Phenotypic *In Vitro* Assessment of Environmental Compound Effects on Cardiomyocyte Physiology Using iPSC Cells

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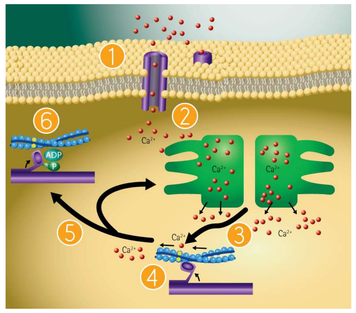
Abstract

A large number of environmental agents, with potential for human exposure, remain inadequately tested for toxicological effects. Hence, there is a pressing need to develop a high-throughput *in vitro* battery of assays to rapidly screen these compounds to facilitate prioritization for more comprehensive testing *in vivo*. Determination of toxic effects of environmental agents on human myocardial performance is critical to properly classify them as cardioactive and/or cardiotoxic. Phenotypic assays using human induced pluripotent stem cell (iPSC) derived cardiomyocytes are an attractive model for testing potential toxic compounds *in vitro* in an HTS screening format. Here we demonstrate advantages of these assays for measuring the impact of pharmacological compounds on the beating rate of cardiomyocytes with the FLIPR® Tetra cellular analysis system. The assays employ calcium sensitive dyes to monitor changes in Ca^{2+} fluxes synchronous with cell beating which allows monitoring and analysis of the beat rate, amplitude, and waveform irregularities, caused by various agents. The assay was tested using commercially available cardiotoxicity library representing different classes of compounds and was found to have an estimated balanced prediction accuracy of greater than 80% for this set of compounds. Using this assay, an 80 compound library containing a diverse set of environmental chemicals (e.g., flame retardants, polycyclic aromatic hydrocarbons, pesticides, and mitochondrial toxicants) and drugs was screened for cardiotoxicity across a 7-point concentration response at 30 mins and 24 hrs of treatment. Positive and negative controls performed as anticipated. After controlling for generalized cytotoxicity, several environmental chemicals such as pesticides (permethrin) and flame retardants (isopropylated phenyl phosphate and 2,2’4,4-tetrabromodiphenyl ether) were identified that inhibited *in vitro* cardiomyocyte function. Such data is amenable to concentration-response modeling and can be utilized to prioritize suspected cardiotoxicants for *in vivo* hazard characterization and mechanistic follow-up studies..

High Throughput Cardiomyocyte Beating Assay

The FLIPR Tetra system with EarlyTox™ Cardiotoxicity Dye can be used to monitor changes in intracellular Ca^{2+} fluxes associated with cardiomyocyte contractions. The system allows automatic addition of reagents and compounds simultaneous with reading from 96, 384, or 1536 wells. Temporal response curves for visualization of beating can be acquired, and analysis done in ~ 2 min per plate making this assay suitable for high throughput screening of compound libraries. A cartoon of the calcium intracellular flux process is shown in Figure 1.



FLIPR Tetra Cellular Analysis System

Figure 1. Roles of calcium ions during a cardiomyocyte contraction-relaxation event.[1,2] **1.** Membrane depolarization occurs, calcium channels open and calcium enters cytosol. **2.** Calcium Induce Calcium Release (CIRC) – Intracellular calcium triggers calcium release from sarcoplasmic reticulum. **3.** Cytoplasmic calcium binds to troponin, activates sarcomere. **4.** Cardiomyocyte contraction occurs. **5.** Removal of calcium by active transport into the sarcoplasmic reticulum and calcium exchange with extracellular fluid. **6.** Cycle repeats

Materials & Methods

iPSC derived iCell® Cardiomyocytes

•Cells were received frozen from Cellular Dynamics International (CDI). Cells were thawed and plated according to CDI protocol and incubated for 14 days. The presence of strong synchronous contractions of cells in the wells under the light microscope was confirmed prior to running experiments.

EarlyTox™ Cardiotoxicity Kt for Ca^{2+} Flux Assay

• The EarlyTox Cardiotoxicity Kit is designed to work with stem cell derived cardiomyocytes or primary cardiomyocytes. In the typical assay a vial from an EarlyTox Cardiotoxicity kit was thawed and diluted in 10 mLs Component B buffer. The dye loading buffer solution was warmed to 37ºC to avoid slowing the beating of the cardiomyocytes upon addition. 25 µL dye was added to 25 µL cells in the plate. Cells were loaded with dye for 2h at 5% CO_2, 37ºC. For 30min assay cells were pre-loaded with dye for 1.5h then treated with compounds for 30min. For 24h assay cells were treated with compounds for 22h, then dye was added for 2 more hours (4x).

