NR and High-Throughput Screening: Putting the Pieces Together

Keith A. Houck, Ph.D.

National Center for Computational Toxicology
U.S. Environmental Protection Agency
Research Triangle Park, NC
Phone: (919) 541-5519
Houck.keith@epa.gov

Course Objectives/Outline

- Screening for NR Ligands
  - Radioligand binding assays
  - Fluorescence polarization binding assays
  - Cellular transactivation assays
  - Functional biochemical assays
  - Biotransformation
- Quantitation of Results
  - Concentration-response curves for nuclear receptor activity
  - Potency
  - Efficacy
  - Partial agonists
- Secondary Screening Methods
  - Orthogonal assays
  - Distinguishing selective receptor modulators
  - Regulation of target gene expression
- Nuclear Receptor Screening and Safety Assessment

Abbreviations

- AF1 activation function 1
- AF2 activation function 2
- AR androgen receptor
- CAR constitutive androstane receptor
- CAT chloramphenicol transferase
- CDCA chenodeoxycholic acid
- DBD DNA-binding domain
- DES diethylstilbesterol
- E_{max} maximum efficacy
- ER estrogen receptor
- ERE estrogen response element
- FRET fluorescence resonance energy transfer
- FXR farnesoid X receptor
- GAL4 yeast transcription factor GAL4
- GR glucocorticoid receptor
- IC_{50} half maximal inhibitory concentration
- K_d equilibrium binding constant
- K_i equilibrium dissociation constant
- K_{off} dissociation rate constant
- K_{on} association rate constant
- LBD ligand-binding domain
- M-CSF macrophage colony stimulating factor
- MMTV mouse mammary tumor virus
- NR nuclear receptor
- Oligo oligonucleotide
- Pol II RNA polymerase II
- PPAR peroxisome proliferator-activator receptor
- PSA prostate specific antigen
- PXR pregnane X receptor
- RANKL receptor activator for nuclear factor κ B ligand
- RA retinoic acid
- RE response element
- RT-PCR real time polymerase chain reaction
- RXR retinoic X receptor
- SA streptavidin
- SPA scintillation proximity assay
- SRC-2 steroid receptor coactivator 2
- TAD transactivation domain
- TR thyroid receptor
- UAS upstream activation sequence
- VDR vitamin D receptor

Assays to Detect Nuclear Receptor Ligand Activity

Radioligand Binding Assays

- Gold standard assay
- Can determine K_i
- Requires source of receptor
  - Partial purification from tissues or cells
  - Recombinant expression--ligand-binding domain often used
- Requires medium- to high-affinity radiolabeled ligand
- High-throughput library screening technically challenging
  - 96-well filter plates and manifolds possible
  - Scintillation proximity assay useful for HTS

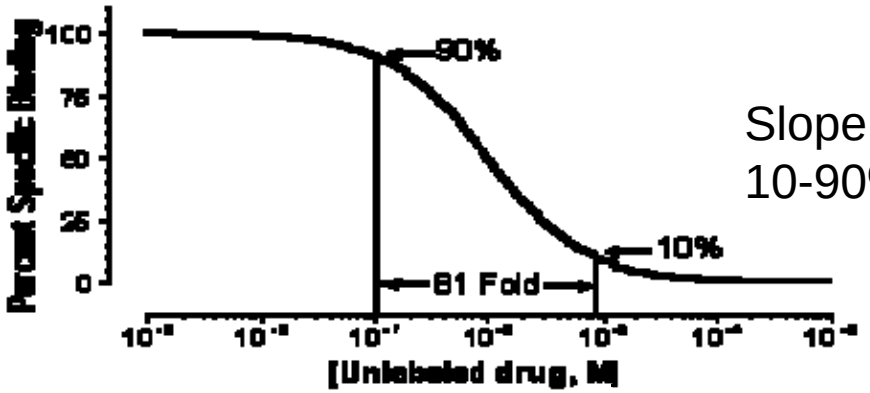
Radioligand Binding Assays

Ligand + Receptor $\Leftrightarrow$ Ligand-Receptor

- Law of mass action
  - Diffusion-controlled collision [Ligand][Receptor]k_{on}
  - Affinity-controlled dissociation [Ligand-Receptor]k_{off}
  - Equilibrium when rate of new complex formation = rate of Ligand-Receptor dissociation
- [Ligand][Receptor]/[Ligand-Receptor]=$k_{off}/k_{on}=K_d$
- K_d expressed in molar units
- Fractional occupancy = [Ligand]/[Ligand] + K_d
  - When [Ligand] = 0, occupancy = 0
  - When [Ligand] is high (many times Kd), occupancy » 100%
  - 4 X K_d is 80% occupancy

Radioligand Binding Assays

- Measures binding of labeled ligand at single concentration in presence of unlabeled compound
- Labeled ligand usually at $\leq K_d$ of radioligand for receptor
- Practical application of law of mass action:



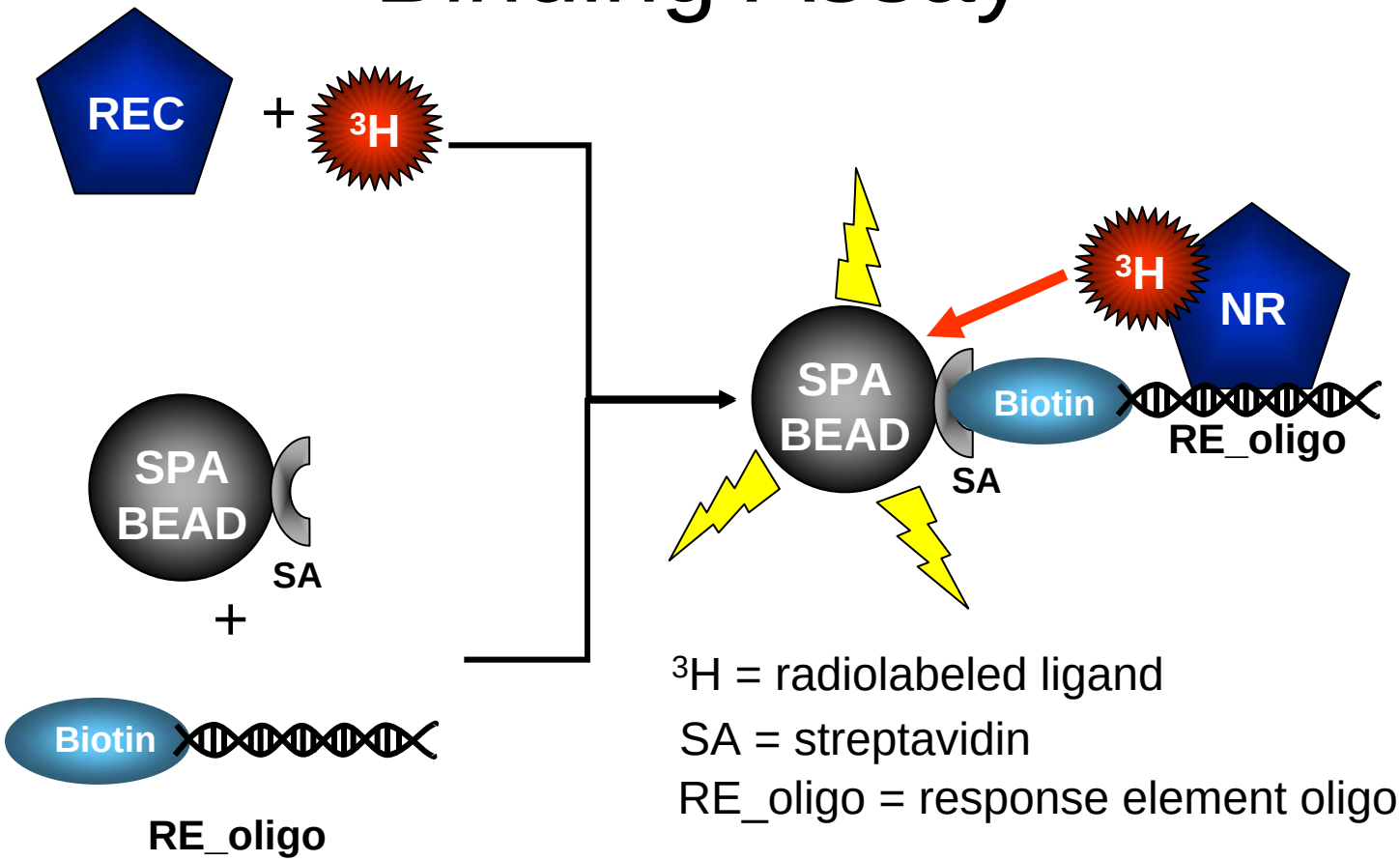
Slope ~ -1
10-90% over two logs concentration

- Fit using non-linear regression to determine IC_{50}
  - Can set top (= no competitor) or bottom (reference competitor)
  - Calculate K_i: $K_i = IC_{50}/(1+[\text{radioligand}]/K_d)$ (Cheng-Prusoff)
  - Example: IC_{50} = 1.0 μM
    radioligand] = 0.01 μM
    K_d = 0.005 μM

K_i = 1.0/(1 + 0.01/0.005) = 0.33 μM

- Agonists do not necessarily follow law of mass action: binding + activation (rare)

