Cancer Research Technology
Aurore Lejeune ● 5th & 6th March 2013, Promega Cell Based Assay Tour

A Novel Cell-Based Screening Approach For The Identification Of FOXA1 Pathway Inhibitors for the Treatment Of Tamoxifen-Resistant Breast Cancer
Presentation Plan

- Introduction to Cancer Research Technology
- FOXA1 Project: Rationale & Target Validation
- Development and Validation of Cell Based Assay
- HTS campaign
- Hit Confirmation & Profiling
Cancer Research Technology
Who we are

Cancer Research Technology (CRT)

- Technology development & commercialisation arm of Cancer Research UK (140 staff)
- Manage the intellectual property from CR-UK funded research
- HQ in London, US subsidiary in Boston, partner in Australian consortium
- Provide technology transfer services, including access to CRT/CR-UK translational resources, to both CR-UK and non CR-UK affiliated institutions worldwide
- Internal drug discovery laboratories (CRT-DL) with major partnerships with AstraZeneca and Teva (formerly Cephalon)
Role of the CRT-DL

- To develop (and de-risk) to *in vivo* proof of principle stage
  - Partner at this stage
  - Maxmise number of projects developed

- HTS, pharmacology, medicinal chemistry, crystallography; project validation function
  - Focus on industry experience and skills to prosecute a maturing portfolio

- Select “novel” targets as priority from CR-UK funded and other academic research
  - Collaborations worldwide with leading academic research groups
  - Discovery alliances with industry (AstraZeneca and TEVA)

CRT bridges the gap between academia and Industry
FOXA1 Project: Rationale & Target Validation
ER Therapy Resistance in Breast cancer: Unmet Clinical Need

Estrogen receptor is the driving transcription factor in 75% breast cancers
- ER antagonist Tamoxifen is first line treatment
- Resistance frequently occurs – intrinsic or acquired

Initially ER positive (75%)
- ER negative (25%)
- Benefit from hormonal therapy
- Develop resistance due to loss of ER
- Develop resistance but maintain functional ER

Up to 83% of patients who are resistant to Tamoxifen may be sensitive to a FOXA1 pathway inhibitor AND
Potential first line treatment for ER+ve breast cancer

Other indications include:
- Molecular apocrine breast cancer
- Castrate resistant prostate cancer

Cancer Research Technology
Advancing Discoveries to Beat Cancer
Jason Carroll – CR:UK PI

Group leader: Nuclear Receptor Transcription – CR:UK CRI (joined 2006)
• World leader in FOXA1 and ER Biology
• Received the award of Cancer Research UK’s Future Leaders in Cancer Research at 2012 NCRI conference.

Many resources and technology to add value to FOXA1 project

Active researching into FOXA1 pathway
• Mapping post-translational modifications of FOXA1
• Determination of FOXA1 protein complexes by novel proteomics methodologies
• Global FOXA1 and ER chromatin binding analysis (ChIP-Seq)
• Pathway signatures, interference and connectivity (gene expression and ChIP-Seq)
FOXA1 is a Pioneer Factor for Estrogen Receptor Binding

**FOXA1**

Estrogen

**ER**

Signalling cascade

**mRNA**

**Protein synthesis**

**Proliferation**

---

**Review**

**FOXA1: master of steroid receptor function in cancer**

Michael A Augello\(^1,2\), Theresa E Hickey\(^3\) and Karen E Knudsen\(^1,2,4,5,6,8\)
In vitro Validation from Carroll Lab (CRI)

ER binding is largely dependant on FOXA1
- FOXA1 knockdown abolishes 95% of all ER genomic binding events
- Knockdown prevents expression of ER target genes
- FOXA1 knockdown reduces cell growth in Tamoxifen sensitive and Tamoxifen resistant cell lines

Hurtardo et al. (2011) Nat. Gen. Carroll lab
FOXA1: Value Proposition and Rationale

**FOXA1/ERα pathway inhibitors designed to overcome acquired resistance associated with endocrine therapy**

- FOXA1 is required for cell viability in endocrine resistant breast cancer models
- Distinct FOXA1 mediated ERα-binding profiles associated with poor clinical outcome
- FOXA1 expression positively correlates with metastatic vs. primary tumour samples
- Identification of robust Biomarker for *in vitro* and *in vivo* investigations
Development and Validation of Cell Based Assay
Screening Cascade to Hit Characterisation

