Comprehensive *in vitro* Proarrhythmia Assay (CiPA) Using Cor.4U Cardiomyocytes with the FDSS in a Calcium Transient Assay

Dr. Ralf Kettenhofen

09.06.2016 Hamamatsu User Meeting
Barcelona, Spain
Content

- The CiPA Initiative - Short Introduction
- Factors Influencing the Calcium Transient Assay
- Customer Report - Drug Development Support
Comprehensive in vitro Proarrhythmia Assay
CiPA - Initiative
CiPA Members

2014-2015 MEMBERS

- Government (17)
- Academia (20)
- Other (2)
- Industry (45)

84 Total Organizations
>250 Individual Participants
COMMITTEE WORKING GROUPS OVERVIEW

- **Proarrhythmia Working Group**: Main objective: Assess proarrhythmic risk
- **Cardiac Biomarkers Working Group**: Main objective: Development and application of biomarkers of CV toxicity
- **Cardiac Stem Cell Working Group**: Main objective: Understanding & characterizing stem cell-derived cardiomyocytes for use in CV safety assessments
- **Integrative Strategies Working Group**: Main objective: Assess predictability of preclinical CV models to human
CiPA Phase I - Pilot Study

- 3 Providers of pluripotent stem cell-derived cardiomyocytes
- 16 Volunteer sites
  - 12 sites; 3 microelectrode array platforms
  - 4 sites; 4 Voltage-sensing-optical (VSO) platforms
  - 8 blinded test compounds; 4 concentrations, 3 triplicates

- Study was accomplished End 2014
- Manuscript for publication is under discussion
CiPA Phase II - Validation Study

- 2 Providers of pluripotent stem cell-derived cardiomyocytes
- 5 core sites (funded by FDA grant)
  - 2 sites; 4 microelectrode array platforms
  - 3 sites; 3 Voltage-sensing-optical (VSO) platforms
- Calcium Transient Assay (potential backup assay)
  - 3 sites: Janssen, Axiogenisis, Merck (USA)
- Compounds:
  - 28 blinded test compounds; 4 concentrations, 6 replicates
  - 4 calibration compounds
- Volunteer non-core test sites:
  - 12 blinded test compounds + 4 calibration compounds
CiPA Phase II - Validation Study

Next Steps

- Myocyte Phase 2 Study Initiated
- Educational Webinars

- Myocyte Phase 2 Study Data Analysis
- Educational Webinars

- Myocyte Phase 2 manuscript submission (close BAA)
- New project scoping

- Myocyte Phase 2 Study Protocol Development
- Educational Webinars kick-off

- Myocyte Phase 2 Study Data Collection
- Educational Webinars

- Myocyte Phase 2 manuscript drafting
- Educational Webinars

Presented for:
Excitation-Contraction Coupling

A) Action Potential

B) Action Potential

- Calcium Transient
- Contractile Motion

Currents:
- Na$^+$
- Ca$^{2+}$ L-type
- Ca$^{2+}$ T-type
- Na$^+$/Ca$^{2+}$-exchanger
- K$^+$ $I_E$
- K$^+$ $I_K$
- K$^+$ $I_{Kr}$
- K$^+$ $I_{Kr'}$
- K$^+$ $I_{Kur}$
- K$^+$ $I_{Kr'}$
- K$^+$ $I_{Kr'}$
- K$^+$ $I_{Kur}$

Time

Amplitude
High Throughput Kinetic Plate Reader Assays
Plate Reader System - Hamamatsu

Both systems can be equipped with a temperature control

Data generated in collaboration with Hamamatsu
Plating Efficiency of Cor.4U Cardiomyocytes on a 384 Well Plate

Recording of Cor.4U cardiomyocytes with the FDSS 7000EX using Cal520 dye (AAT Bioquest).
Assay Optimisation
Important Factors Influencing the Calcium Transient Assay with hiPSC-derived Cardiomyocytes
Calcium Transient Assay - Important factors

- The calcium dyes
- Dye loading time
- Assay stability over time (assay window)
- Wash vs. non-wash
- Signal to noise ratio
  - Medium / buffer
  - Quencher
- Addition of organic anion transporter (e.g. probenecid)
Dye-induced Morphological Differences and Changes of Cor.4U Cardiomyocytes’ Calcium Transients

Cal520 (AAT Bioquest)
Calcium 5 Assay Kit (Molecular Devices)
ACTOne (Codex)
Results - Cal-520

10 µM | 5 µM | 2.5 µM | 1.0 µM | 0.5 µM

- FLIPR Calcium 5 and Codex ACTOne reveal a slowed rise of the calcium transients from 80% to 100%.
- There is obviously a change in calcium transients which potentially indicates the start of toxic events at an early time point.
- Calcium transient durations are increased with the FLIPR Calcium 5 dye and the ACTOne dye at concentrations tested compared to Cal-520 dye (see also quantitative analysis).
### Results - Cal-520

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Graphs</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µM</td>
<td><img src="image1" alt="Graph" /></td>
</tr>
<tr>
<td>5 µM</td>
<td><img src="image2" alt="Graph" /></td>
</tr>
<tr>
<td>2.5 µM</td>
<td><img src="image3" alt="Graph" /></td>
</tr>
<tr>
<td>1.0 µM</td>
<td><img src="image4" alt="Graph" /></td>
</tr>
<tr>
<td>0.5 µM</td>
<td><img src="image5" alt="Graph" /></td>
</tr>
</tbody>
</table>