Scintillation Proximity Radioligand Binding Assay



^{3}H = radiolabeled ligand

SA = streptavidin

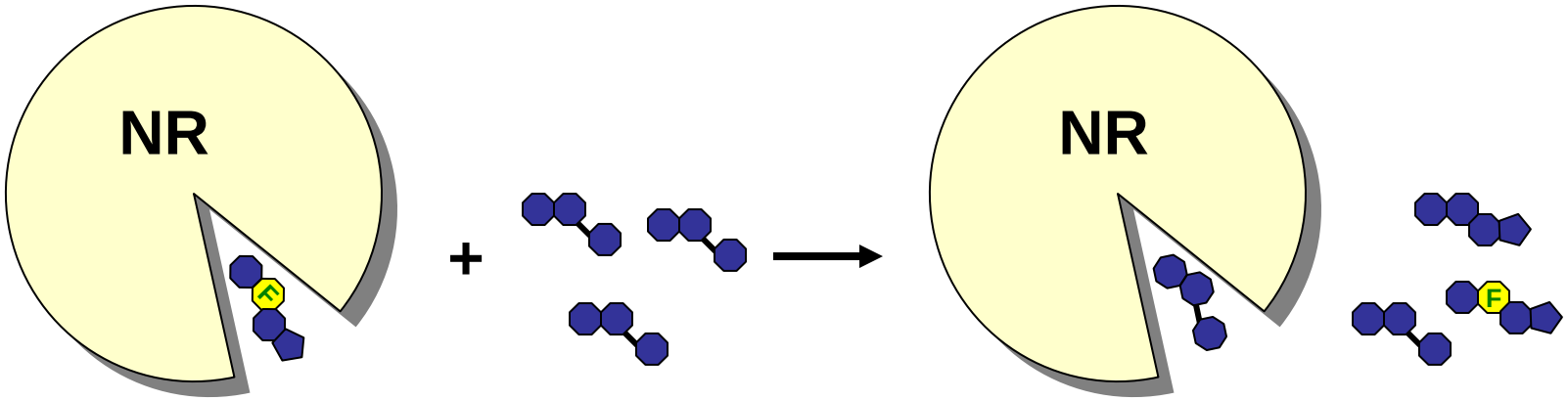
RE_oligo = response element oligonucleotide

- Requires purified receptor and labeled ligand
- Homogenous format—no separation step
- Detects agonists and antagonists
- Issues with avidity limit accurate quantitation of K_i

Fluorescence Polarization Binding Assay

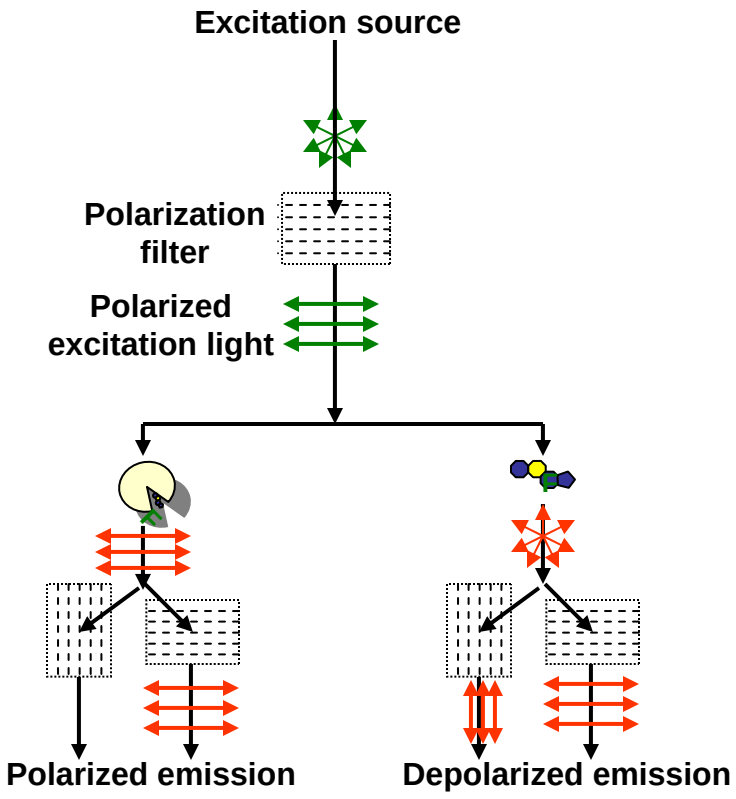
- Requires purified receptor
- Requires fluorescent ligand
- Simple, homogeneous
- Often [receptor] is high causing poor pharmacology for potent ligands
- Does not distinguish agonist from antagonist

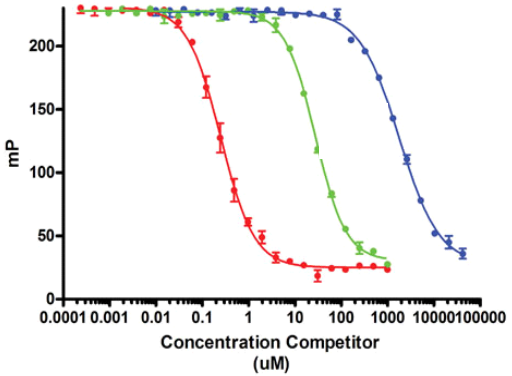
Fluorescence Polarization Binding Assay



High Polarization

Low Polarization





- No excess fluorescent ligand
- Cheng-Prusoff causes > 10-fold overestimation of K_i
- Other correction methods better suited:
  - http://spotlite.nih.gov/assay/index.php/Section5:Practical_Use_of_Fluorescence_Polarization_in_Competitive_Receptor_Binding_Assays

Cellular Transactivation Assays

- Functional cellular assay
  - Agonist
  - Antagonist
  - Partial agonist
  - Inverse agonist

- Multiple formats
  - Endogenous promoter
  - Cotransfection assay
  - Mammalian one-hybrid (GAL4-LBD system)
  - Mammalian two-hybrid

- Molecular biology tools—no protein purification or labeled ligand required

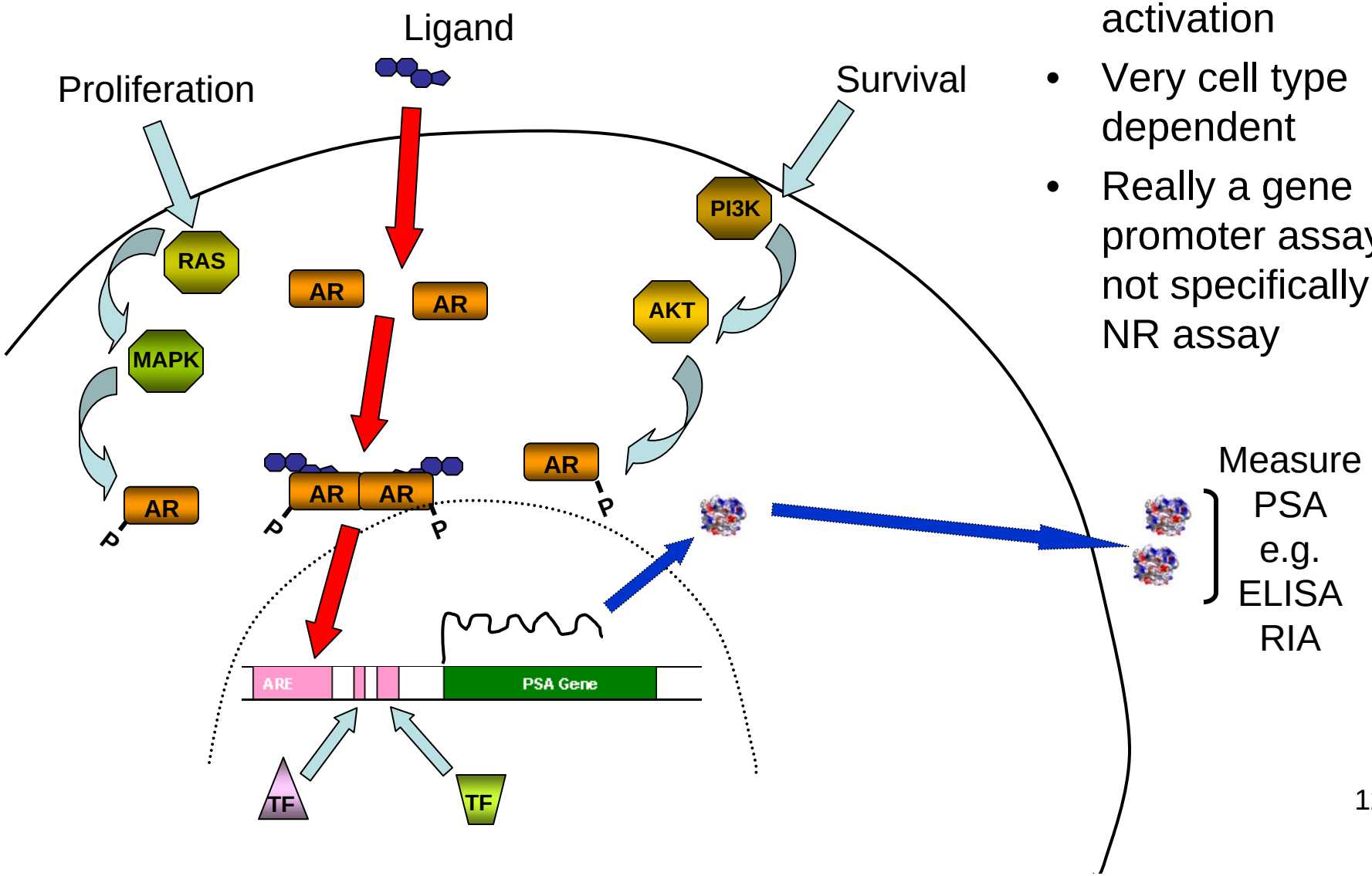
- Disadvantages
  - Cellular assay susceptible to cytotoxicity
  - Bell-shaped concentration-response curves not uncommon
  - Differences in coregulator environment in varied cell types my influence results

