

Table 1. Nuclear receptors and their potential indications (cont'd.)

NR	SYMBOL	ENTREZ GENE NAME	POTENTIAL INDICATIONS
NR1H4	FXR	Nuclear receptor subfamily 1, group H, member 4	Dyslipidemia, liver disease
NR1I1	VDR	Vitamin D (1,25- dihydroxyvitamin D3) receptor	Osteoporosis, psoriasis, cancer, inflammation
NR1I2	PXR	Nuclear receptor subfamily 1, group I, member 2	Xenobiotic metabolism
NR1I3v1	CAR1	Nuclear receptor subfamily 1, group I, member 3 variant 1	Xenobiotic metabolism
NR1I3v2	CAR2	Nuclear receptor subfamily 1, group I, member 3 variant 2	Xenobiotic metabolism
NR1I3v3	CAR3	Nuclear receptor subfamily 1, group I, member 3 variant 3	Xenobiotic metabolism
NR2A1	HNF4A	Hepatocyte nuclear factor 4 alpha	Diabetes, dyslipidemia
NR2A2	HNF4G	Hepatocyte nuclear factor 4 gamma	
NR2B1	RXRA	Retinoid X receptor alpha	Diabetes, cancer
NR2B2	RXRB	Retinoid X receptor beta	Diabetes, cancer
NR2B3	RXRG	Retinoid X receptor gamma	Diabetes, cancer
NR2C1	TR2	Nuclear receptor subfamily 2, group C, member 1	Cancer, male fertility
NR2C2	TR4	Nuclear receptor subfamily 2, group C, member 2	Obesity
NR2E1	TLL	Nuclear receptor subfamily 2, group E, member 1	Neurodegeneration
NR2E3	PNR	Nuclear receptor subfamily 2, group E, member 3	Retinal degeneration
NR2F1	COUPTFA	Nuclear receptor subfamily 2, group F, member 1	Breast cancer, neural development
NR2F2	COUPTFB	Nuclear receptor subfamily 2, group F, member 2	Cancer, angiogenesis

Table 1. Nuclear receptors and their potential indications (cont'd.)

NR	SYMBOL	ENTREZ GENE NAME	POTENTIAL INDICATIONS
NR2F6	EAR2	Nuclear receptor subfamily 2, group F, member 6	Uterine/gynecological disorders
NR3A1	ESR1	Estrogen receptor 1 (ER alpha)	Breast cancer, osteoporosis, cardiovascular disease, gynecological disorders, Alzheimer's
NR3A2	ESR2	Estrogen receptor 2 (ER beta)	Prostate cancer, osteoporosis, obesity, cardiovascular disease, Alzheimer's
NR3B1	ESRRA	Estrogen-related receptor alpha	Osteoporosis, dyslipidemia
NR3B2	ESRRB	Estrogen-related receptor beta	Fertility
NR3B3	ESRRG	Estrogen-related receptor gamma	Fertility
NR3C1	GR	Nuclear receptor subfamily 3, group C, member 1	Arthritis, asthma, immunosuppression, obesity, diabetes
NR3C2	MR	Nuclear receptor subfamily 3, group C, member 2	Hypertension, CHF
NR3C3	PGR	Progesterone receptor	Contraception, cancer, osteoporosis
NR3C4	AR	Androgen receptor	Frailty, prostate cancer, sexual dysfunction, osteoporosis
NR4A1	NGFIB	Nuclear receptor subfamily 4, group A, member 1	Drug abuse, cancer, schizophrenia, manic-depression, psychoses, neurodegeneration, immunomodulation
NR4A2	NURR1	Nuclear receptor subfamily 4, group A, member 2	Parkinson's, schizophrenia, manic-depression, cancer
NR4A3	NOR1	Nuclear receptor subfamily 4, group A, member 3	Drug abuse, cancer, immunomodulation
NR5A1	SF1	Nuclear receptor subfamily 5, group A, member 1	Adrenal disease, disorders of steroid metabolism
NR5A2	LRH-1	Nuclear receptor subfamily 5, group A, member 2	Breast cancer, fertility, dyslipidemia
NR6A1	GCNF	Nuclear receptor subfamily 6, group A, member 1	Fertility/contraception
	AhR	Aryl Hydrocarbon receptor	Xenobiotic metabolism

This particular set of compounds were developed as potential RORγ inverse agonists. As shown in Table 1, this NR is an emerging therapeutic target that has been proposed to play a role in osteoporosis and immunosuppression. The test compounds elicited dose-dependent effects on RORγ, albeit with varying efficacy and potency (see Figure 3 and Table 2). From this analysis of a single receptor, decisions can be made on which compounds meet minimal criteria for an effective drug and can be benchmarked relative to a reference compound (in this case ursolic acid). Several compounds had EC50 values in nM or low μM concentrations (i.e. IBI21014-01, -10, -17) with peak activity equal to or greater than the reference compound (all except -17, -06, -15). By examining the Peak Activity/EC50 (P/E) the compounds can be evaluated by both parameters, much in the manner that intrinsic activity is defined for enzymes (Vmax/Km). Using this approach the top potential candidates are IBI2014-01, -10, -04, -02, and -03. This analysis has merit as a means to create scaffolds for future compound development (i.e. use IBI21014-1 as base molecule to modify) and can be performed for thousands of compounds in a relatively short period of time. However, without knowledge of off-target effects there is no good indication as to the overall specificity or safety.

