High content analysis for detection of drug-induced structural cardiotoxicity

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Cardiovascular toxicity is a major cause of drug attrition

<table>
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<tr>
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<th>Phase I</th>
<th>Phase I-III</th>
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<th>Nervous system</th>
<th>Immunotoxicity</th>
<th>Gastrointestinal</th>
<th>Reprotox</th>
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The various toxicity domains have been ranked first by contribution to products withdrawn from sale, then by attrition during clinical development. Note general agreement between pairs of equivalent studies.

Diversity of drug-induced cardiovascular toxicity

Functional CV tox
- Cisapride
  - QT prolongation
- Haloperidol
  - ↓CM contractility

Structural CV tox
- Fenfluramine
  - Vavulopathy
- Trastuzumab
  - Necrosis/apoptosis
- Sunitinib
  - Sorafenib

Indirect CV tox
- Sunitinib
  - Bevacizumab
    - ↑Blood pressure
- Rosiglitazone
  - Odema
- Rofecoxib
  - Thrombosis

Arrhythmia
Cardiomyopathy
Heart failure
Myocardial Infarction

Withdrawn (W)
Restricted/label warning (R)
### In vitro cardiac models

<table>
<thead>
<tr>
<th>Type</th>
<th>Model</th>
<th>Screen potential</th>
</tr>
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<tbody>
<tr>
<td>Cell lines</td>
<td>rat H9c2</td>
<td>Limited cardiac phenotype, non-human</td>
</tr>
<tr>
<td></td>
<td>mouse HL-1</td>
<td>Atrial, non-human</td>
</tr>
<tr>
<td>Primary</td>
<td>Freshly isolated dog/rat CMs</td>
<td>Not amenable to long term culture</td>
</tr>
<tr>
<td></td>
<td>Human primary cardiomyocytes (various vendors)</td>
<td>Limited cardiac phenotype</td>
</tr>
<tr>
<td></td>
<td>neonatal cultured rat CMs</td>
<td>Low yield, non-human</td>
</tr>
<tr>
<td>Stem cell derived</td>
<td>Human ESC derived (Cytiva\textsuperscript{TM}, GE Healthcare Life Sciences)</td>
<td>Assay ready, large scale production</td>
</tr>
<tr>
<td></td>
<td>Human ESC derived (hES-CMC\textsuperscript{TM}2D/002, Cellectis)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mouse ESC derived (Cor.AT, Axiogenesis AG)</td>
<td>Assay ready, large scale production</td>
</tr>
<tr>
<td></td>
<td>Human iPS derived (iCell Cardiomyocytes, Cellular Dynamics International)</td>
<td>Assay ready, large scale production</td>
</tr>
<tr>
<td></td>
<td>Human iPS derived (ReproCardio, ReproCELL Inc.)</td>
<td></td>
</tr>
</tbody>
</table>
Stem cell derived cardiomyocytes

General features

**Spontaneously beat**

**Cardiomyocyte markers**

- Mixed myocyte population
  - Ventricle-like AP: 82%
  - Atrium-like AP: 18%
  - Nodal-like AP: <1%

- Embryonic phenotype

1. Images courtesy of GE Healthcare [hESC]
High content analysis (HCA) for detecting drug toxicity *in vitro*

- HCA is a drug discovery method that uses images of living cells as the basic unit for molecular discovery.
- Enables investigation of multiple pathways at the single-cell level yielding mechanistic information.

**Cytotoxicity**
- Cytotoxicity assays measure cell death.
- ATP levels deplete as cell number decreases.
Tracking cardiomyocyte health with HCA & cytotoxicity
hESC derived (Cytiva™, GE Healthcare)

Cryopreserved

384 well plate

Incubate for 72 h (media changed every other day)

Add compound - 6 point concentration response (0.03 µM - 100 µM)

H9c2 cells

passaged

Treat for 72 h, re-dose after 48 h

Fluorescently label cells (TMRE, ER Tracker™, Flou-4, TOTO-3®)

Imaging-based technology (ImagXpress Molecular Devices)

ATP assay

Generate concentration response curves and IC₅₀ values

IC₅₀ values normalised to total Cmax (Therapeutic Index, TI)

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Assay cut-off defined using ROC curve
Example drugs and compound classification

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Marketed drugs were categorised based on the occurrence and manifestation of cardiotoxicity reported in the FDA approval package.