Cellular Transactivation Assays

System	Receptor	Reporter	Example	Comments
Endogenous gene expression	Endogenous	Endogenous	GR/TAT	High background, limited number of cell types expressing receptor
			AR/PSA	Specific in prostate cancer cells
Exogenous reporter gene	Endogenous	CAT/luciferase/GFP/ β-galactosidase/β-lactamase	ERE; MMTV	Specificity dependant on promoter. ER relatively specific, GR responds to mineralocorticoids, androgens, progestins acting through their receptors
Cotransfection	Exogenous	CAT/luciferase/GFP/ β-galactosidase/β-lactamase	ER/ERE	Specificity dependant on promoter. Receptor overexpression may help.
GAL4 Cotransfection	Exogenous	Exogenous	ERα-LBD/ UAS_Luciferase	High specificity; primarily AF2 activation only

Endogenous Reporter Gene Assay

- AR/PSA

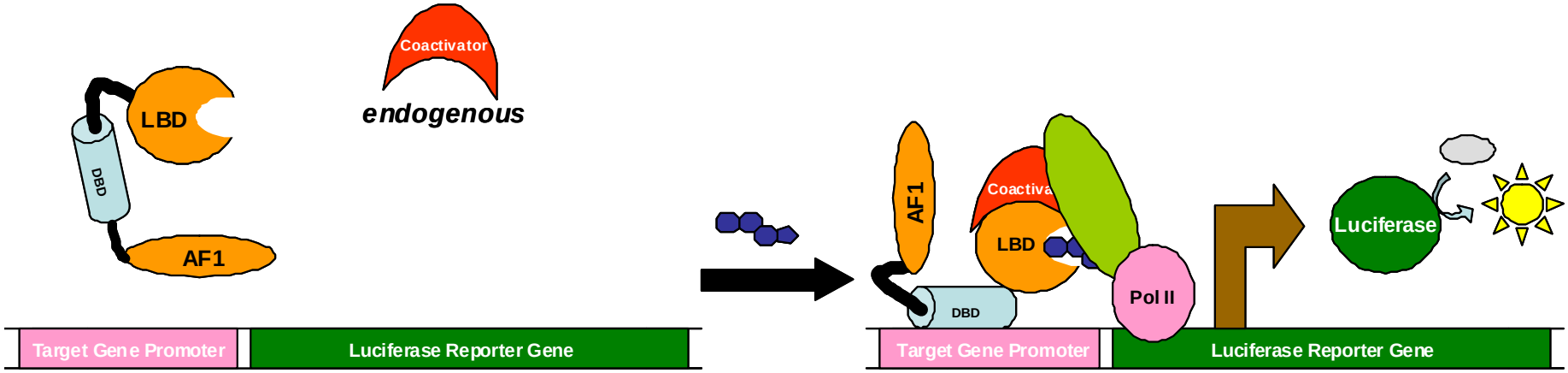


- Measures multiple means of activation
- Very cell type dependent
- Really a gene promoter assay, not specifically NR assay

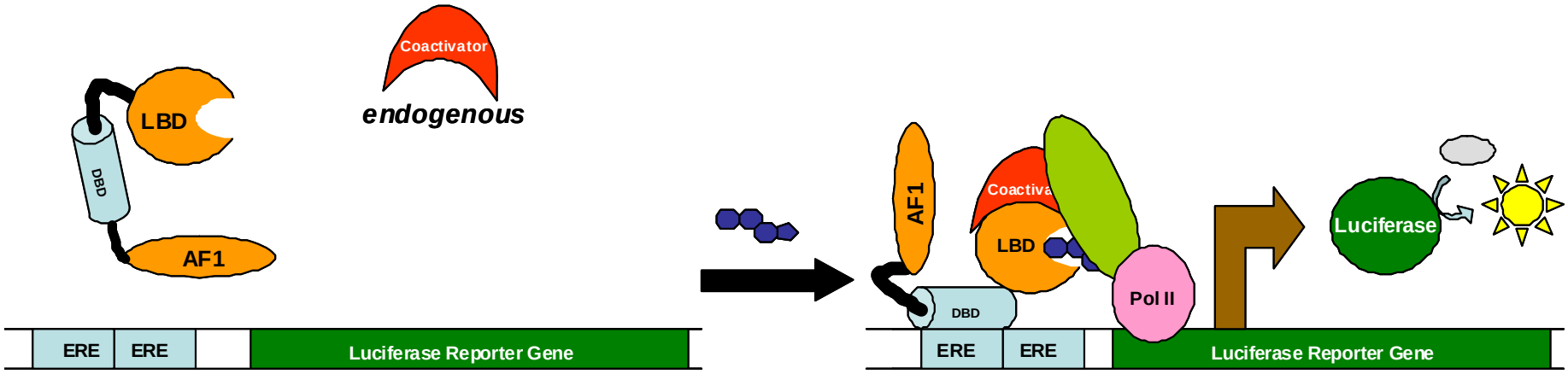
NR Cotransfection Assay

- Two DNA vectors required:
  - Receptor expression vector
    - Full length, native sequence
    - AF1 and AF2 domains present
  - Reporter gene vector:
    - NR response element
    - Larger target gene promoter region
- Can be less specific than GAL4 systems due to:
  - Promiscuity of response elements
  - Other transcription factor binding sites in promoter
- Also reports AF1 activity