Figure 3 RORγ inverse agonism

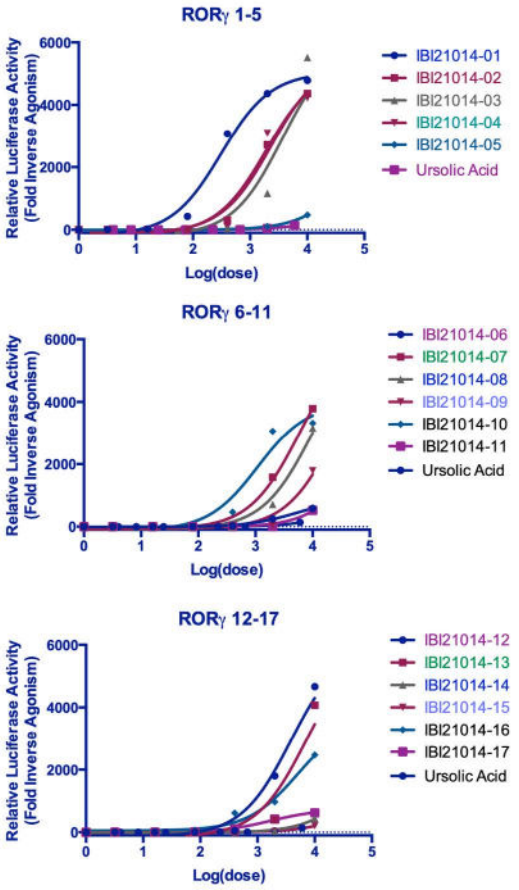


Table 2. RORγ Inverse Agonism

CMPD.	EC_{50}	EC_{50} RANK	PEAK	PEAK RANK	PEAK/EC_{50}	P/E RANK
IBI21014-01	346.5	1	5075	11	14.65	1
IBI21014-02	2567	5	5572	7	2.17	4
IBI21014-03	3896	6	5999	1	1.54	5
IBI21014-04	2130	4	5310	9	2.49	3
IBI21014-05	99996	14	5175	10	0.05	15
IBI21014-06	6036	10	938.6	17	0.15	13
IBI21014-07	5994	9	5999	1	1.00	7
IBI21014-08	10152	12	5999	1	0.59	10
IBI21014-09	26397	13	5999	1	0.23	12
IBI21014-10	1214	2	3988	13	3.29	2
IBI21014-11	100000	15	5491	8	0.05	14
IBI21014-12	4067	7	5999	1	1.48	6
IBI21014-13	7508	11	5999	1	0.80	9
IBI21014-14	100000	15	4622	12	0.05	16
IBI21014-15	100000	15	2199	16	0.02	18
IBI21014-16	4364	8	3514	14	0.81	8
IBI21014-17	1785	3	742	18	0.42	11
Ursolic Acid	100000	15	2310	15	0.02	17

Making critical decisions about which compounds to pursue in future studies or what types of potential liabilities exist from your drug leads can be a challenge when faced with this amount of information. For that reason, we have developed a simple evaluation tool that may assist in this process, the IBIPlot™ (Figure 4). This method takes advantage of the classification schemes described above and can clearly delineate not only which receptors are affected by a compound but also what pathways or functions may be influenced. In this example, the receptors are classified based on transcriptional networks[12]. The scale is set from fold change 1-10 (10 being the outermost ring) and is examined at the highest concentration of each compound, and each compound can be examined at a single dose. The IBIPlots of the top five candidates (10 μM) are shown in Figure 5. Note that since RORγ activity results in inverse agonism, the desired activity is depicted in the lower panel of figures (the last bar in the purple shaded Xenobiotic and Bile Acid Metabolism group). All compounds exhibits significant PXR activation (top figures) to varying degrees ranging from 4.8 fold (IBI21014-01) to 24.5-fold (-02). Compound IBI21014-10 also exhibited AhR agonistic activity (3.3-fold). In addition to being an RORg inverse agonist, compound IBI21014-01 was an AhR and PGR antagonist, and an RORγ inverse agonist. IBI21014-02 was an RXRβ antagonist while the other top candidates had no other NR inverse agonism or antagonism properties.

