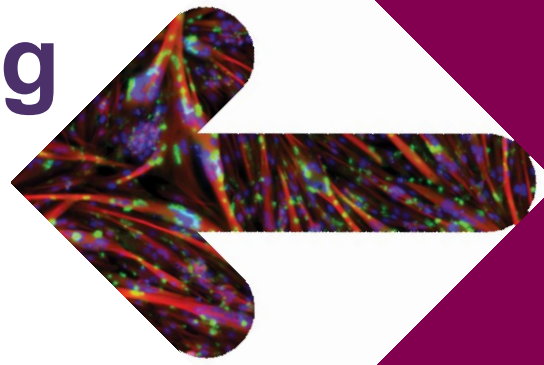
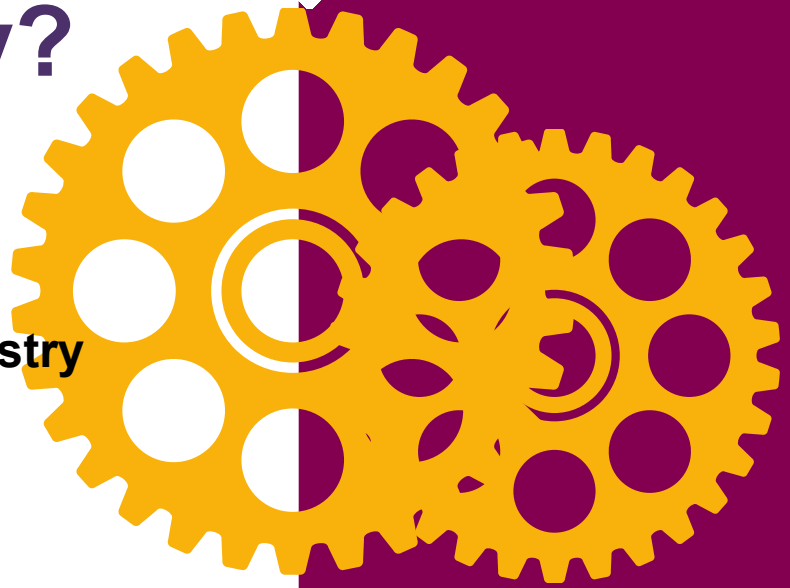
Phenotypic vs target screening approaches – black or white or shades of grey?





Graeme Walker

The Society of Chemical Industry
Choosing the Right Target in Drug Discovery
London, May 15th, 2013

AstraZeneca

Discovery Science competencies

How can we maximize value and impact into drug discovery projects?

- Crystallography; first structures, iterative structures
- NMR and other biophysical techniques

- Fragment Chemistry
- Chemical Biology

- Computational biology
- Cheminformatics
- Predictive Chemistry
- Compound collection enhancement

- Compound management
- Biobanks (clinical samples)

- Hit generation
- SAR screening
- Ion channel centre of excellence

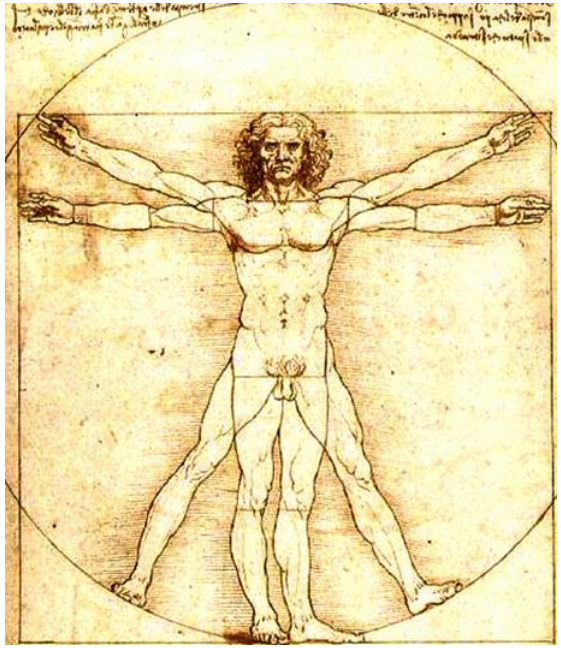
- Proteins
- Cells
- Transgenics
- Antibodies

- Project support
- Statistical qualification

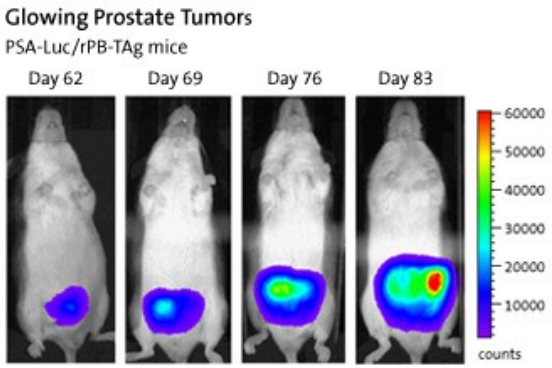
- Assay development
- High content biology assays

Phenotypic versus Target approaches

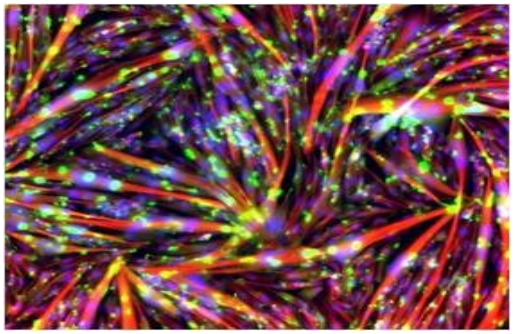
What do we mean?



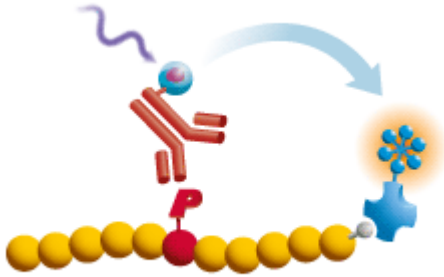
Patient



Animal Models



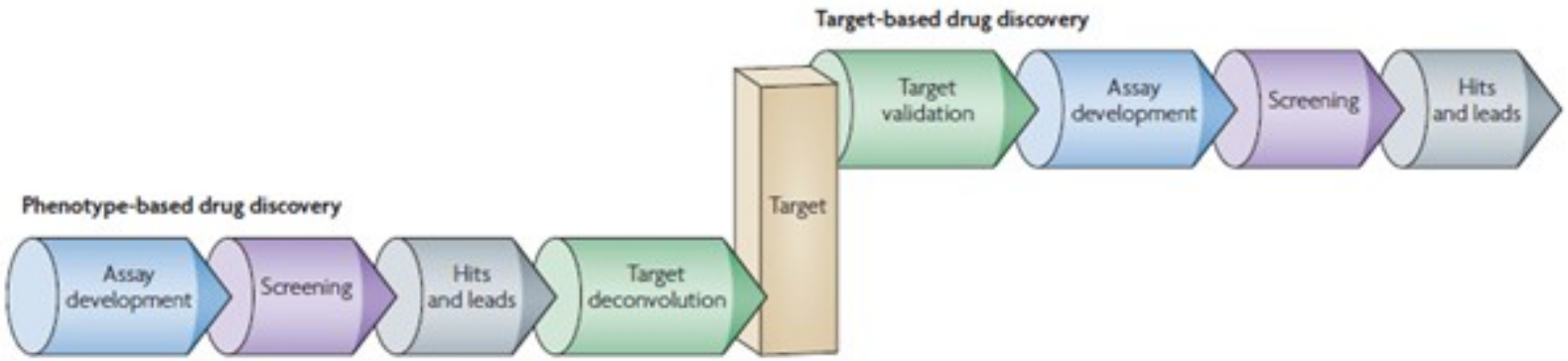
Cellular Systems



Isolated Enzyme

Target Directed (TDD) vs Phenotypic Drug Discovery (PDD)

- Targets are identified and validated (?)
- Typically use recombinant proteins or cells over-expressing the target of interest
- Assay throughput is usually high
- Screens used are to measure the compound's effect on the target of interest
- **Need to confirm compound effects in biological effect assay**



Terstappen *et al*, Nature Reviews Drug Discovery, 2007, 6, 891-903

- Targets are unknown
- Ideally use native human cells
- Assay throughput is usually low
- Screens used to measure the desired biological effect in cells, tissues or whole organisms where multiple, biologically relevant targets and pathways are simultaneously interrogated
- Activity in phenotypic screening might be translated to human disease more effectively than that in target-based screens
- **Need to do target deconvolution to identify target**