NR Cotransfection Assay



Promoter-driven

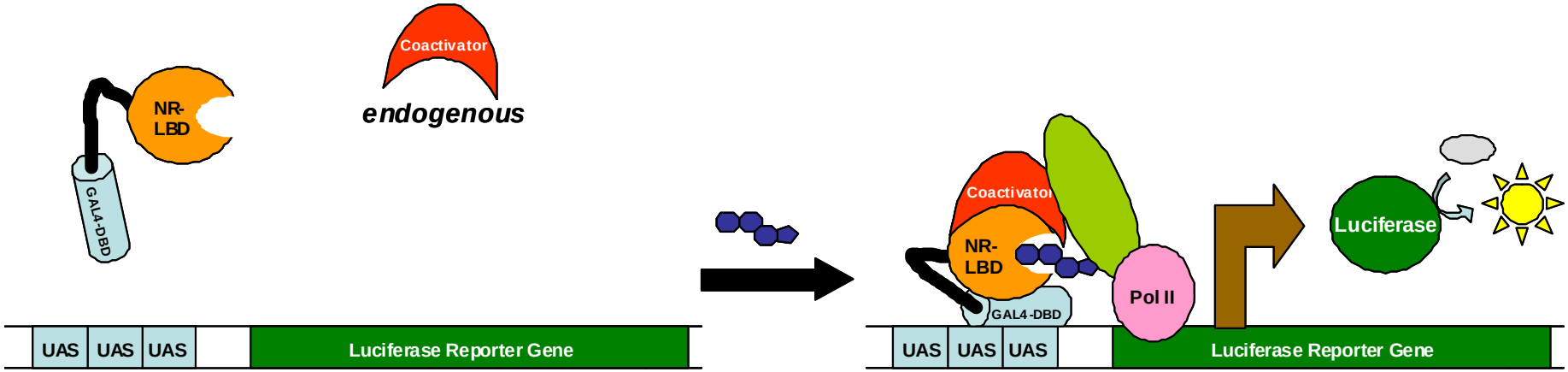


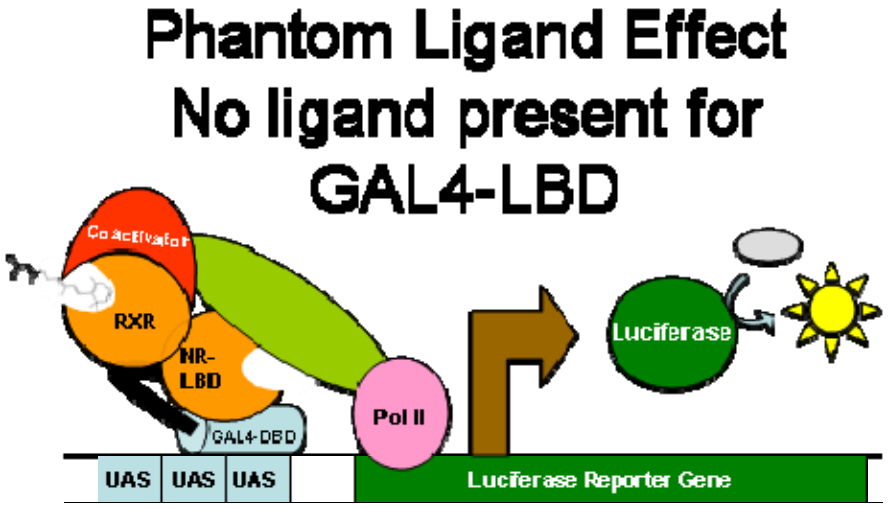
Response element-driven

Mammalian One-Hybrid GAL4-LBD Assay

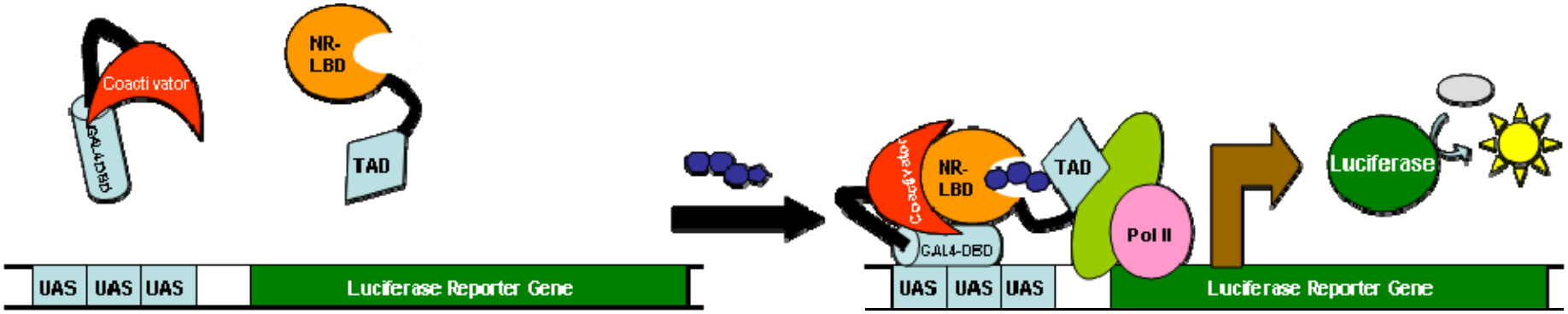
- Two DNA vectors required:
  - GAL4-DBD_NR-LBD
  - UAS_Reporter Gene
- Sensitive and specific for NR ligands binding to LBD
- No AF1 present
- Homologous system (same reporter gene, same GAL4-DBD) useful for comparison between receptors
- May be sensitive to “phantom ligand effect” (for RXR heterodimers)

Mammalian One-Hybrid GAL4-LBD Assay





Mammalian Two-Hybrid Assay

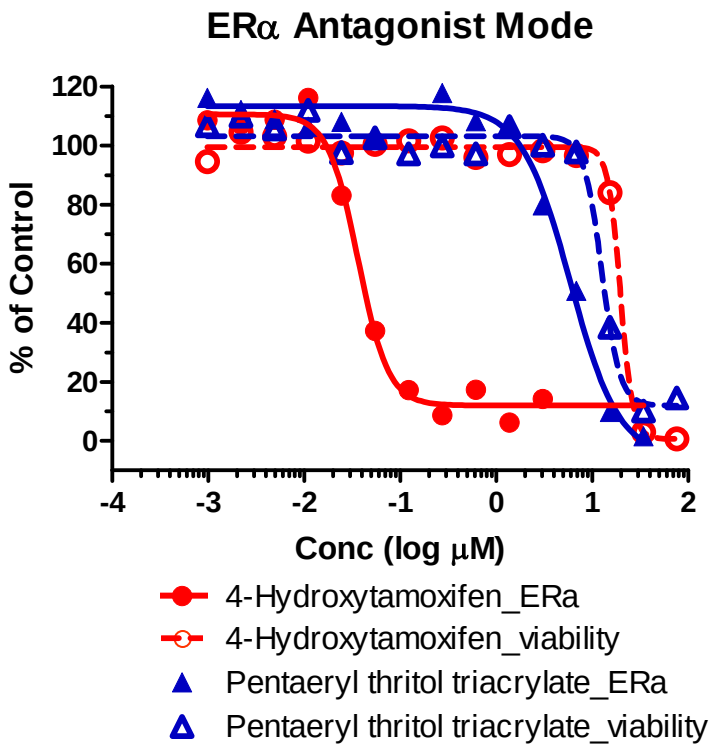


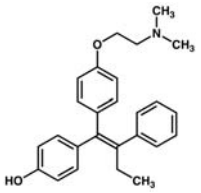
- Provides signal amplification due to TAD from strong transcription factor, e.g. NFκB
- Provides ability to look at specific coactivators

Transactivation Assays: Antagonist Format

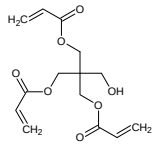
- Cellular reporter assay run in the presence of EC_{50}-EC_{80} of agonist
- Competitive inhibitor should displace agonist and reduce signal
- Confounded by cytotoxicity which also reduces signal
- Can normalize with second, constitutive reporter gene, e.g. Renilla luciferase or measure cytotoxicity by other means
- Should confirm with orthogonal assay
- Inverse agonists possible, e.g. CAR and androstenol