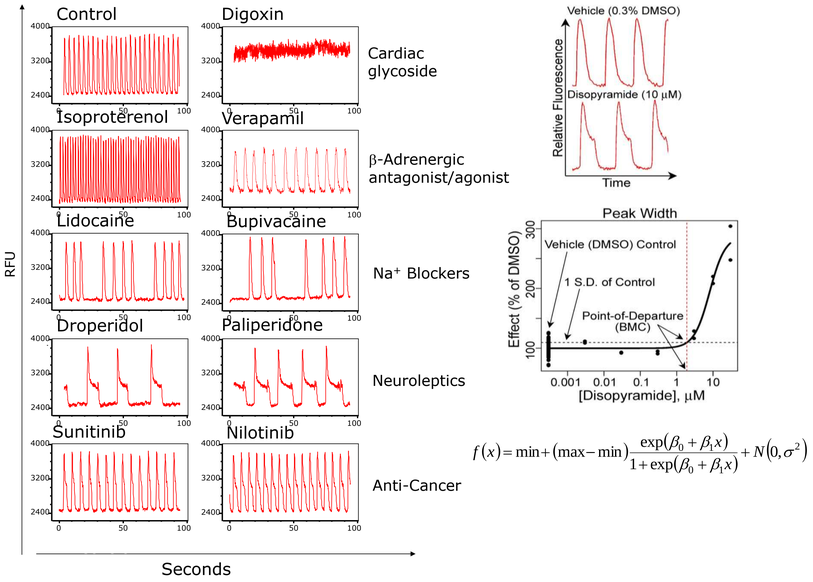
• Reference compounds are included in the kit that demonstrate a variety of effects upon the iPSC cardiomyocytes. The compounds were made up in Component B buffer so that a 5X concentration is added either off line or on the FLIPR Tetra system during detection. The compound plate was also warmed to 37ºC prior to addition to minimize temperature effects upon the cells.

References

1: Bers et al., Nature **415**, 198-205, 2002.
2: Bolton, T. J Physiol **570,** 5-11, 2006.
3. Benchmark dose technical guidance. US EPA, 2012
4. Robin et al., BMC Bioinformatics **12,** 77, 2011.
5. Reif et al., Bioinformatics **29**, 402-403, 2013.

Toxicity Library Screen Results

A diverse set of 80 environmental chemicals (e.g., flame retardants, polycyclic aromatic hydrocarbons [PAHs], pesticides, mitochondrial toxicants) and drugs was screened for cardiotoxicity across a 7-point concentration response at 30 min and 24 h of treatment. Positive and negative controls performed as anticipated. After controlling for generalized cytotoxicity, a number of environmental chemicals such as pesticides, PAHs, and flame retardants were identified that inhibited in vitro cardiomyocyte function. Such data is amenable to concentration-response modelling and can be utilized to prioritize suspected cardiotoxicants for in vivo hazard characterization and mechanistic follow-up studies.



$$f(x) = \min + (\max - \min)\frac{\exp(\beta_0 + \beta_1 x)}{1 + \exp(\beta_0 + \beta_1 x)} + N(0, \sigma^2)$$

Figure 2. Left: Representative signal traces from beating cardiomyocyte assay for different compound classes. Data acquired on FLIPR Tetra system. Right: Concentration-response profiles were modeled using logistic modeling to derive a benchmark concentration (BMC) point-of-departure value, based on one standard deviation departure from the estimated baseline in vehicle (dimethylsulfoxide)-treated cells.

ToxPi Safety Ranking

Comparisons of *in vitro*-derived effect levels to Cmax has been suggested as a surrogate metric for ranking compounds with respect to their potential *in vivo* cardiotoxicity. However, this approach is not amenable to drugs or chemicals that lack the necessary pharmacokinetic studies. We used an alternative approach to integrate multiple parameters collected in this study to rank chemicals in the screened library for their overall cardiosafety. Each compound’s BMC values for the effects on beat rate, peak shape (spacing, amplitude, rise, decay, width), and cell viability were analyzed and visualized using the ToxPi approach,[5] which generates transparent graphical rankings to facilitate decision making (see Figure 4). The larger the slice (scaled from 0 [minimum BMC] to 1 [maximum concentration tested], the less effect the compound had on the endpoint measured. The numbers are then summed across slices to give an overall ToxPi cardiosafety score from 0 (predicted to be more cardiotoxic) to 7 (predicted to be more cardiosafe).