Target-driven/directed Drug Discovery (TDD)
vs
Phenotypic Drug Discovery (PDD)

TDD: The ability of compounds identified in target-driven approaches to modify disease progression in patients is not known *a priori* and may not be related to the biochemical activity of the compound *in vitro*

PDD: Simultaneously interrogating multiple, biologically relevant molecular targets and pathways to discover compounds that modulate relevant biological processes in a target/mechanism agnostic fashion

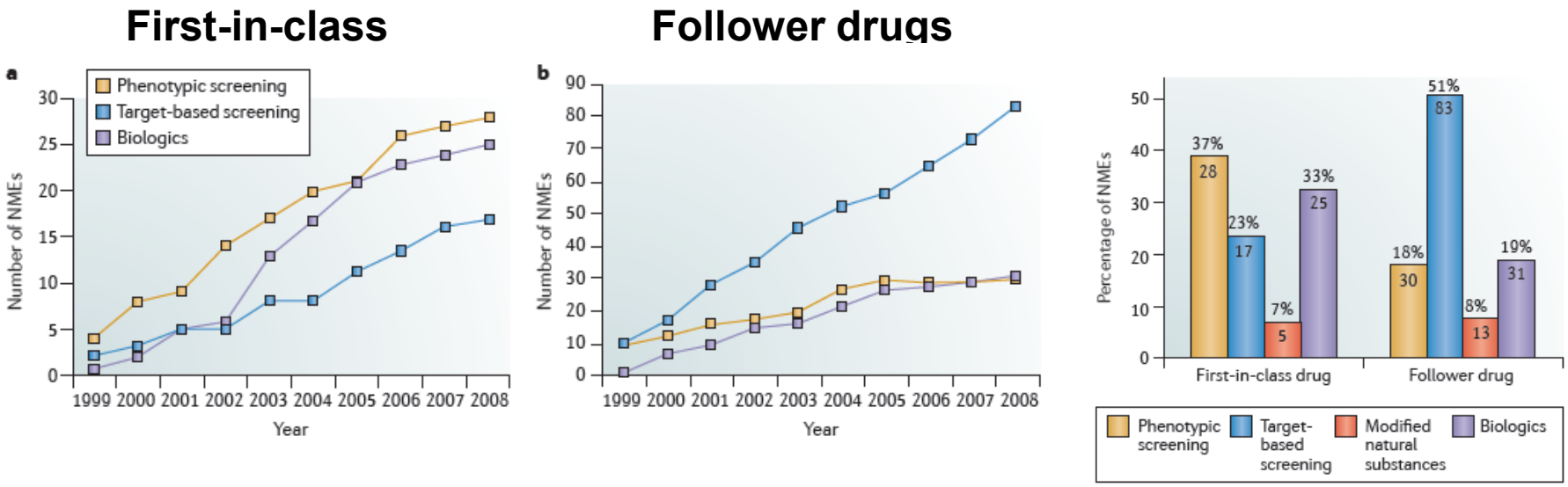
- novel functions for well-studied proteins
- discover new pathways of therapeutic value

Novel target discovery
Increase chemical diversity
Novel compound mechanism of action

Why Phenotypic Discovery?

- Analysis of drug discovery over the last ten years is a story of massively increasing expenditure with less delivery of new drugs to the market
- Some of this can be explained by tougher regulatory policies but the fact remains that unless this improves we will not be able to replace the loss of previous “blockbusters”
- One possible explanation is that target-directed drug discovery driven by molecular biology and HTS has not delivered to expectations
- Over the last ten years most First In Class (FIC) small molecule drugs have still come from phenotypic screens rather than target directed screening
- Efficiency vs effectiveness?
- Followers (fast or slow)/Best In Class (BIC) tend to use target based approaches

Drugs discovered by target-based and phenotypic-based approach between 1999 and 2008

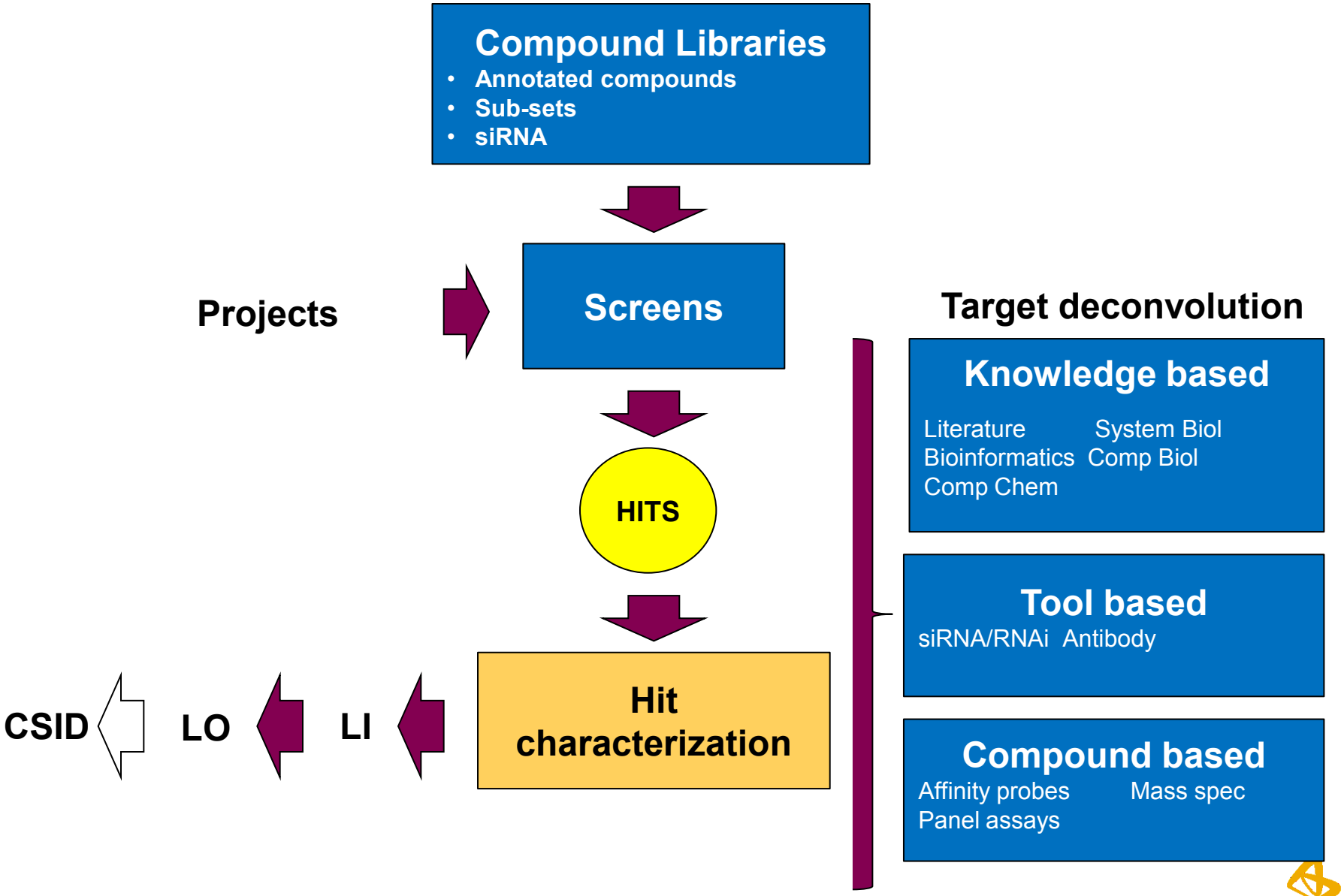


Swinney and Anthony, **Nature Reviews Drug Discovery, 2011, 10, 507-519**

Main problems with phenotypic screening are:

- Phenotypic assays have generally been low throughput
- This necessitates deconvolution of target for HTS
- Cell based (phenotypic assays) are now much better – good enough to drive Med Chem?

Phenotypic Discovery Cascade



Bridging the gap

Grey area between Target and Phenotypic approaches?