Examples of Antagonist Format Assays

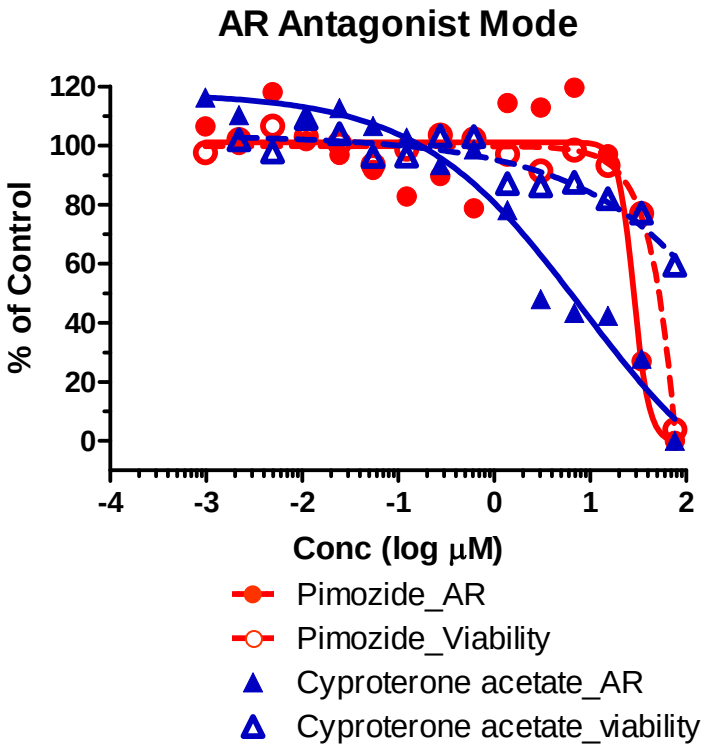


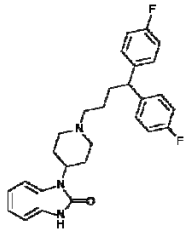


4-hydroxytamoxifen

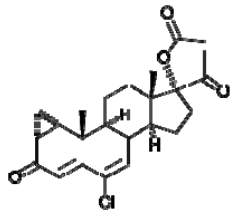


Pentaeryl thritol triacrylate





pimozide



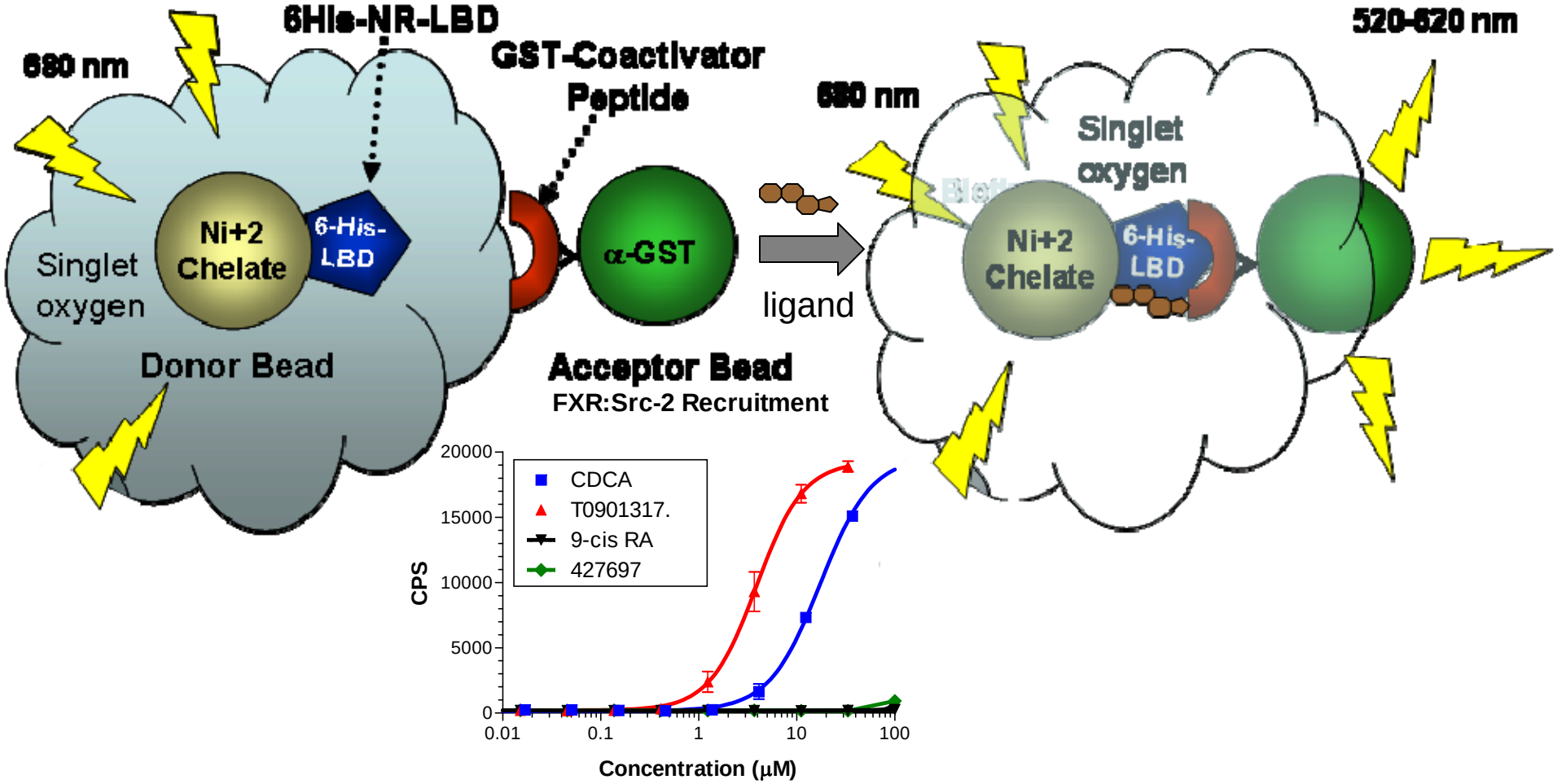
cyproterone

Functional Biochemical Assays

- Based on co-regulator recruitment following ligand binding
- Reports functional activation of receptor:
  - Distinguishes agonist from antagonist
  - Can be used to show co-regulator preference
- Requires purified receptor (LBD)
- Variety of assay formats that measure protein:protein interactions
  - FRET
  - AlphaScreen
- Co-regulator can be recombinant protein or synthetic peptide
  - Requires nuclear receptor interacting domain (containing LXXLL motif for coactivator, LXXXIXXXI/L for co-repressor)
  - Can be as short as 8 amino acids

Coactivator Recruitment Assay

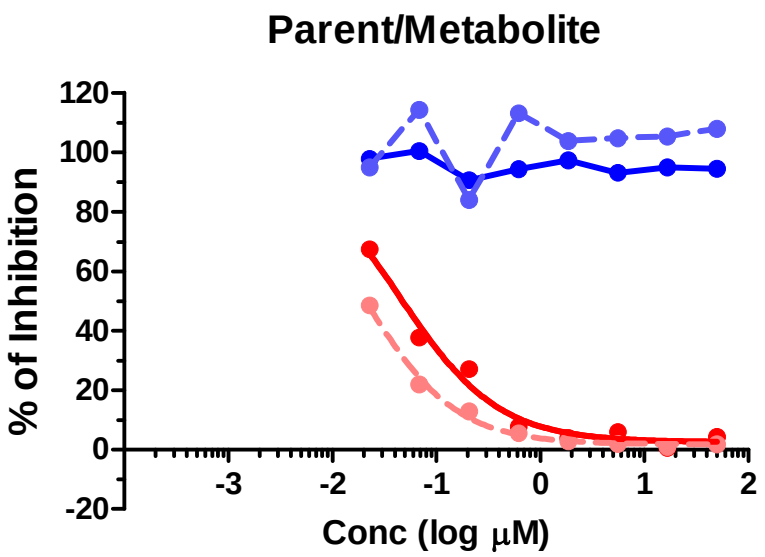
AlphaScreen Format



	CDCA	T0901317.
Bottom	163.1	206.2
Top	19666	19259
LogEC50	1.242	0.5892
HillSlope	1.663	1.816
EC50	17.45	3.884

Biotransformation: In vitro Screening's Achilles' Heel

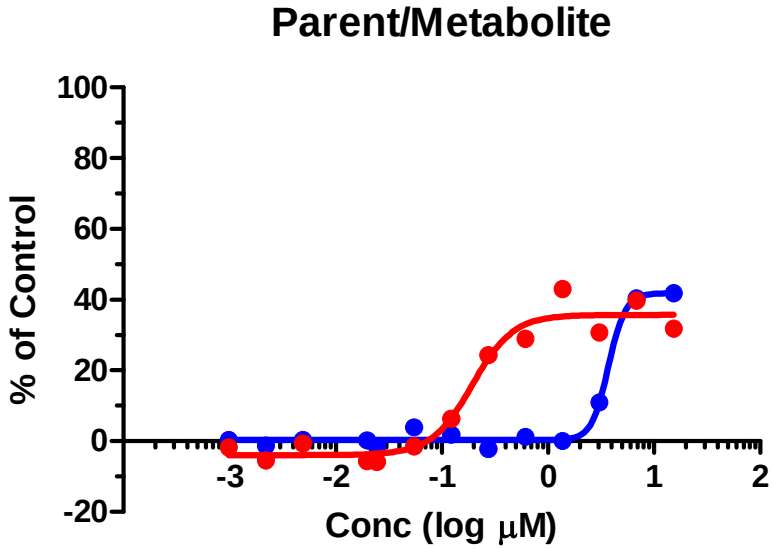
ERα radioligand binding assay



Methoxychlor (human ERα)
Methoxychlor (bovine ERα)
HPTE (human ERα)
HPTE (bovine ERα)

	HPTE (hERα)	HPTE (bERα)
Bottom	2.448	1.725
Top	= 100.0	= 100.0
LogIC50	-1.349	-1.697
HillSlope	-0.9197	-0.9877
IC50	0.04476	0.02009

ERα cellular (HEK293) transactivation assay



Methoxychlor
HPTE

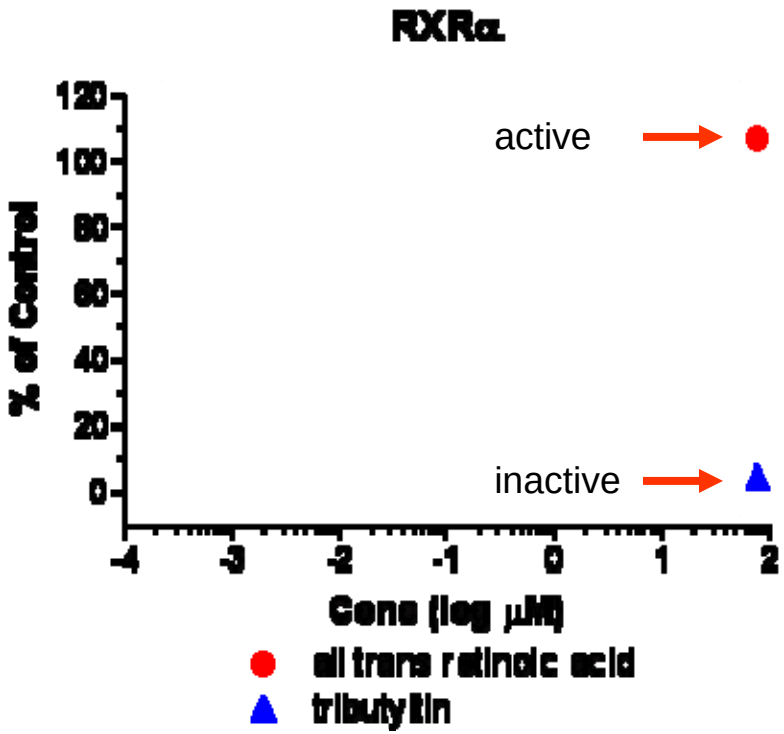
	Methoxychlor	HPTE
Bottom	0.3017	-3.912
Top	41.86	35.70
LogEC50	0.5714	-0.7131
HillSlope	5.466	2.258
EC50	3.727	0.1936

Quantitation of Results: Concentration Response Curves for Nuclear Receptor Activity

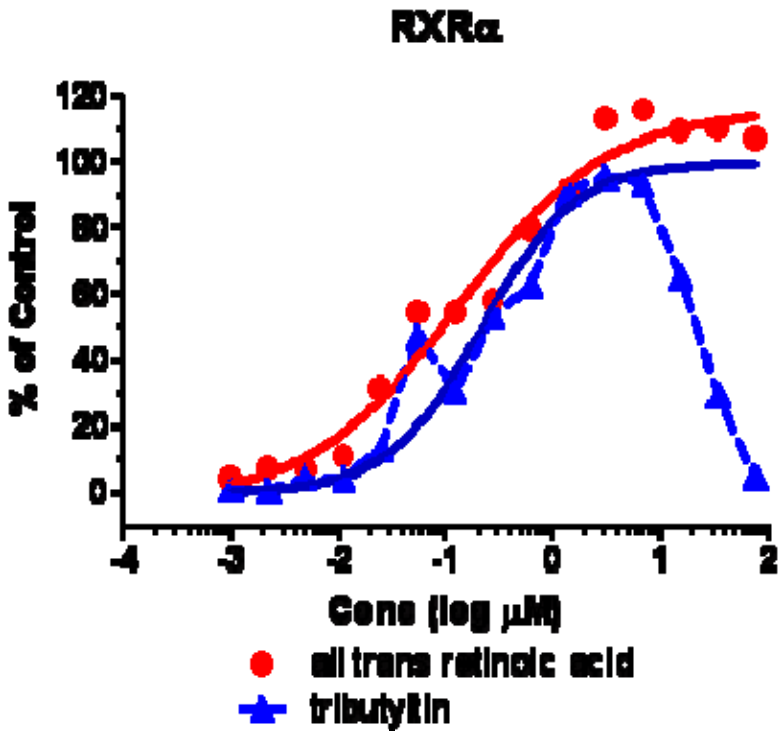
- Bell-shaped curves not uncommon
- May require initial screening at multiple concentrations
- Antagonist format confounded by cytotoxicity
- Partial agonists discernable
- Hill equation fitting generally appropriate