Aims of Hit Identification campaign

• Identify and validate novel inhibitors of the FOXA1 pathway

• Screen 150,000 compounds from CRT’s chemical libraries

• Confirm Hits through primary reporter assay

• Counterscreen for general inhibition of transcription, luciferase/quenching and other Interference effects

Primary Screen

Hit Identification

Counter-screens

Hit Validation

ER dependency

Phenotypic Assay

PD Biomarker Assay

Hit characterisation

Hit Validation

LCMS QC batch02 plus “Analogue by catalogue”

1) CMV-driven Reporter (MCF7)
2) Luciferase Interference

Biochemical ERα Binding Assay

2D Viability
FOXA1/ERα +ve (MCF7)
FOXA1/ERα –ve (MDA-MB-231)

Endogenous PD biomarker TFF1 and XBP (MCF7)
Cell-Based Primary Screen for FOXA1 Pathway Inhibitors

Estrogen

Signalling cascade

FOXA1/ER binding sites

Minimal promoter

mRNA

Protein synthesis

Custom-made reporter assay developed in Carroll lab

Cancer Research Technology

Advancing Discoveries to Beat Cancer
Validation of Reporter Cell-based assays

Specificity for Primary and Counterscreen reporter assays established via siRNA Knockdown

- Reduction of luciferase activity by siRNA FOXA1 (and Fulvestrant) depletion in Primary assay, confirming its role in regulation of reporter transcription
- FOXA1 siRNAs or Fulvestrant had no effect on the CMV assay activity
**Principle of Reporter Cell-based assay**

**Tissue Culture Facilities**

- Cell Expansion in MultiLayer TC Flask
  - ~ 5x10^7 cells total/run

7 days prior screening

**Bulk Dispenser**
- Thermo MultiDrop Combi

Screening Day 1

**Acoustic Dispenser**
- Labcyte ECHO550

Screening Day 2

**Thermo MultiDrop Combi**
- + Reader BMG Pherastar

Screening Day 3

**Automated Compound transfer from**
- 3mM Compound stock 100%DMSO to cell plates

**Automated Steady Glo dispensing**
- + Luminescence Reading

Data Analysis

**Dotmatics Vortex**
- + IDBS Activity Base

Advancing Discoveries to Beat Cancer
Miniaturisation and Automation of Primary reporter assay

- Optimisation of cell density and stability of luminescent signal were critical parameters to establish a HTS-compatible assay. Selection of Steady-Glo reagent to quantify reporter signal.
- Automation of process relies on compatibility of cell-based assay and limitations imposed by use of automation.

**Timecourse of reporter signal & growth curve**
- Luminescent signal (RLu) vs. Time (h)
  - Viability
  - Reporter

**Signal stability in Primary reporter assay**
- Steady Glo Luminescent signal (RLu) vs. Time (h)
Miniaturisation and Automation of Primary reporter assay

- High variability of signal (CVs > 10%) observed within profile of full plates resulted in lack of robustness and dramatically affected Z factor.
- Troubleshooting focused on various technical aspects of High Throughput process but none would decrease variability of signal.

**Profile of 384well plate, Reporter Assay:**

Steady Glo

**Profile of 384well plate, Viability Assay:**

Cell Titre Glo

SG CV = 14%

CTG CV = 5%
Miniaturisation of Reporter Cell-based assays

Validation of Primary and Counterscreen reporter assays

- Use of tool reference compounds, Fulvestrant as control compound, to establish robust and repeatable 3-fold signal window across Assay Development runs.

![Diagram](chart.png)

- Green Diamonds: Fulvestrant potency (pEC50) in Primary reporter assay.
- Plain green line: average pEC50 value.
- Dash green line: 3fold acceptance range in Primary reporter assay.

- Red Squares: Fulvestrant Maximum Inhibition in Counterscreen assay.

Cancer Research Technology
Advancing Discoveries to Beat Cancer
• Screening a set of 3000 compounds from CRT’s chemical library, in 3 independent experiments.

• Despite high variability of signal across plates (CV ~ 8 to 15%), 3 robust automation trials identified 2 active compounds (red) with good reproducibility and Fulvestrant as control compound.