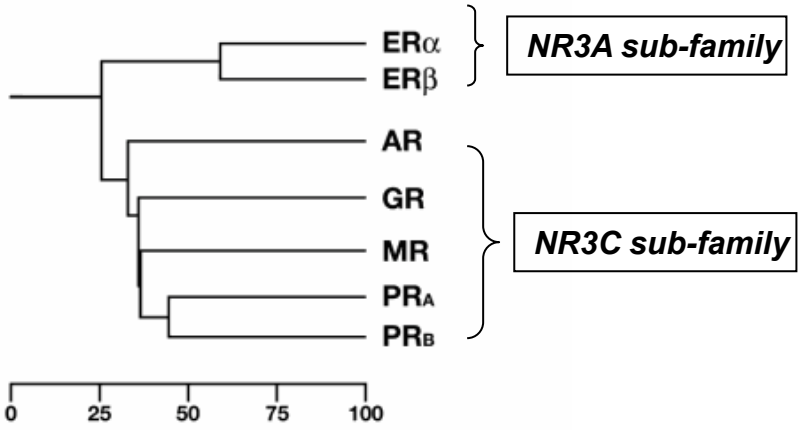
- Pathway specific approaches
  - Monitor specific pathways then identify upstream target(s)
- Other approaches to improve chances of technical success in a project
- Build cellular assays which are more predictive of in-vivo and clinical response
  - Use of primary and stem cells
  - Use of 3D cell culture systems
  - Combine target and phenotypic end-points
- Design cascades to identify compounds with a specific molecular mechanism of action with greater disease relevance
  - Covalent inhibitors
  - Down-regulators of targets

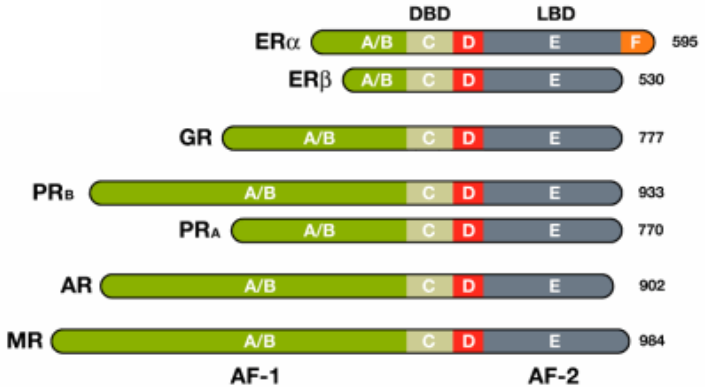
Selective Estrogen Receptor Down-Regulator (SERD) Project

Identifying compounds with a different molecular mechanism of action

ERα basics and link to breast cancer

- Member of the nuclear receptor superfamily
  - Steroid hormone receptor (ERα, ERβ, PR, GR, MR and AR)
- Ligand-activated transcription factor which regulates expression of estrogen responsive genes
- Natural ligand is estradiol
- Normal role is in female reproductive function and maintaining bone density.
- Role in breast cancer - ~75% of cancers are ER and /or PR +ve in postmenopausal women
  - Candidates for endocrine treatment
- ERα is a key transcriptional regulator in driving ER+ve breast cancer proliferation
- Estrogen link first identified by Sir George Beatson in the 19th century
  - Ovarectomy leads to a reduction in breast tumour size





ERα versus ERβ
DBD – 95% homology
LBD – 53% homology

Evolving endocrine treatments

- Current treatments
  - block ERα signalling using antagonists such as Tamoxifen
  - inhibit synthesis of Estrogens - aromatase inhibitors (Anastrozole)
  - removing ERα with an ERα-downregulator (SERD) (Fulvestrant)

- Tamoxifen has been the mainstay of endocrine treatment for many years
  - Antagonist on breast; partial agonist on bone and endometrium
  - Selective Estrogen Receptor Modulator (SERM)

- Subsequent 2nd generation SERMS
  - Raloxifene, Lasofoxifene, Bazedoxifine

- Third generation aromatase inhibitors
  - Anastrozole

- Fulvestrant - SERD
  - Pure anti-estrogen; no agonist effects; novel mode of action

SERMS and SERDs

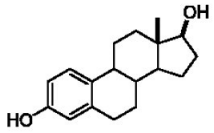
SERM = Selective Estrogen Receptor Modulator

SERD = Selective Estrogen Receptor Down-regulator

Mechanistically different classes of ER modulators

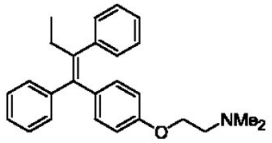
Agonists:

Estradiol

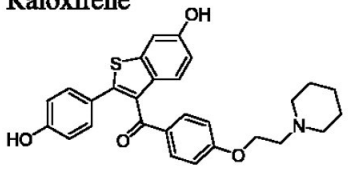


SERMs:

Tamoxifen

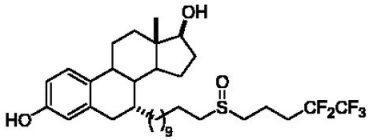


Raloxifene

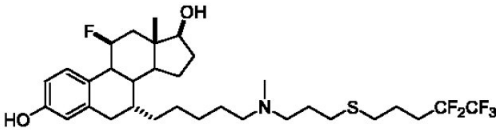


SERDs:

ICI 182,780

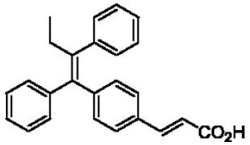


ZK-703



Mixed Function SERM / SERDs:

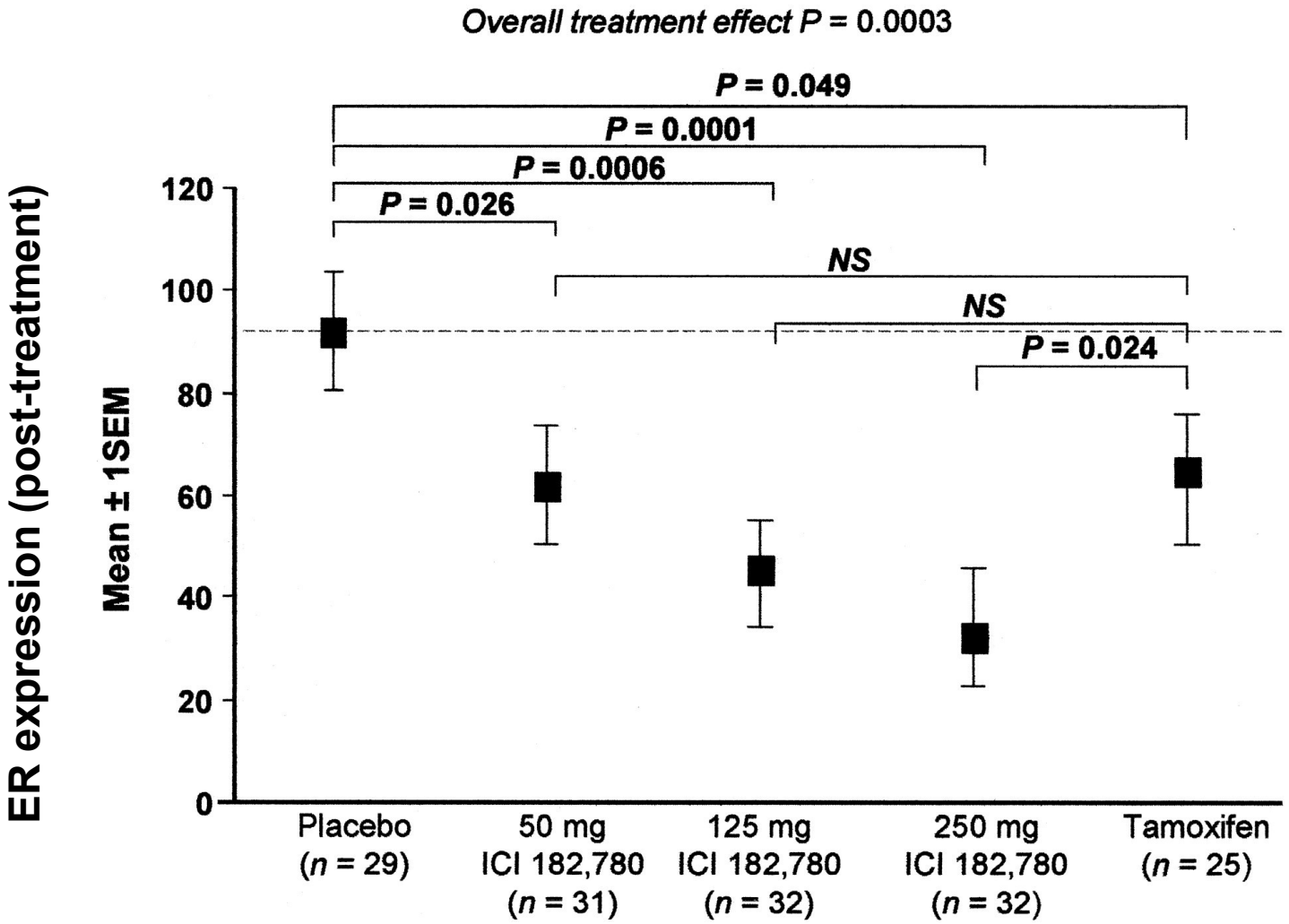
GW5638



Issues with SERMS led to a desire for a "pure anti-estrogen" agent

McDonnell, D. P. Clin Cancer Res 2005;11:871s-877s

Fulvestrant binds the ER, blocks hormone signalling and increases receptor degradation