Importance of Testing in Concentration-Response Format

Single Concentration Testing



Multiple Concentration Testing

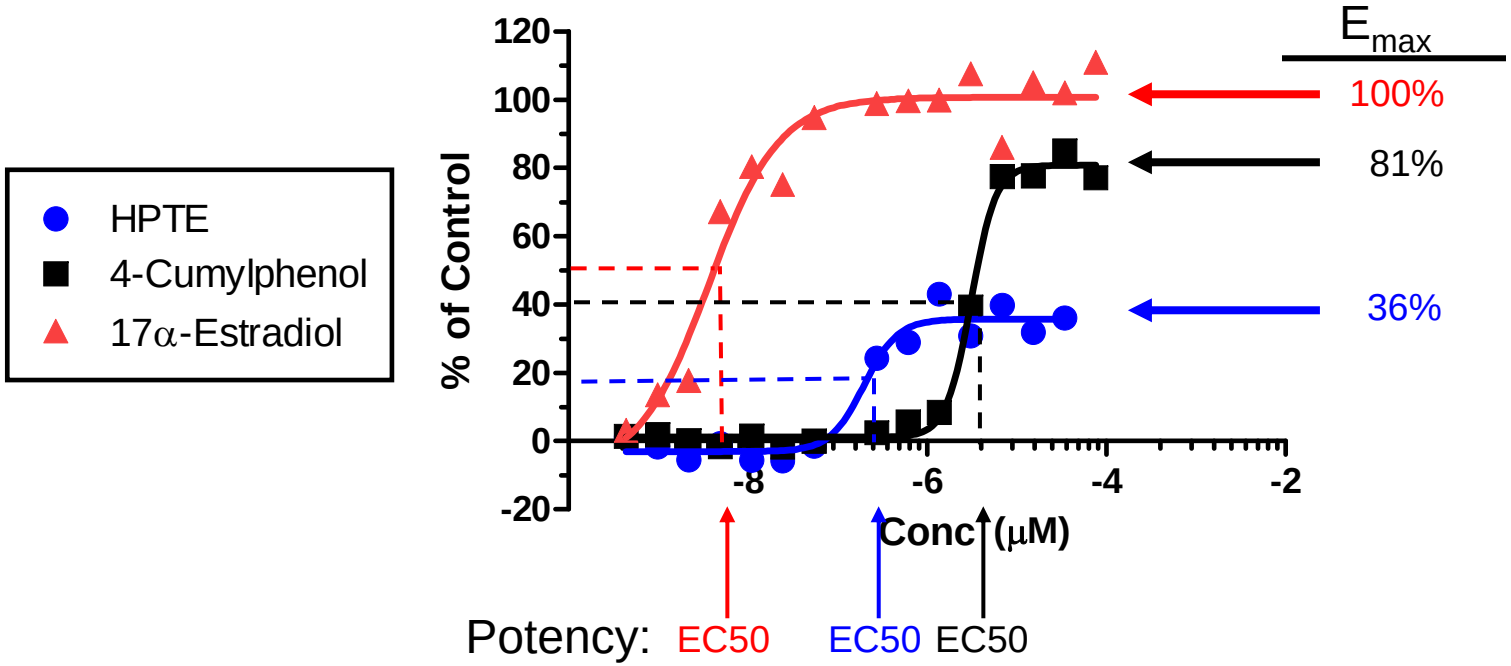


Quantitation of Results: Transactivation Assays

- Usually best to normalize results to reference compound, e.g. % of control
- 3 or 4 parameter logistic curve fit acceptable
  - Under appropriate conditions, top or bottom can be fixed
  - For agonists, often not possible to fix top if maximum efficacy unknown
- Best not to preset Hill coefficient
  - Can cause loss of information
  - Generally around 1.0 for NR-mediated activity and in the absence of cytotoxicity
- Can measure relative and absolute EC_{50}
  - Absolute: molar concentration that increases (reduces) activity to 50% of a reference compound
  - Relative: molar concentration that increases (reduces) activity to 50% of the maximum effect of that compound

Efficacy vs. Potency

Estrogen Receptor α Transactivation Assay



Potency: Estradiol >> HPTE > 4-cumylphenol

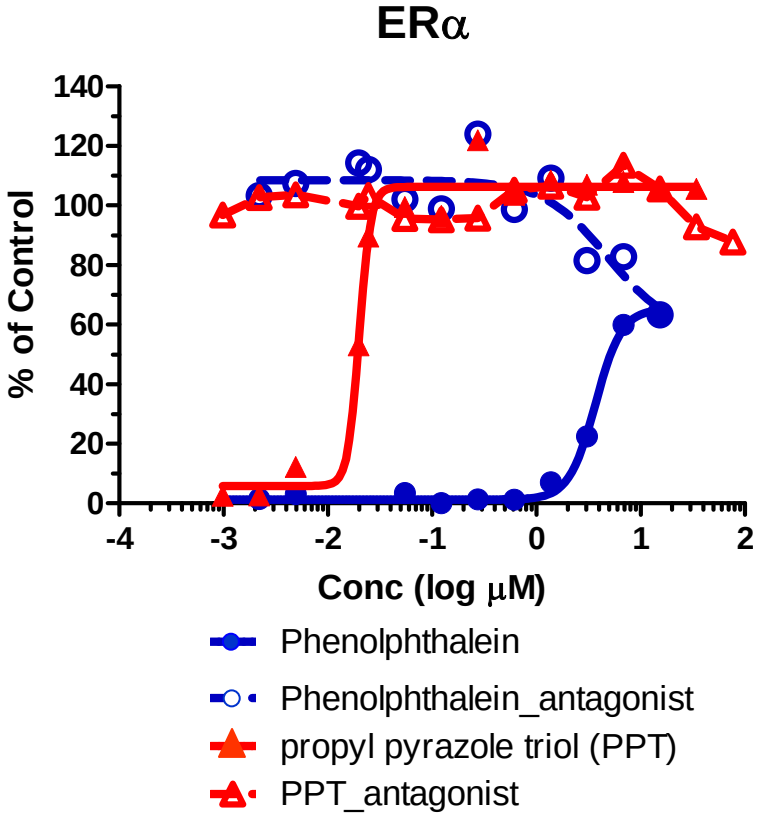
Efficacy: Estradiol > 4-cumylphenol >> HPTE

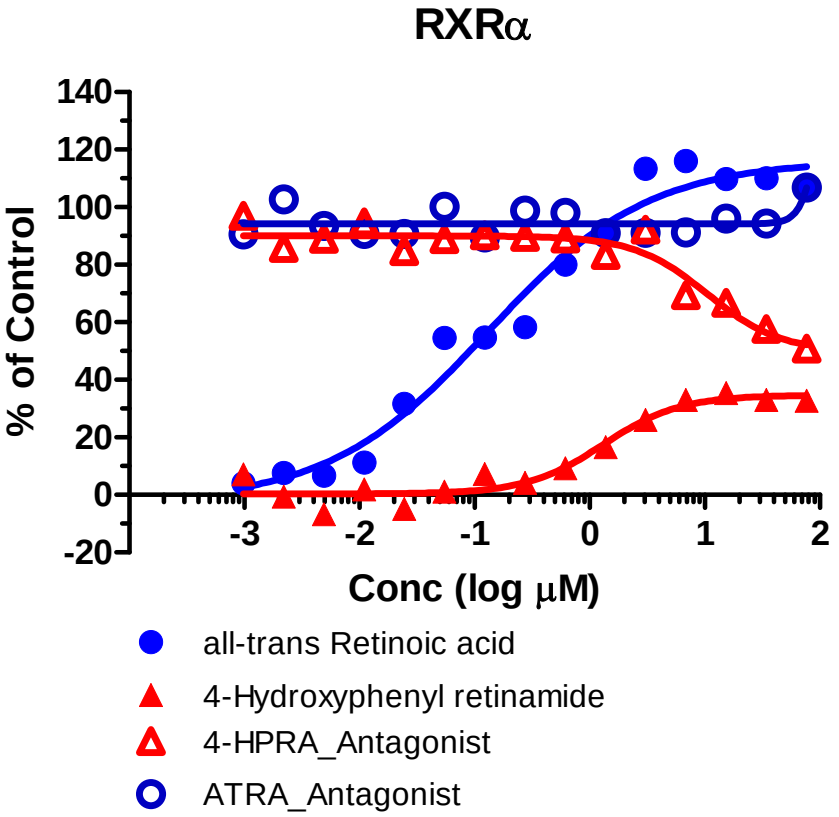
Interpreting the toxicology is challenging!