**Clinical Set**

- **Structural cardiotoxins (15)**
  - Amiodarone
  - Amphotericin B
  - Bortezomib
  - Clozapine
  - Cyclophosphamide
  - Dasatinib
  - Doxorubicin
  - Fluorouracil
  - Idarubicin HCl
  - Imatinib
  - Isoproterenol
  - Lapatinib
  - Mitoxantrone
  - Sorafenib Tosylate
  - Sunitinib

- **Functional cardiotoxins (9)**
  - Anagrelide HCl
  - Buspirone HCl
  - Cisapride Hydrate
  - Ketoprofen
  - Levofloxacin
  - Minoxidil
  - Nifedipine
  - Terfenadine
  - Voriconazole

- **Non-cardiotoxins (8)**
  - Acyclovir
  - Donepezil HCl
  - Erlotinib
  - Gemfibrozil
  - Mebeazole
  - Naringenin
  - Praziquantel

**Preclinical Set**

- **Structural cardiotoxins (19)**
  - Degeneration
  - Necrosis
  - Hypertrophy

- **No evidence of structural cardiotoxicity (18)**
  - Multiple organ toxicities e.g. liver, bone marrow, kidney

AZ Compounds that have been tested in repeat dose rat or dog toxicity studies of up to 1 month duration with histopathology in multiple organs and PK data.

34 +ve

35 -ve
72 h gives optimum detection of structural cardiotoxins

<table>
<thead>
<tr>
<th>Compound</th>
<th>6 h IC₅₀ (μM)</th>
<th>24 h IC₅₀ (μM)</th>
<th>72 h IC₅₀ (μM)</th>
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<tr>
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<td>ATP MMP</td>
<td>Ca²⁺ mobilization</td>
<td>ER integrity</td>
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<tr>
<td>Sunitinib Malate</td>
<td>51.63 83.46</td>
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<td>100</td>
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</table>

Most relevant end point

![Graph showing ATP, MMP, Ca²⁺ mobilization, and ER integrity responses to different concentrations of Sunitinib Malate over time.](image)
hESC-CM assay performance compared to H9c2

Most sensitive end point

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Cut-off (μM)</th>
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<tr>
<td>72 h hESC-CM</td>
<td>74</td>
<td>74</td>
<td>10</td>
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<tr>
<td>72 h H9c2</td>
<td>58</td>
<td>62</td>
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hESC-CMs give improved detection of clinical and pre-clinical structural cardiotoxins
HCA endpoints in hESC-CMs provide extra sensitivity

hESC-CM most sensitive assay

- Clinical cardiotoxins
- AZ cardiotoxins
- Line of unity

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Importance of being organotypic
Provides the ability to distinguish between organ toxicity and intrinsic cytotoxicity in vitro

In vivo exposure value ($C_{max}$)

Cut offs
10µM 100µM

Intrinsic cytotoxicity
Organ-specific toxicity exhibited *in vitro*?

Same concept applies for pre-lethal HCB parameters
**Importance of the beating phenotype**

**Pharmacological inhibition of spontaneous beating prevents toxicity**
hESC-CM assay reveals broad mechanisms of cardiotoxicity
Anticancer agents associated with cardiotoxicity

- **Type I (permanent damage)**
  - Cytotoxic anticancer agents
    - Doxorubicin (anthracycline)
    - Daunorubicin, epirubicin (anthracycline)
    - Mitoxantrone (anthracenedione)
    - Cyclophosphamide (oxazophorine alkylating agent)
  - Associated with increased cardiovascular mortality

- **Type II (reversible damage)**
  - Targeted anticancer agents
    - Trastuzumab (monoclonal antibody)
    - Sunitinib (tyrosine kinase inhibitor)?
    - Lapatinib (tyrosine kinase inhibitor)?

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In *vitro* end points indicate potential clinical outcome?

Sub-lethal changes in *vitro* ≈ Type II cardiotoxicity/ potential reversible damage?