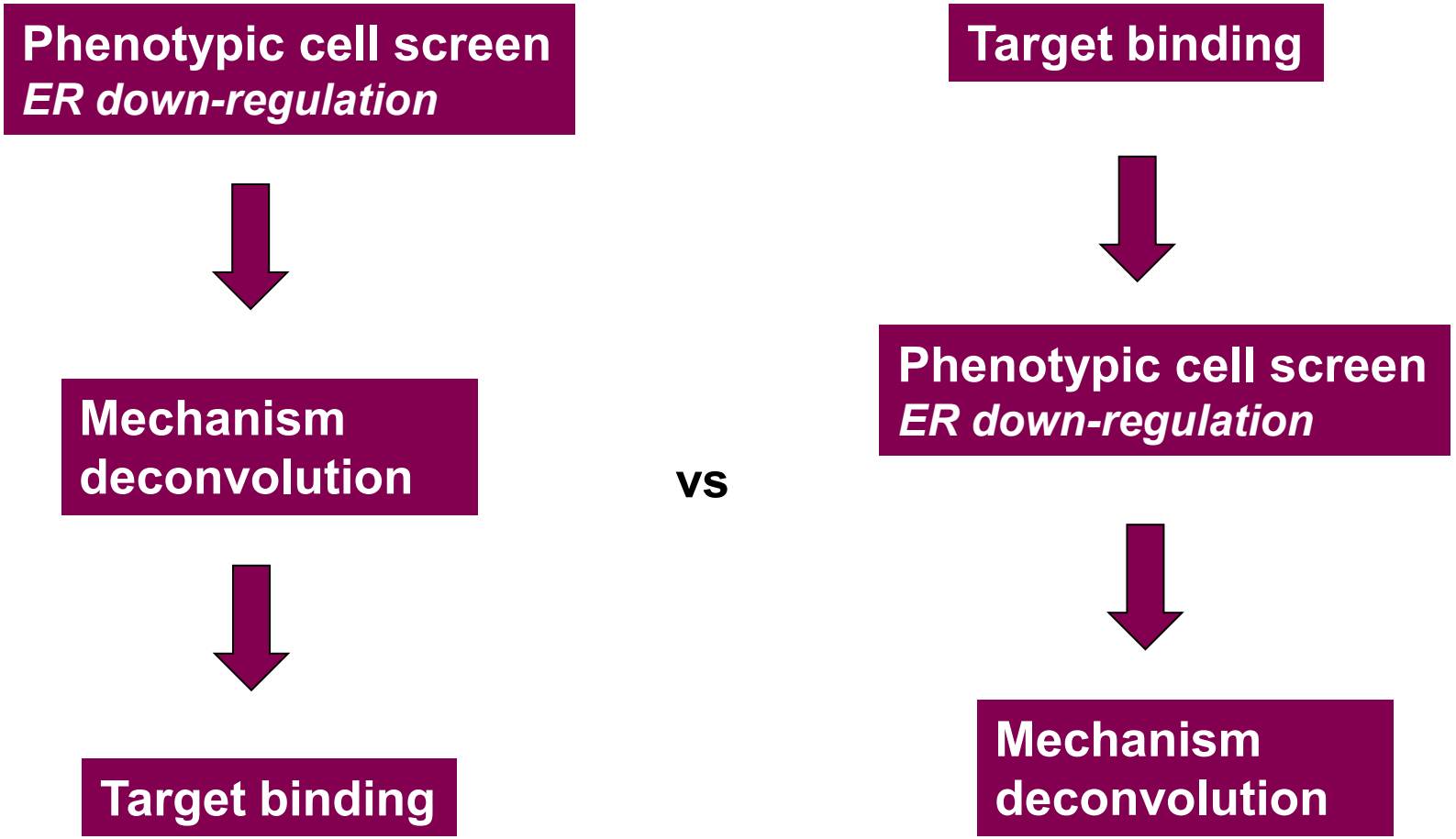


Robertson, J. F. et al. Cancer Res 2001;61:6739-6746

Why a novel SERD?

- Fulvestrant is approved as a second line therapy in ER positive PM women after anti-estrogen therapy failure
- Evidence that down-regulation is linked to efficacy
  - Higher dose regimens recently approved
  - $160m sales in 2006 (Anastrazole $1.7b) - 250mg dose
  - 500mg dose approved Sep 2010 – 2011 sales $546m
- Once monthly 5ml injection – oral route preferred
- Ongoing clinical trials to evaluate dose scheduling and combinations with Anastrazole
- Novel oral agent with greater efficacy desired

Lead Generation strategy?



Lead Generation Strategy

- Within AZ many previous efforts had targeted ER α, or β, as well as estrogen-related receptors
  - External literature and competitors
  - Analyse collection to identify non-steroid (non-tamoxifen), non-phenol ER binders - early start-points for chemistry
- Structural information is available, and steroid-pocket binding hypotheses are available for ER and GR
  - Establish ERα structural system in house
- Sub-set HTS biochem screen
  - Identify novel binders then modify them into down-regulators
- Build cellular cascade to drive SAR and understand mechanism of action of compounds
  - Multiple mechanisms of ERα-downregulation exist
  - Cellular cascade assays are required which can differentiate down-regulation via direct binding to the ERα , agonist feedback or off target mediated down-regulation

ERα Ligand Binding Domain (LBD)-GST Assay Overview

ERα LBD-GST Time Resolved-Fluorescence Resonance Energy Transfer (TR-FRET) Competitive Binding Assay - Invitrogen
A tracer & antibody-based HTS method for identification of ERα ligands.

Binding of the tracer (fluorescent steroid) to ERα is detected by TR-FRET from the terbium-labeled antibody (donor) to the tracer's fluorophore (acceptor).

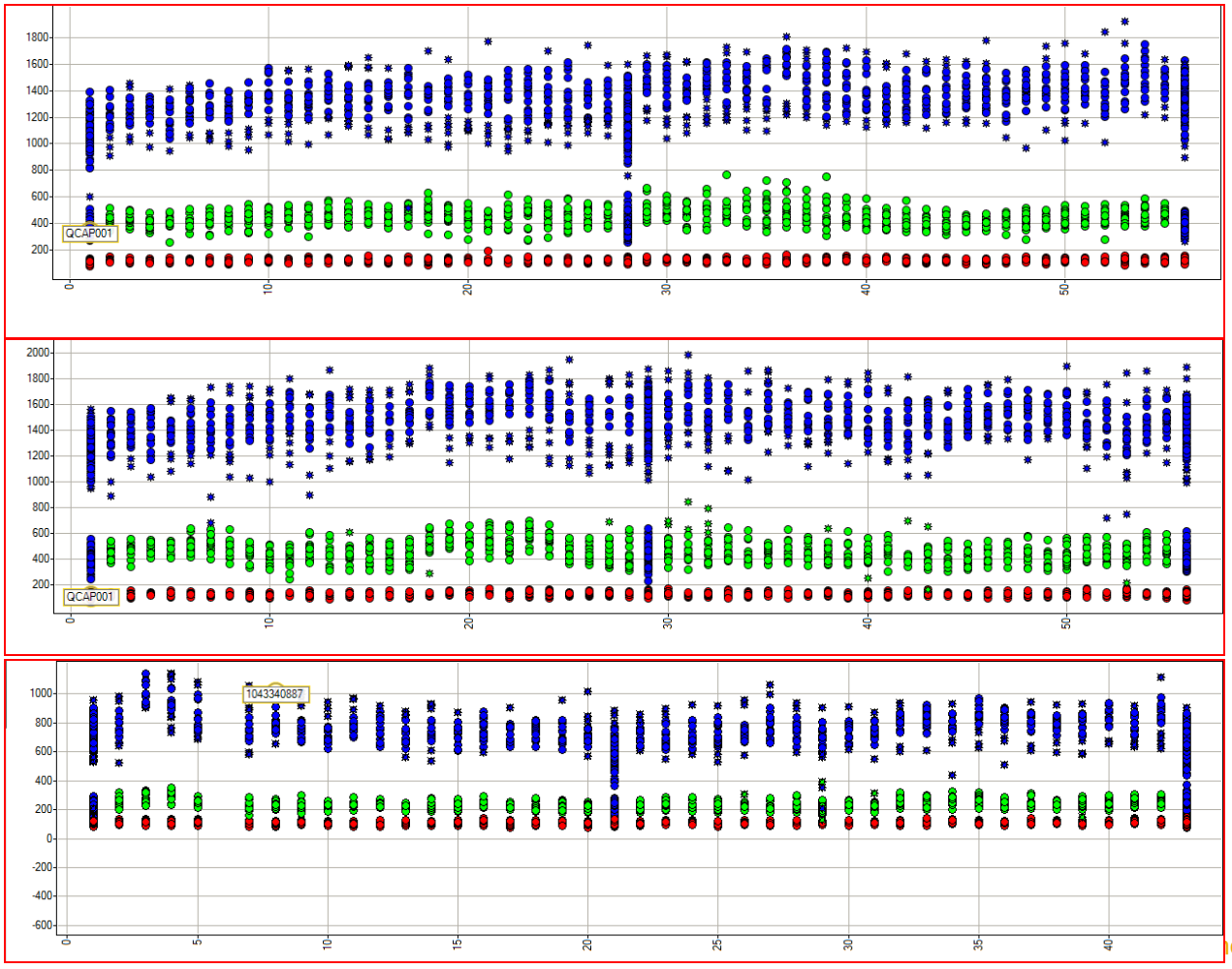
ERα ligands can be identified by their ability to displace the tracer and disrupt the TR-FRET signal.