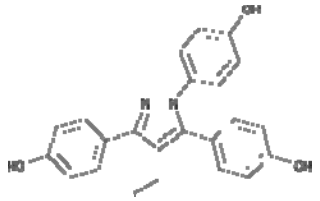
Partial Agonists

- Produce only a fraction of the complete response seen with a full agonist
- Likely due to differences in co-regulator binding affinity
  - specific receptor conformation induced by structure of ligand
  - varied co-regulator:receptor affinities
  - varied co-regulator cellular/tissue concentrations
- Would expect to see selective modulator effect *in vivo* (and toxicity?)
- Difficult to predict *in vivo* responses
- At saturating concentrations, could antagonize effects of a full agonist

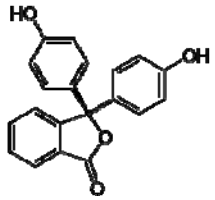
Effects of Partial vs Full Agonists in Agonist and Antagonist Mode Assays



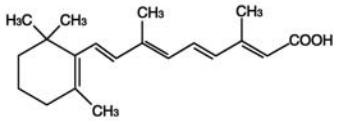




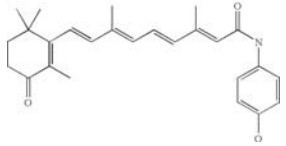
Propyl pyrazole triol



phenolphthalein

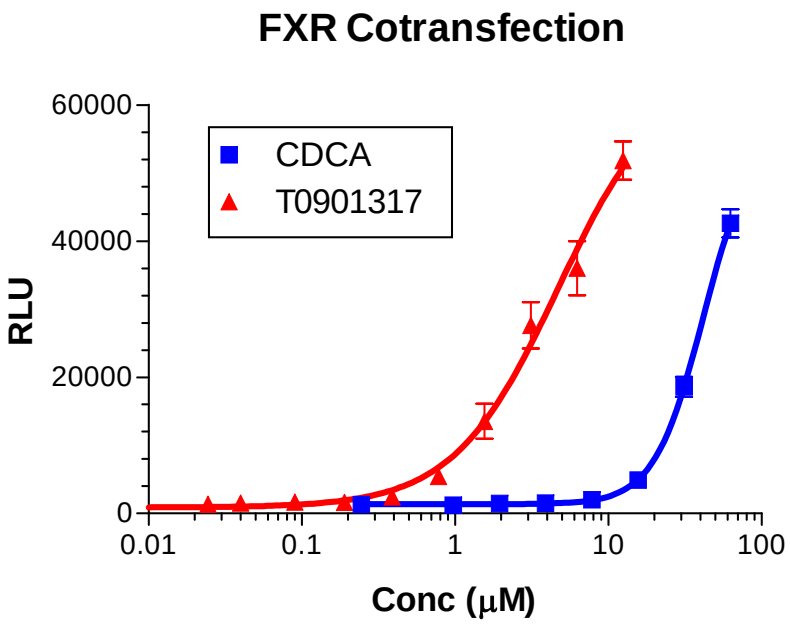


all-trans retinoic acid

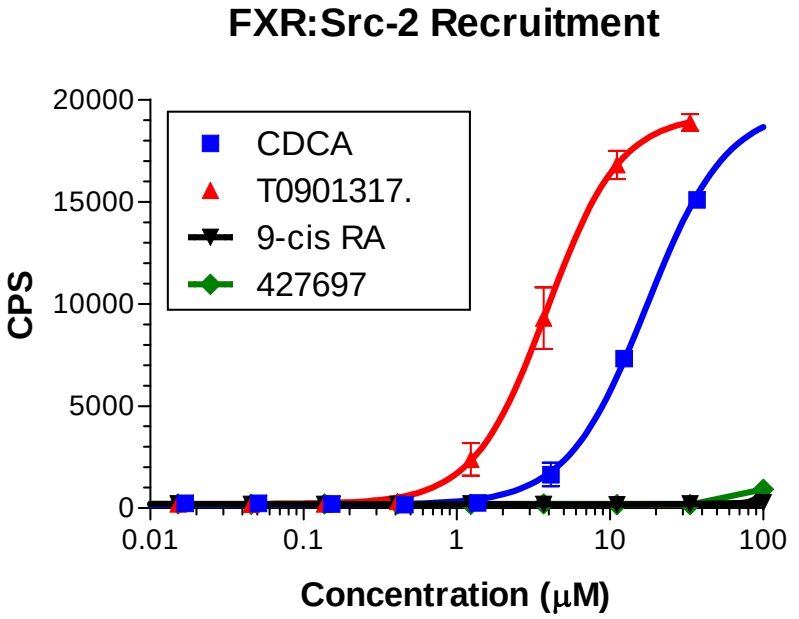


4-hydroxyphenyl retinamide

Secondary Screening Assays: Utility of Orthogonal Assays



	CDCA	T0901317
Bottom	1363	874.2
Top	56419	65078
LogEC50	1.621	0.6711
HillSlope	2.710	1.279
EC50	41.75	4.689



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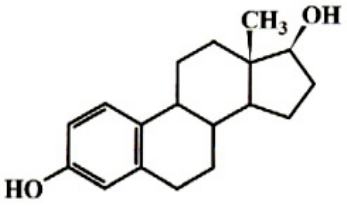
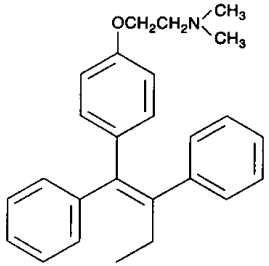
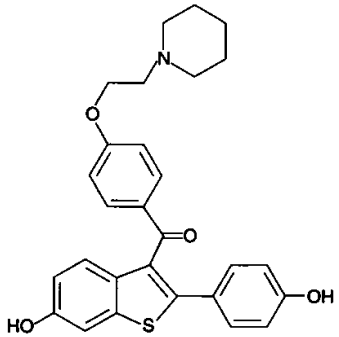
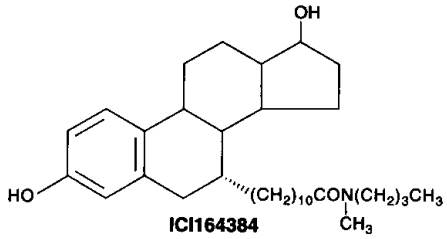
EC50	17.45	3.884

- Combination of cellular transactivation and binding or co-regulator recruitment assays eliminates many possible artifacts

Assays for Distinguishing SERMs

- Panels of cellular efficacy assays
- Panels of co-regulator recruitment assays
- Gene expression assays, particularly *in vivo*
- *In vivo* efficacy models
- Success lies in reference compounds for comparison of response profiles

Tissue-specific Activity of ER Ligands

Agonist		Partial Agonists				Antagonist	
17β-Estradiol		Tamoxifen		Raloxifene		ICI164384	
							
Breast	+	Breast	-	Breast	-	Breast	-
Uterus	+	Uterus	+	Uterus	-	Uterus	-
Bone	+	Bone	+	Bone	+	Bone	-

- Cellular assay panel:
  - Breast: inhibition of estradiol-stimulated proliferation of MCF7
  - Uterus: Stimulation/inhibition of alkaline phosphatase expression in Ishikawa cells
  - Bone: inhibition of RANKL and M-CSF induction of RAW264.7 differentiation to osteoclasts