Cytotoxic in *vitro* ≈ Type I cardiotoxicity/, Potential for permanent damage?
Building a Molecular Toxicology project support tool kit

1. Next generation RNA sequencing of in vitro models and tissue from human and preclinical species

2. Knock down of target with siRNA by Nucelofection in hESC-CMs

3. Functional verification of knock down in hESC-CMs (radiolabelled assay)

4. Assess impact of target knock down on cardiotoxicity in hESC-CMs

Known drug transporters

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Adenosine uptake

Rate (pmol/mg/min)

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<tr>
<td>Sunitinib + dipyridamole</td>
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Sunitinib | Sunitinib + NBTI | Sunitinib + dipyridamole
Project issue solutions

Target assessment
- RNA sequencing of key CV tissues (rat, dog, monkey, human)
- siRNA knockdown of toxicity targets
- Structurally similar inactive cps

Screening
- hESC-CM assay
- H9c2-CM assay
- mESC-CM assay
- Additional HCB and gene expression endpoints
- Label free technologies

Species translation
- Robust models, equivalent to human CMs for rat, dog, monkey *
- Translatable biomarkers

Patient setting
- Hypoxic environment
- Altered fuel substrates
- *In vitro equivalent of combination therapies
- iPS derived CM from susceptible genotypes*

Available

Several years away
Project issue solutions

Project A: 1 week rat studies have identified severe cardiac necrosis with the lead compound

- Develop a follow up screen to aid compound selection without this toxicity

**Target assessment**
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**Patient setting**
- Hypoxic environment
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- *In vitro* equivalent of combination therapies
- iPS derived CM from susceptible genotypes*
Project issue solutions

Project B: Two structurally distinct candidate drugs have caused cardiac necrosis in the 1 month dog GLP study
  • Is the mechanism target related?
  • Is it species specific?

Target assessment
  • RNA sequencing of key CV tissues (rat, dog, monkey, human)
  • siRNA knock down of toxicity targets
  • Structurally similar inactive cps

Screening
  • hESC-CM assay
  • H9c2-CM assay
  • mESC-CM assay
  • Additional HCB and gene expression endpoints
  • Label free technologies

Species translation
  • Robust models, equivalent to human CMs for rat, dog, monkey
  • Translatable biomarkers

Patient setting
  • Hypoxic environment
  • Altered fuel substrates
  • In vitro equivalent of combination therapies
  • iPS derived CM from susceptible genotypes
Project issue solutions

Project C: Clear evidence that there is a cardiac liability associated with the target in the rat. However, there is literature to suggest a human-specific protective mechanism.

- Is there an *in vitro* strategy to test this?
- Can the patient disease environment be modelled *in vitro*?

---

**Target assessment**
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**Screening**
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- mESC-CM assay
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- iPS derived CM from susceptible genotypes
Indirect CV toxicities

**Functional CV tox**
- QT prolongation
- ↓CM contractility

**Structural CV tox**
- Vavulopathy
- Necrosis/apoptosis

**Indirect CV tox**
- ↑Blood pressure
- Odema
- Thrombosis

**Heart failure**
- Arrhythmia
- Cardiomyopathy
- Hypertophy
- Myocardial Infarction

Drugs:
- Torcetrapib
- Rosiglitazone
- Rofecoxib
Tissue engineering opportunities for modelling complex CV tox mechanisms

- Role of Cardiac microvascular endothelial cells (CMEC) in CV toxicity
  - Collaboration with Dr Mike Cross, MRC Centre for Drug Safety Science, University of Liverpool
  - Investigating effect of CV toxins on CMEC toxicity
  - CMEC-CM co-culture system (spheroids)

- Interaction with non-CV organs
  - Modular bioreactors e.g. QuasiVivo™ system (Kirkstall Ltd)
  - Ability to link multiple cell culture units
Summary

• Human stem cell derived cardiomyocytes are a step change in *in vitro* assessment of drug induced cardiotoxicity
• They distinguish between cardiac-specific and inherent cytotoxicity, reinforcing the importance of organotypic in vitro models.
• Application of HCB provides abroad insight into relevant mechanisms of toxicity
• Challenge is to develop assays that detect non-degenerative structural changes and new in vitro models for indirect toxicities
• Currently developing the assay for routine compound screening
• Immediate application is for projects with structural cardiotoxicity issues
Acknowledgements

• MRC Centre for Drug Safety Science, Liverpool:
  - Stephanie Ravenscroft
  - Dr Mike Cross
  - Professor Kevin Park

• AstraZeneca:
  - Dr Amy Pointon
  - Sarah Dawson
  - Stephanie Roberts
  - Dr Najah Abi Gerges
  - Dr Chris Pollard