Tracer bound
TR-FRET occurs

Tracer displaced
Loss of TR-FRET

HTS follow up – control well data

100k sub-set screen ; 6k followed up in concentration response format
0.5k < 1uM IC50 ; 2.5k < 10uM ; 15 distinct series ; 7 confirmed by X-ray



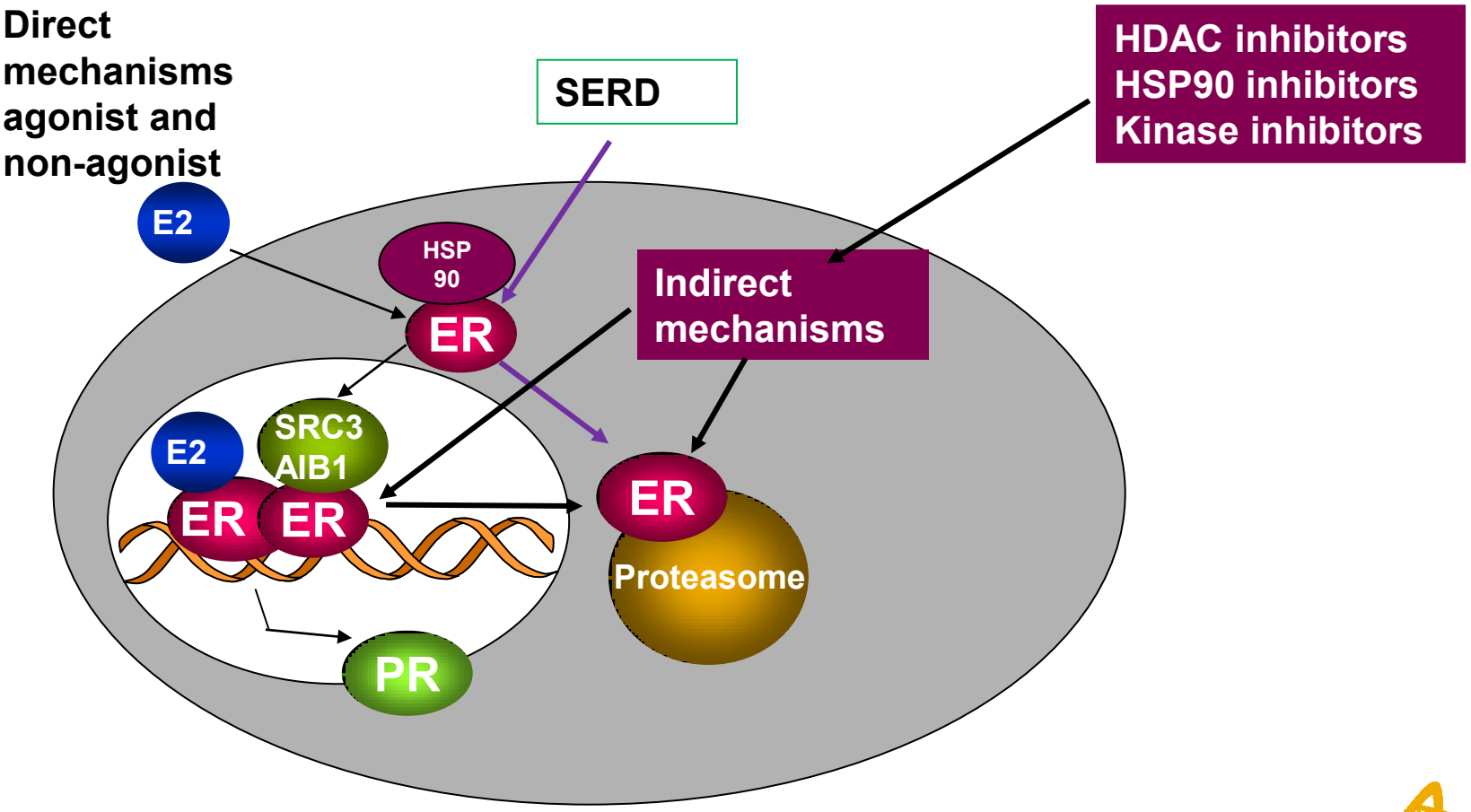
Run 1
Z' 0.73

Run 2
Z' 0.75

Run 3
Z' 0.68

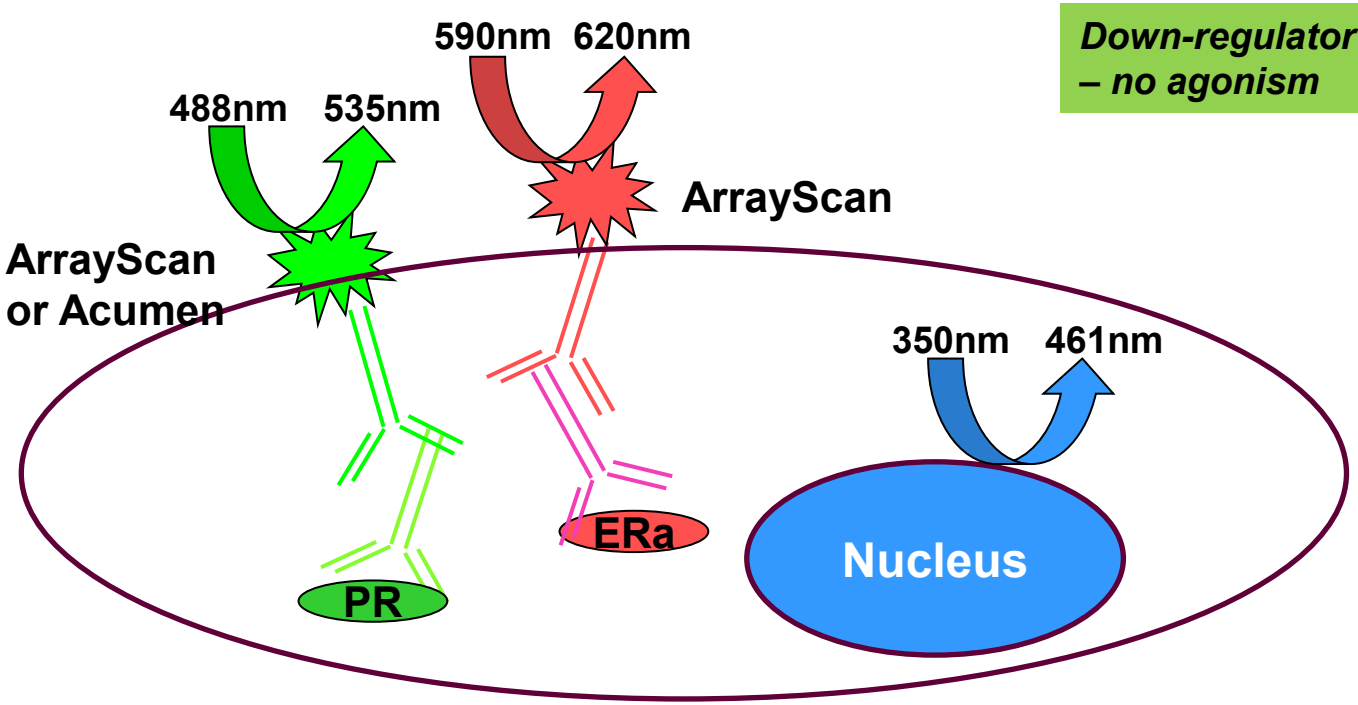
Multiple mechanisms of ERα regulation exist – Mediated via ligand binding to ERα or other targets