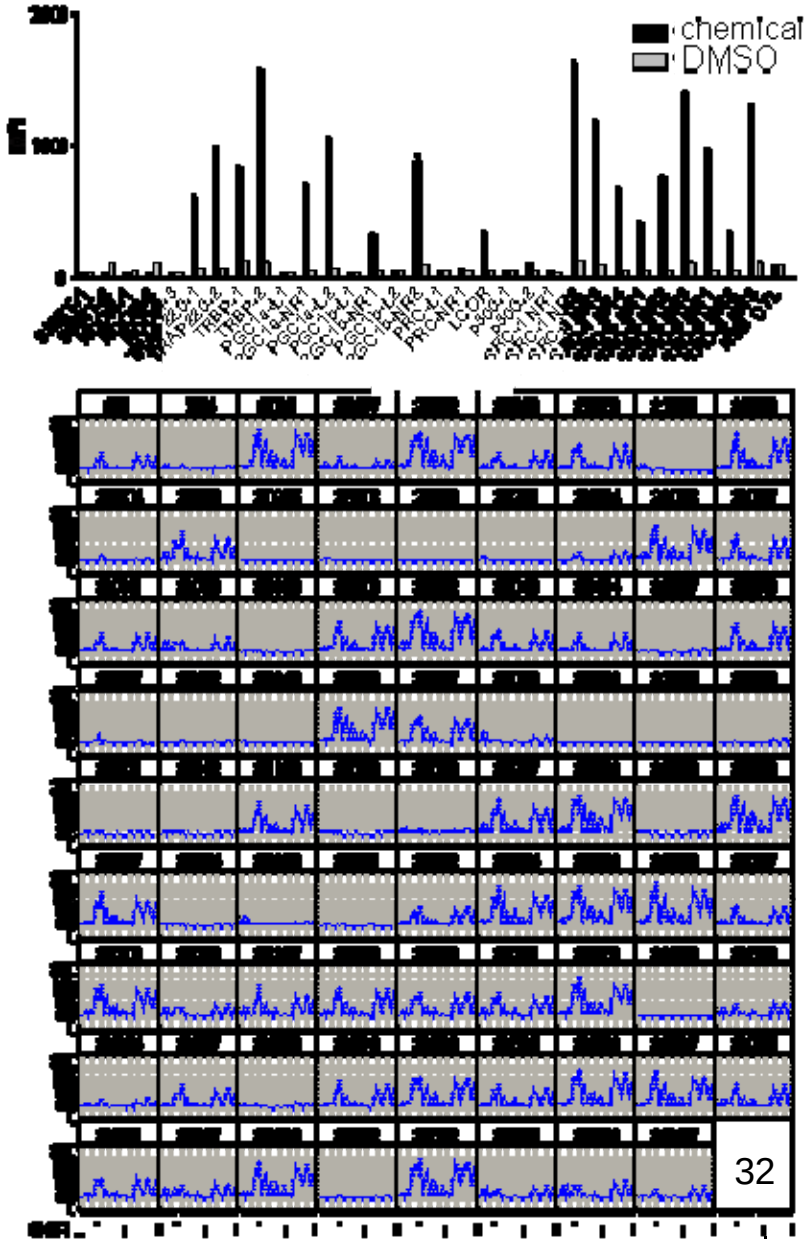
Coregulator Panel Screening

Coactivators – LXXLL consensus sequence

Src1_1	Biotin-KQQDALMDQEGMEEQTGHKLVQLLTTADQQL
Src1_2	Biotin-KQQQQCNGMEMETKMKCLMKLVQEQMDI
Src1_3	Biotin-KQQKLADAMSSSMMQHQLLKYLLMDMDKLA
Src1_4	Biotin-KQQNGPLQCQSPTSQSQTPQAQQPQMLQQLLTE
Src2_1	Biotin-KQQEDAQQQMMLEQMDQTKLLQLLTTMDQHE
Src2_2	Biotin-KQQNQQQTMDTQMKMMKCLMKLLQDQMPVD
Src2_3	Biotin-KQQVTIHQEPVSMMKCMALLKYLLMDQTMDI
Src3_1	Biotin-KQQDAMEQEPPLMMKCMKCLQLLTCMDDQD
Src3_2	Biotin-KQQSTMMMMSLAQMMKCLMKLLQEQMPMAE
Src3_3	Biotin-KQQVVHQEQLPPMKCMKALLKYLLMDQPMDA
TRAP220-1	Biotin-KQQPPQEHDPSMPQPTKYLTKLLQLTCDQQS
TRAP220-2	Biotin-KQQTPPPMMMACPTMKCMKLMKLLMDMALQDF
TRBP-1	Biotin-KQQQEQPPMKCPTLTSPLLKLLQSDIDACE
TRBP-2	Biotin-KQQMMMLPPMKCPTLEAPTKLLQQLLMDMAPF
PGC1a-L1	Biotin-KQQEDPQKLPKCDKMMAALLAFLTKTLDQLP
PGC1a-MRL	Biotin-KQQMMPQQTPPQMAKPPKLMKLLAAPMTQL
PGC1a-L2	Biotin-KQQAMSIDQQQKPQLKPCMKLLKTLTTSSPME
PGC1b-L1	Biotin-KQQTMPQQSELPQDQMKMALLAKLVKTLDQIP
PGC1b-MRL	Biotin-KQQAPKMMAMALPMPDKLMKLLQKLLATDTPTS
PGC1b-L2	Biotin-KQQQDSKAPKMQQSPSCTKLMKKLTSAQCCLQ
PGC1b-MR2	Biotin-KQQSPQMMMKCMKAIPKCLMKLLAQDVLCDV
PRC-L1	Biotin-KQQQKLQMSMAMDPPKLVKALTKIADNDD
PRC-MRL	Biotin-KQQDKIPQSKAPMPKQQKLMKLVTLMKTPPE
LCOR	Biotin-KQQAMPVTTGPTAKTQPPVLMKLLMAQPQKPL
p300-1	Biotin-KQQATEQQDIMKCPQVMQQALQKLLKTLNSNS
p300-2	Biotin-KQQAQTMGMPQDAAMMKQLMKLLNQSNSPL
p48	Biotin-KQQAEQLMMPPVAQTQSQKTLKVLLNADKDKE
RIP140-Box	Biotin-KQQMETEQQPPMKTKKPVIKYKLLQEQQQKEP
RIP140-8	Biotin-KQQMMEPMSPQPPPPMMTVLMKLLMQMQPPVP
RIP140-9	Biotin-KQQEQGMEGMLMKMMPPMTVLMQLLLSMCVTD

Corepressors – LXX I/H IXXX I/L consensus sequence

SMRT-1	Biotin-KQQVKQHGRVVTLAQHSSEVTQDYTRHPQQL
SMRT-2	Biotin-KQQQSHASTNMQLSAIRKALMQKYDQNPEESPP
NCOR-1	Biotin-KQQVPRTHRLITLADHCQIITQDFARNGVSQQ
NCOR-2	Biotin-KQQFADPASHLQLEDIRKALMQSFDQKVEDHQ
NCOR-3	Biotin-KQQKTTITAANPDVETRQIASDIDARERQSQ
NCOR-Box	Biotin-KQQEDRPSSTQSTQFPYNPLTMPMLSSTPPTPI



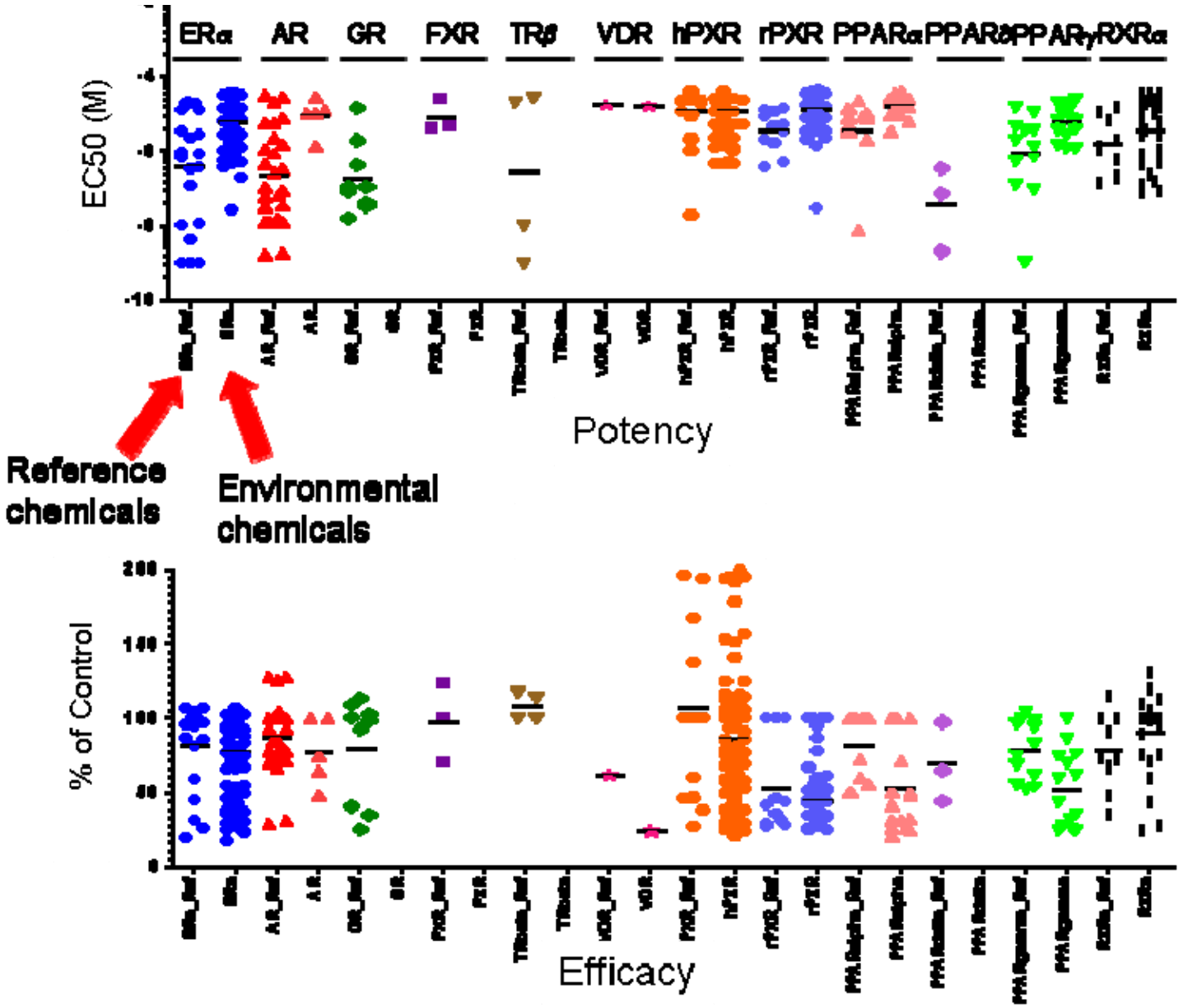
Efficacy Assays

- Target gene expression assays
  - RT-PCR on focused NR target genes *in vitro* or *in vivo*
    - *Need to define relevant genes*
  - RT-PCR arrays
    - *Collection of target genes that may help define toxicity pathways*
  - Whole-genome microarrays
    - *Too much information?*
- Animal studies
  - Hershberger assay
  - Uterotrophic assay
  - Multigeneration assay
  - Pubertal assays

Nuclear Receptor Screening and Safety Assessment

- Screen against panel of cellular assays in agonist and antagonist format:
  - Endocrine-related receptors
    - ER, AR, TR, PR, GR
  - Drug-Drug interaction
    - PXR, CAR
  - Liver carcinogenesis-related receptors
    - PPARα, CAR
  - Others of potential importance
    - RXR, RAR
- Confirm actives in additional assay format
- Consider and accommodate species differences in NR ligand preferences
- Compare potency and efficacy relative to reference ligands with known *in vivo* activity, e.g: DES, fibrates, rifampicin, TCPOBOP, all-trans retinoic acid

Environmental Chemical Library (Tox21) Profiling: NR Transactivation Assays



Summary

- Variety of possible strategies for detection of nuclear receptor activity
- Important to confirm actives with orthogonal format early
- Compare to reference ligands for potency and efficacy
- Don’t ignore the pharmacology!
- Consider lack of biotransformation for *in vitro* assays
- Role of selective NR modulators (partial agonists) in possible toxicities remains to be understood