At higher concentrations of Cal-520 the slope at 80% to 100% starts to slow as well.
Quantitative Analysis of Non-Wash Cal-520 Calcium Transients Recorded from Cor.4U Cardiomyocytes
- Beat rate is higher in Cal-520 Assay compared to the both other dyes, especially at the lowest dye concentration.
- Beat rate decreases with increasing dye concentrations.
Results - Cal-520

- Cal-520 calcium transient PW30% and 80% increase over time in the highest concentrations (toxic effect?).
- FLIPR Calcium 5 and ACTOne dye PW80% values are almost twice as high compared to the lowest Cal-520 concentration (=> toxic or unphysiological?).
Results - Cal-520

- Calcium Transient amplitudes from Cal-520 increase over time (max after 3 hours) although no probenecid was added.
- FLIPR Calcium 5 and ACTOne dye amplitudes reach a maximum after 60 min.
Wash Assay Using

Cal-520™, AM (AAT Bioquest)
and
ACTOne (Codex)
Results

Calcium Transient Amplitude

- Amplitude of Cal-520 calcium transients is absolutely stable during after 4 hours.
- ACTOne amplitudes are decreased after 3 hours.
- 0.5x ACTOne peak width at 80% are doubled compared to 2.5 µM Cal-520 (and also 0.25x ACTOne) and almost 3x the values of 0.5 µM Cal-520.
Dye Effect on GPCR Agonist Pharmacology with Cor.4U Cardiomyocytes
Results

Calcium 5 Assay Kit Dye

- Right shift of isoproterenol increased beat rate with the Calcium 5 Assay Kit dye
- Cal520: More physiological isoproterenol effect
Conclusion

- Choice of the right calcium dye is important
- Cal520 at low concentrations revealed to be the most physiologic dye
  - Long-term stability (assay window)
  - Calcium transient and beating parameters
- No quencher is required for Cal520 when the right assay medium/buffer is chosen
- Washout is required for Cal520
Support of Pharma Drug Development

Dr. Thomas Licher, Sanofi Frankfurt, Germany

http://axiogenesis.com/resources/presentations/webinar.html

10.02.2016 Axiogenesis webinar
Internal software tool

- In-House software tool to calculate the relevant parameters for the “Cardioscore”
  - Visualization of all time points at once
  - Combination of raw data, calculated data and fluorescence traces
Cardio-SAR support

- **Project support**
  - 2 GPCRs, 2 Kinase, 1 Ion channel - till now, more coming

- 881 compounds were tested in single dose @10 µM
- Used as series prioritization, SAR optimization tool and filter for Purkinje fiber experiment
Case study I: “Cardiotox” measurements – SAR for Kinase

- Good correlation between ion channels activity and iPSC
- For SAR6, better correlation between ion channels and iPSC than FIP
Case study II: “Cardiotox” measurements generate comprehensible SAR and guide selection of GPCR agonist with reduced AP impact

- **Objective**: Identify compounds early in the chemical optimization to reduce “cardiotox” risk.
  - Lead compound SAR1 showed undesired effect in Purkinje fiber study (APD$_{50}$ shortening)

SAR1

**Add Polarity**

AP shortening in Purkinje fiber (10 μM)

**Linker region**

- **Linker1**
- **Linker2**

**Tail Groups**

- **Tail1**
- **Tail2**

**Head Groups**

- **Acids, high polarity**
- **Weak Bases**
- **Bases, lipophilicity**

**Heat map Cardioscore**

- EC$_{50}$ > 10 μM
- EC$_{50}$ ~ 10 μM
- EC$_{50}$ < 10 μM

**RA2**

**RA3**

- **No marked AP impact (30 μM)**
- Pre-candidate nomination planned for February 2016

10.02.2016 Axiogenesis webinar, Dr. Thomas Licher,
Case study II: Cardiomyocyte EC50 vs. hERG-Inhibition (patch clamp) Analysis for 43 GPCR agonists

- Reasonable correlation of cardiomyocyte EC50 with % hERG-inhibition
- Only 4/43 hERG-blockers under-predicted
- Some "over-prediction" may indicate response to non-hERG affinities, which would add value to the assay
Summary and Next Steps

- **Summary of results**
  - Measurement of the Ca²⁺-oscillations of spontaneously beating hiPS-derived cardiomyocytes in 384 format with "standard" fluorescence imaging reader.
  - Identification of Na⁺-, Ca⁺-, hERG and mixed channel blockers
  - Single dose data for more than 850 compounds available
  - Good correlation with Purkinje experiments and hERG testing
  - Establishment of "cardiomyocyte" SAR

- **Plans for the next 6 months**
  - Further support projects with cardiomyocyte SAR
  - Development of a fluorescence-based membrane potential assay as an "action potential"-readout
  - Extend knowledge of the MoA of tool compounds
    - Evaluate effects of a set of 48 "CIPA" compounds

- **Critical issues and general points**
  - Pharmacological QC in house is a must
  - Stem cell assays are cost intensive: miniaturization very important
Thank you!