Post HTS – identifying novel ERa down-regulators was feasible but vast majority had "agonist" profile



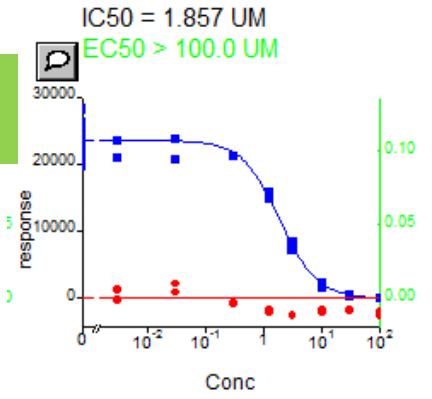
Multiplexed MCF7 cell assay

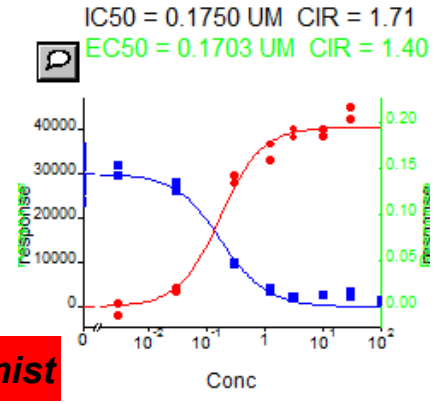
- Immunofluorescence quantified on the ArrayScan (ER) and Acumen (PR) platforms
- Total levels of ERα and PR are detected by specific antibodies then by labelled secondaries
- Detection after 24 hour cpd treatment to enable PR signal to be induced
- Finalized assay uses cryopreserved cells, 384 well format and automated antibody staining
- Has been modified to read-out as a functional antagonism assay – pre-dose with E2
- Full validation package completed successfully.



Literature cpd.

Down-regulator – no agonism





Agonist

Assay Overview - ~300 cpds/run in 12 point duplicate concentration response format

Day 1

Seed cryopreserved MCF-7 cells in 384 well plates.

Day 2

Automated compound dispensing using Integrated Echo workcell followed by 24 hr incubation.

Day 3

Fix cells (20mins).
Automated antibody staining using AutoElisa workcell (1hr permeabilization & incubate with primary antibodies to ER and PR overnight)

Day 4

Automated antibody staining using AutoElisa workcell (1hr incubation with secondary antibodies & Hoechst).
Read plates on Acumen for PR detection and Arrayscan for ERα detection

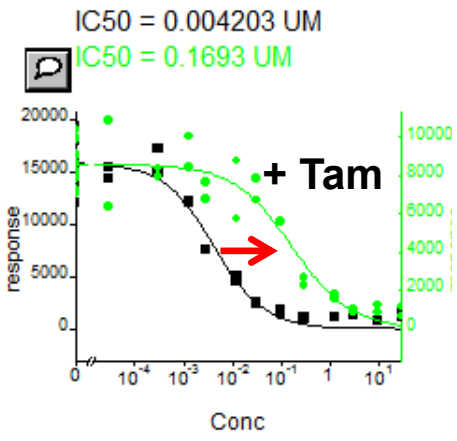
Mechanism of Action assay

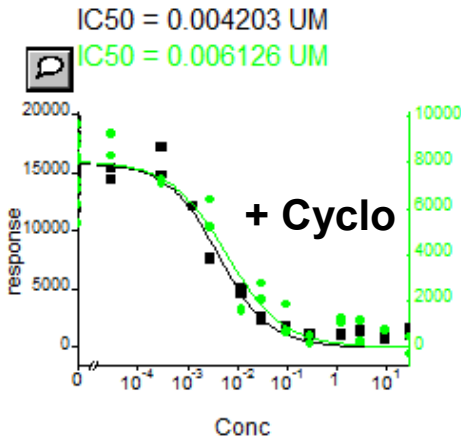
- Comprises 3 tests in parallel to distinguish direct binding down-regulators of ERα from off target mechanisms.
- Tamoxifen binding stabilises ERα
- Cells are pre-treated with 250nm Tamoxifen or DMSO (control) or 25ug/ml Cycloheximide for 1hr prior to compound dosing and then incubated for 5 hrs.
- ERα only is detected on the Acumen or ArrayScan platform – timecourse is too short to have measureable PR induction.
- Potential SERD compounds will compete with Tamoxifen for binding to ERα and become less potent (0.5 log shift in potency for control compounds).
- No shift in potency is seen with off-target down-regulators and indeed some off-target compounds are inactive at 5 hours.
- Protein synthesis is implicated in the agonist induced down-regulation mechanism
- The cycloheximide treatment blocks protein synthesis in the cell which helps further to discriminate indirect and undesirable mechanisms of down-regulation.
- For on target compounds, the potency is largely unchanged in the presence of cycloheximide and this arm also verifies that activity is seen against pre-existing ERα.

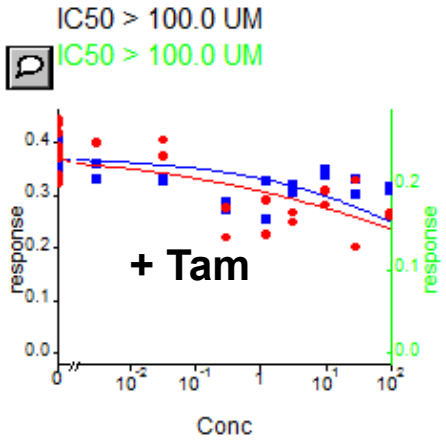
MCF7 MOA cell assay

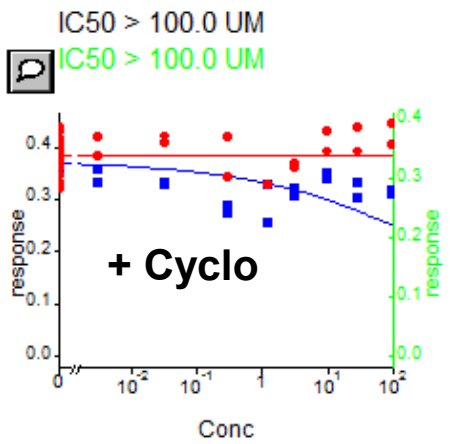
ER detected following DMSO, Cycloheximide and Tamoxifen treatment plus compound

- Immunofluorescence quantified on the Acumen or ArrayScan platform
- Total levels of ERα are detected by a specific antibody then by labelled secondary antibody
- 5 hour timepoint – 1 hour pre-incubation - 3 conditions, vehicle, + 250nm Tamoxifen, + 25ug/ml Cycloheximide
- On target – +Tam IC50 shifts ; Off target - +Tam IC50 no shift or inactive at 5 hours
- + Cycloheximide – compound active versus extant ERα and IC50 non significantly shifted









On - target – Novel SERD

Off - target – HDAC inhibitor

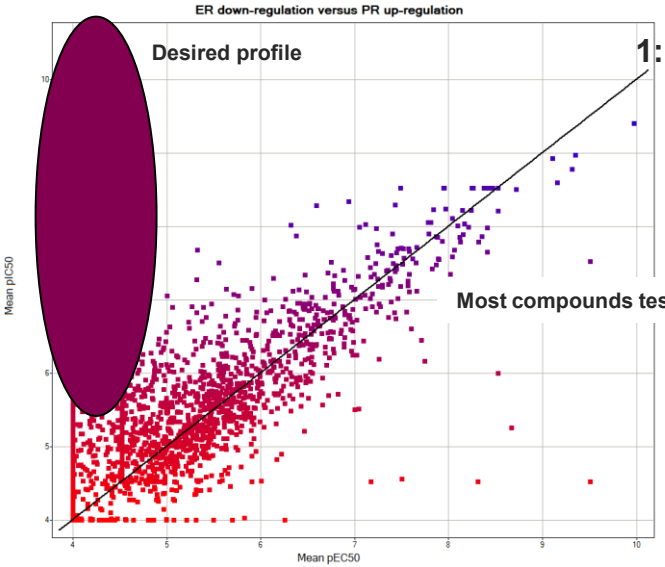
Impact on project – effective and timely compound triage