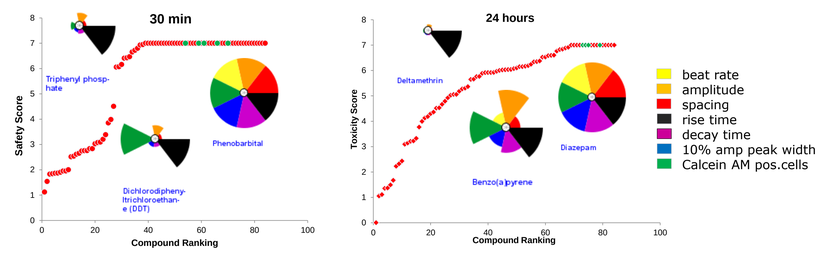
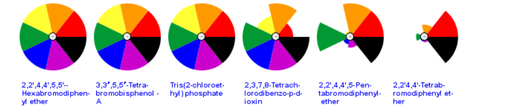


Figure 4. The analysis of the data for both 30 min (A) and 24 hrs (B) time points showed that non-toxic compounds were ranked high for safety, while toxic compounds were ranked low.

Flame Retardants

Chemical	30 min	24 hr
Triphenyl phosphate	2.83	7
Phenol, isopropylated, phosphate (3:1)	1.84	5.91
Tricresyl phosphate	1.82	5.91
tert-Butylphenyl diphenyl phosphate	2.74	4.36
Triphenyl phosphate	1.86	4.51
Isodecyl diphenyl phosphate	1.90	4.18
2- ethylhexyl diphenyl phosphate (EHDP)	3.39	2.22
2,2',4,4',5,5'-Hexabromodiphenyl ether	7	7
Tris(2-chloroethyl) phosphate	7	5.66
3,3',5,5'-Tetrabromobisphenol A	7	4.92
2,3,7,8-Tetrachlorodibenzo-p-dioxin	6.07	5.76
2,2',4,4',5-Pentabromodiphenyl ether	4.50	3.21
2,2'4,4'-Tetrabromodiphenyl ether	2.65	3.22



Bench Mark Concentration Analysis

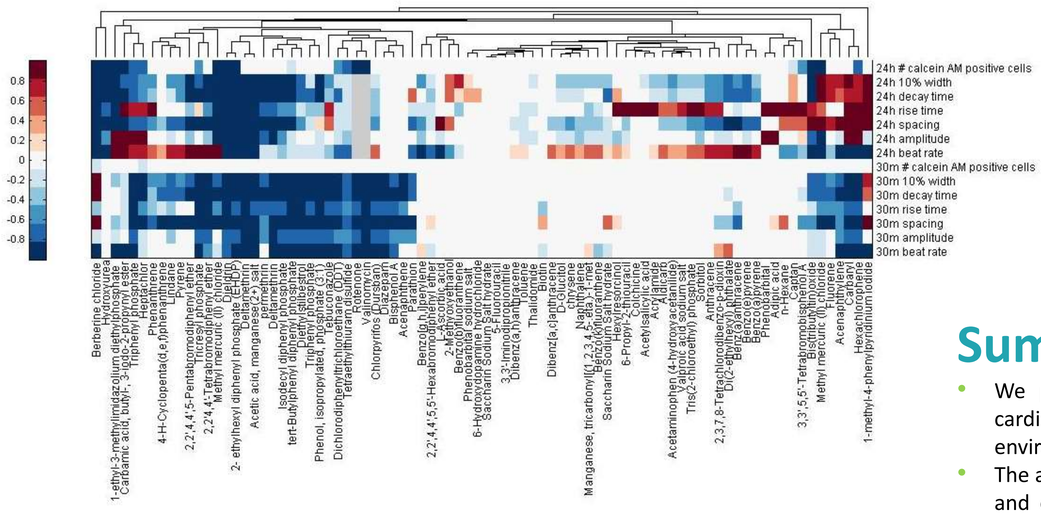


Figure 3. Hierarchical clustering of BMC values. BMC scaled to the maximum dose (scale of 0 - 1)
Red = increasing effects; Blue = decreasing effects; White = no effect; Gray = BMD could not be obtained due to low viability

Dose-response information is critical for safety ranking. However, IC_{50}/EC_{50} analysis is limited by the need for high/low asymptotes. High concentration response plateaus are often not present, leading to a need for an improved analysis method. To overcome this limitation, we propose applying the Benchmark Concentration (BMC) analysis method developed by EPA for analysis of toxicity testing[3,4] to the multi-parametric beating cardiomyocyte assay. A number of physiological parameters of cardiomyocyte beating, such as beat rate, peak shape (amplitude, width, raise, decay) and regularity were collected. Viability was examined at 60 min and 24 h using the # of Calcein AM positive cells. Cutoff for viability was +/- 3 standard deviations from the DMSO mean. BMC values were used for cardiotoxicity classification.

Summary

- We present here an assay model for in vitro assessment of cardiotoxicity hazards that is well-suited for automated screening environments
- The assay system is amenable to concentration-response modelling and can be utilized to prioritize suspected cardiotoxicants for in vivo characterization and mechanistic follow-up studies

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