Figure 4. IBIPlot™ Key

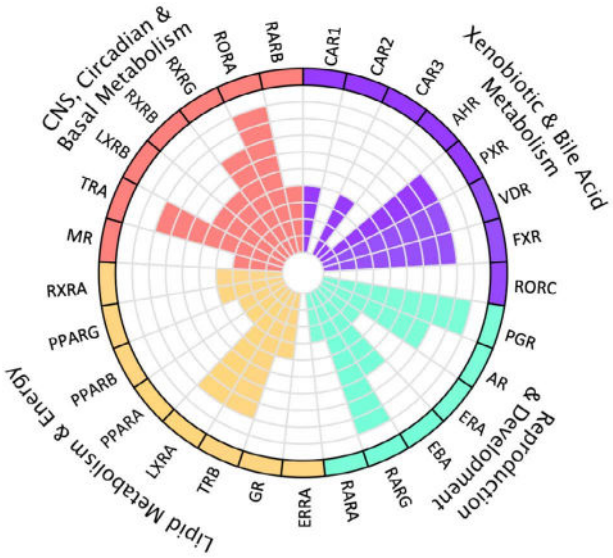
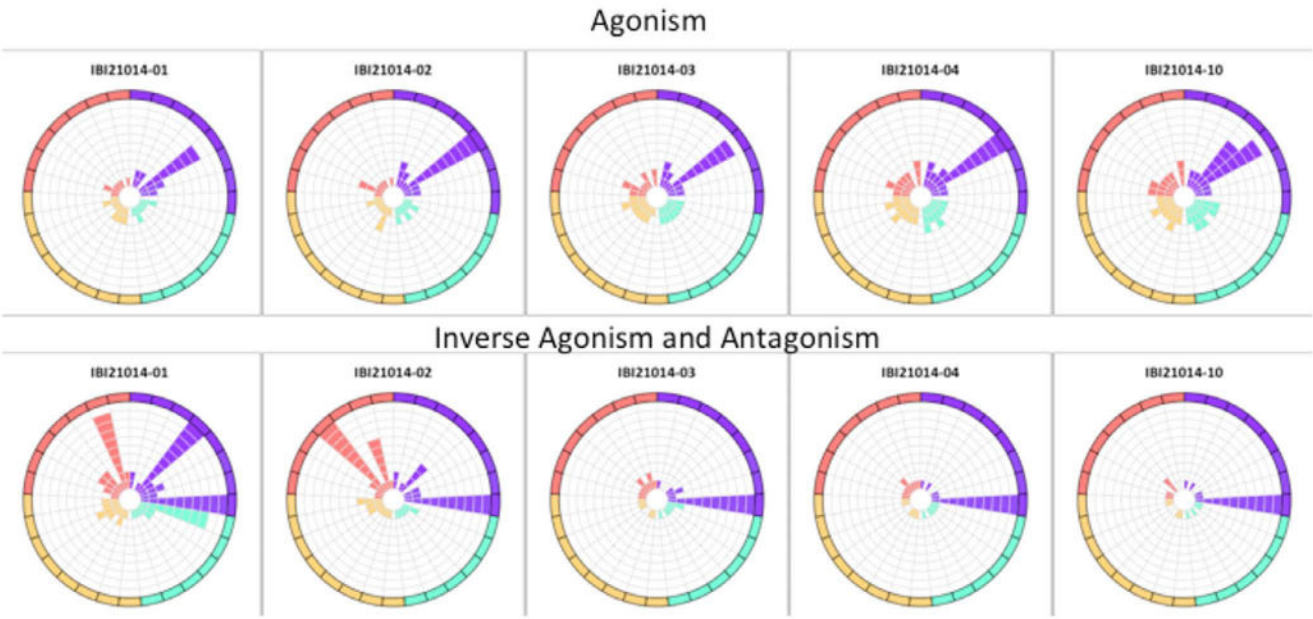


Figure 5. IBIPlots™ of top five candidates. See Figure 4 for key



With this information it is much easier to make critical decisions on the fate of the compounds under consideration. For example, compound IBI21014-01 was the top ranked as an RORγ inverse agonist and had the least amount of PXR activation, but it also had lower specificity than the other candidates. The question can be raised, are these off-target events potential harmful or can they be an asset? For example a dual RORα/γ, as seen with IBI2104-01, may be advantageous due to there similar biological niche. One must also consider the potency and efficacy of the off-target events and rank the candidates accordingly. Obviously, there are no black-and-white answers to the best candidate, but with the information provided in a profiling screen such as that described above, the decisions are made with a great deal of information. This reduces the risk of making a poor decision and can save both time and money in the drug discovery process.

6. References

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