5k compounds

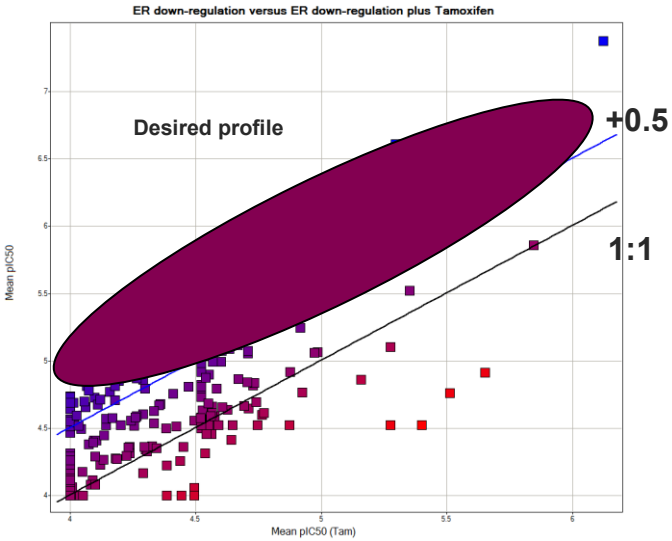
ERα down-regulation ; PR up-regulation

300 compounds

ERα down-regulation
Plus Tamoxifen/cyclohex/DMSO control



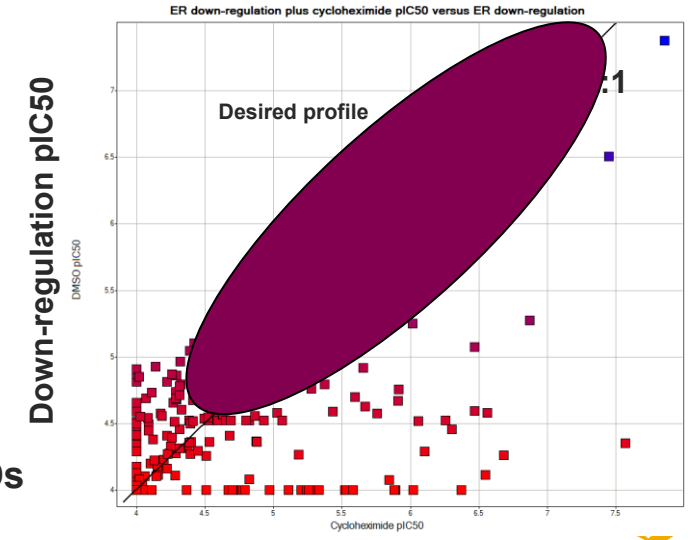
Agonist pEC50



Down-regulation pIC50 + Tamoxifen

Desired profile
~100 compounds

Novel confirmed SERDs



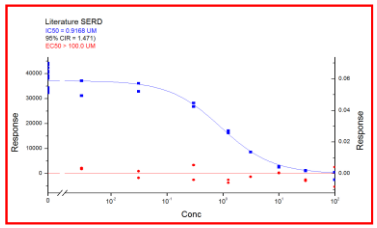
Down-regulation pIC50 + Cycloheximide

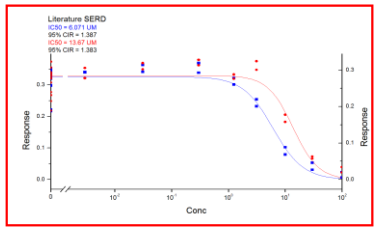
Compound Profiles

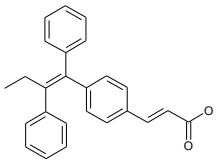
Overlay of ERα down-regulation with PR up-regulation after 24 hrs

Overlay of ERα down-regulation in presence and absence of 250nmTamoxifen after 5 hrs

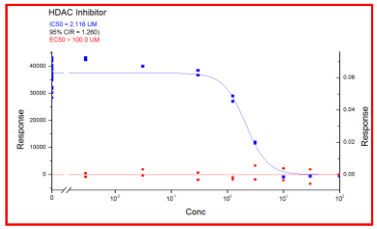
Literature SERD compound
ER down-regulator ✓
PR up-regulator ✗
Competitive with tamoxifen ✓
Active at shorter time point ✓
The desired profile for a novel SERD

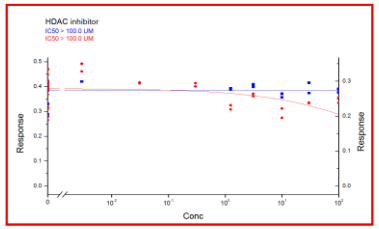


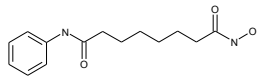




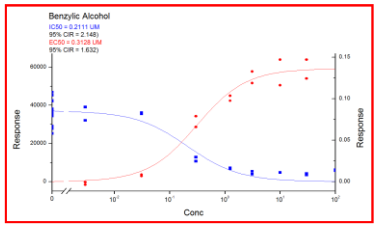
HDAC Inhibitor
ER down-regulator ✓
PR up-regulator ✗
Competitive with tamoxifen ✗
Inactive at shorter time point ✗
An undesired profile for a SERD

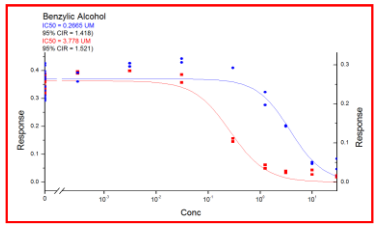


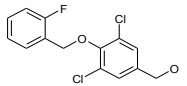




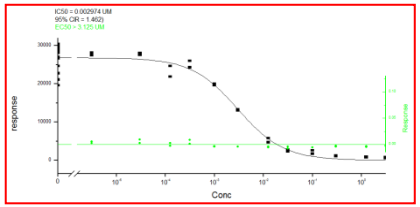
Benzylic Alcohol
ER down-regulator ✓
PR up-regulator ✓
Competitive with tamoxifen ✓
ER agonist down-regulator profile
An undesired profile for a SERD

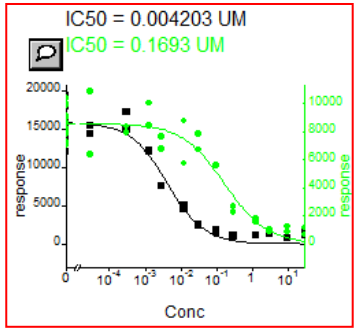






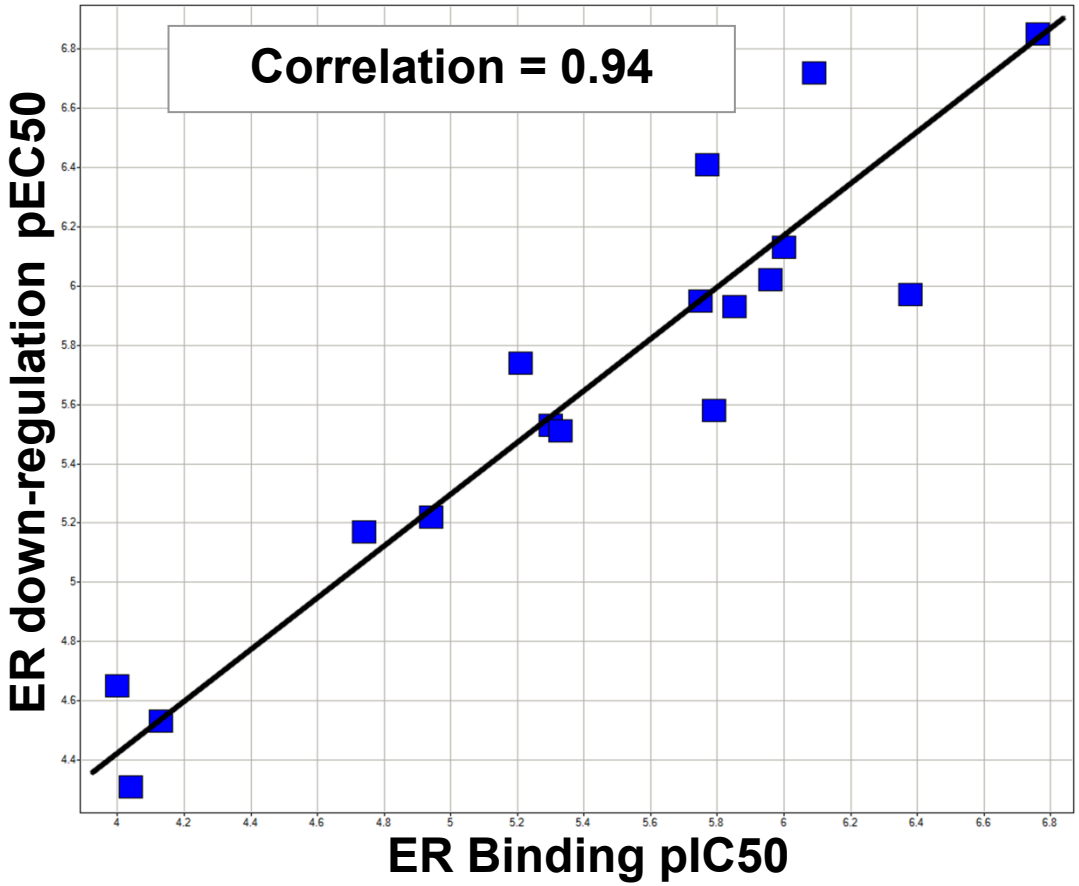
Novel SERD 1
ER down-regulator ✓
PR up-regulator ✗
Competitive with tamoxifen ✓
Active at shorter time point ✓
Desired profile for a novel SERD