• Completing the panel of reference compounds with 2 anti-estrogenic compounds RU58668 and ZK164015, the decision to progress to full HTS was made.
HTS Campaign
**QC of HTS campaign**

- QC criteria are calculated & compared to thresholds established through Assay development.
- Included on each plate at a single dose, potency of reference compounds were monitored.
- Tracking of assay performance confirmed process robustness and maintenance of good quality of screening data.

![Tracking of reference compounds potency](image)

- 1.2nM ZK164015 (EC90)
- 1.2nM RU58668 (EC40)

Colours for different runs
Profile of HTS campaign

- Scatter plot of 150,000 compounds at 3uM/0.1%DMSO from various CRT chemical collections.

% Inhibition Profile during HTS campaign

Green : Calbiochem library
Pale green : Natural product-like set
Yellow : Kinase library
Orange : LOPAC library
Red : Diverse CRT set
Grey : Fulvestrant Reference 3uM
HTS primary campaign

Investigations for data analysis

Review of different approaches for hit selection

- Controls-based % Inhibition, current method for HTS analysis in CRT

- MAD-score, statistical method independent of plate controls. Valid for a non normal distribution of values & no biases within distribution of values across row/columns.

- Approach: Use MAD-score to make a case for plate to plate within one run, generalise approach to full campaign. Compare results from % Inhibition to MAD-score, qualitatively and quantitatively.
Analysis of HTS campaign

- Dual approach for data analysis used to select actives: MADscore AND % Inhibition.
- Selection of 405 actives for reconfirmation, above 68% Inhibition during Primary campaign.

Red: ZK164015 1.2nM
Yellow: RU58668 1.2nM
Green: Unknowns
Hit Confirmation & Profiling
FOXA1 screening Cascade: Hit Confirmation

Primary Screen

1) FOXA1/ER-dependent Luciferase Reporter Assay MCF7

2) CMV-driven Luciferase Reporter MCF7

Counter-screens

2) Luciferase Interference studies

End of Hit Identification

Threshold
Confirmation of active compounds

Correlation between Primary vs Counterscreen assay

- Dose Response profile was established to evaluate potency.

![Correlation of potency in primary and CMV Assays](image-url)

Gradient colours:
- Potency in Primary Assay
Confirmation of active compounds

Correlation between Primary vs Interference assay

• Dose Response profile was established to evaluate potency.

Correlation of potency in primary and Luciferase Assays
Confirmation of active compounds

Establishment of early Structure Activity Relationship

- Identification of 3 chemical clusters with range of potency in Primary Reporter assay, from 0.1-10uM.

Example: Profiling of Compound A, confirmed hit

Log C

%Inhibition

0.0001 0.01 1
-80 -50 -20 10 40 70 100 130 160

Primary
CMV
Luciferase
ER Binding

Advancing Discoveries to Beat Cancer
Despite a challenging Assay Development phase, a robust process using a validated biological system could be established.

The cell-based HTS campaign was successful with identification of 3 potential chemical series, that will be profiled through our screening cascade for hit characterisation.
FOXA1 project

Lessons learnt

- Facing uncertainty at early stage of the project, support from PI/TiVal Biologist, trust in CRT’s Automation Processes and quality of reagents, i.e. Promega Steady Glo reagent, were critical to progress to HTS.

- Within compound screening, the quality of data should not be negotiable. But the criteria that qualify data quality may vary, based on our knowledge/biology of the target and our ability to establish relevant tools to investigate them.
Acknowledgements

**FOXA1 Project Team**

**CRT**
- Jon Roffey
- Julie Stock
- Ai Ching Wong
- Michele Barnard
- Jennifer McKelvie
- Dan Liu
- Stephen Myatt
- Matthew Farren

**CRUK:CRI**
- Jason Carroll
- Kelly Holmes

**SCM team**
- Susan Young
- Mathew Rushbrooke
- Cristina Alli
- Fabrice Turlais
- Sheila McLoughlin
- Christian Dillon
- Christopher Ireson
- Angus Lauder
- Katherine Ewings
- Neil Jones
- Martin Swarbrick

**Technical support**
- Matthew Cook, Promega
- Carl Jarman, Labcyte