Correlation of ER α Binding and Down-regulation

The ERα down-regulation assay developed shows a very good correlation with ERα binding for the lead novel series with a correlation coefficient of 0.94.



Correlation among cellular down-regulation, antagonism and anti-proliferative effects for the novel series – some literature SERDs were better antagonists than SERDs

Novel SERD versus Fulvestrant

Multiplex ERα PR assay

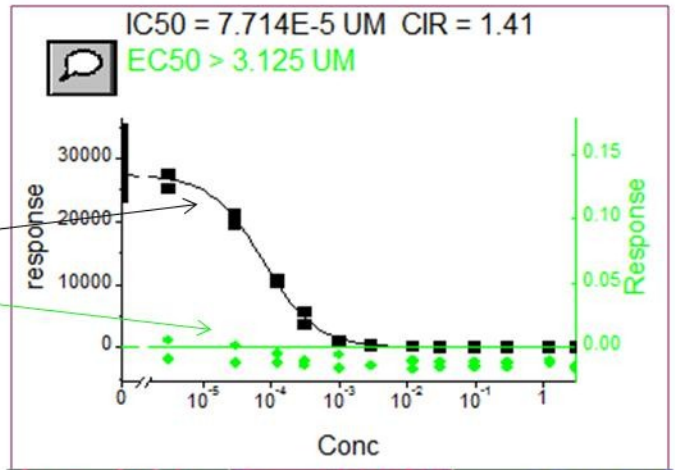
Multiplexed ERα PR assay

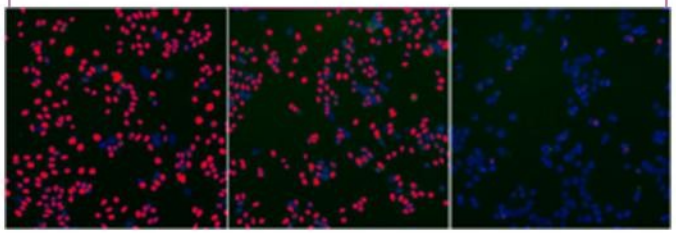
Black – Decrease in ER levels (down reg)

Green – No increase in PR levels (agonism)

ArrayScan images illustrating level of ERα staining

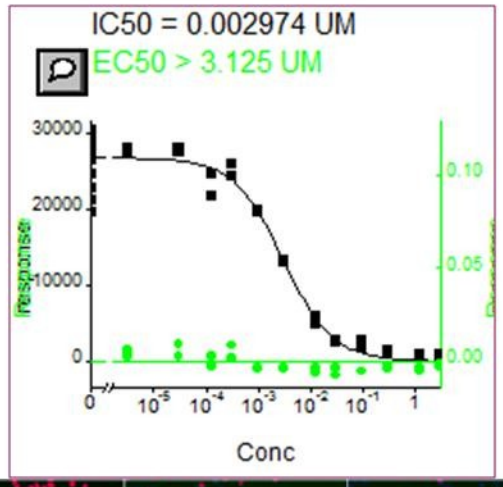
Fulvestrant

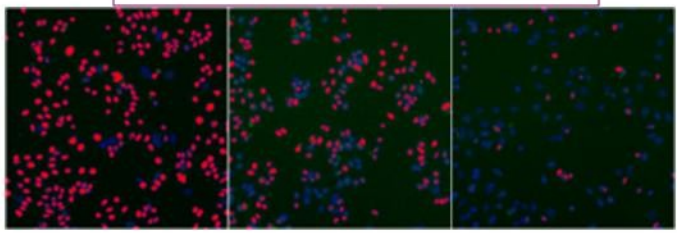




Max ERα response | 50% effect | 100% effect

Novel SERD





Max ERα response | 50% effect | 100% effect

Summary - Cell assays available to discriminate different compound profiles and drive SAR chemistry

Do compounds reduce ERα levels in MCF7 cells?

Are they acting via an agonist mechanism?

Are compounds active at 5 hours as well as 24 hours?

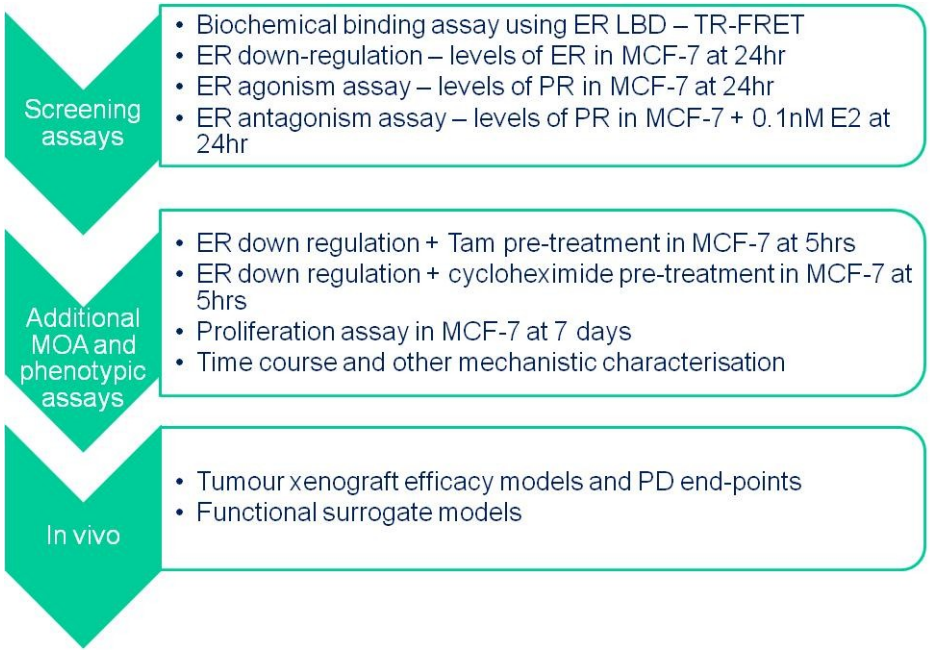
Do compounds work by binding to ERα?

Are compounds able to reduce levels of existing ERα?

Do compounds antagonise ERα mediated signalling?

Are compounds anti-proliferative in an ERα driven cell line?

How do novel SERDs compare to Fulvestrant?



Summary and Conclusions

- Resurgence in interest in phenotypic screening approaches
  - Relative lack of success using target directed approaches perceived to be primarily due to a lack of target validation
  - Developments in cellular reagents and assay platforms enable more predictive and robust phenotypic assays

- Phenotypic approaches require significant investment in target deconvolution
  - Projects are uncomfortable not knowing the molecular target
  - Target identity not necessary for drug registration

- Advances in cell reagents mean that target directed approaches can utilise assays which more accurately reflect disease biology and may ultimately lead to greater project success
  - Examples where human stem cell derived assays predict in vivo and adverse effects better than recombinant cell assays

- Opportunities exist to identify Best In Class molecules with different molecular mechanisms of action

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  - Per-Erik Stromsted and Ryan Hicks

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Confidentiality Notice