Assessing the Cardiotoxicity of 31 Compounds Using a Multiplexed Kinetic Image Cytometry® (KIC®)-based Assay: Harnessing the Predictive Power of Human iPSC-Cardiomyocytes for Early Drug Development

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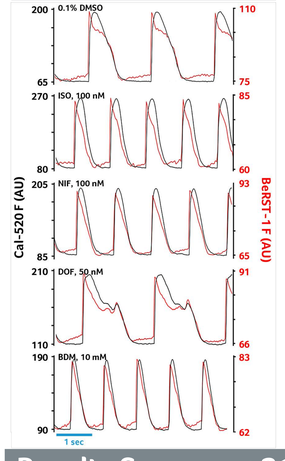
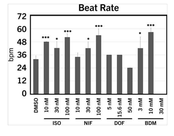
- Cardiotoxicity **(arrhythmias + structural cardiomyocyte damage**, *i.e.*, **myopathy)** is a leading cause of drug failures clinically and post-FDA approval, and of adverse drug reactions (ADRs).
- **Animal studies are laborious, expensive, and poorly predictive** of clinical outcomes (arrhythmia 55.4%[1]–85.4%[2,3]; myopathy 50%[4]–78.5%[5]).
- **Single-cell Kinetic Image Cytometry® (KIC®)** of hiPSC models captures cellular heterogeneity ("averaged out" by whole-well/-field platforms) for higher **sensitivity** & **clinical predictivity** (arrhythmia: 90–95%[6-10]; myopathy: 95.6%[11]).
- **KIC® of patient-derived hiPSCs** scales to "**clinical trials in a dish**"(CTiD) of 100s of candidate drugs for **high efficacy** and **safety** across gene mutations for a fraction that of animals.
- By multiplexing **Ca^{2+}, voltage, mitochondrial membrane potential, and contraction** in metabolically-matured hiPSC- CMs[12], our multiplexed assay **correctly predicted clinical outcomes of 30/31 drugs** (classes: 6 Ca^{2+}/SR, 10 plasma membrane/ion channel, 8 mitochondrial/myosin, 7 negative).
- hiPSC models enable **highly scalable**, **less costly, physiologically relevant** screens that are **more clinically predictive than animals.**

Automated KIC® Measurements: Examples from Assay Development

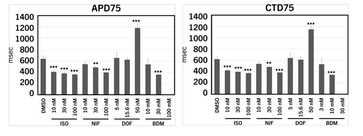
Single-Cell CaT/AP



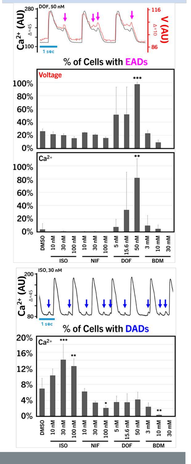
Frequency



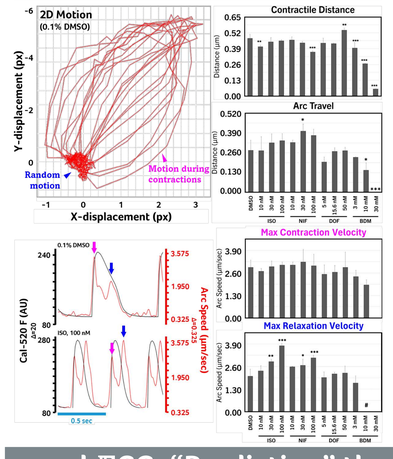
Peak Duration



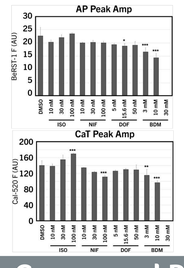
EADs/DADs



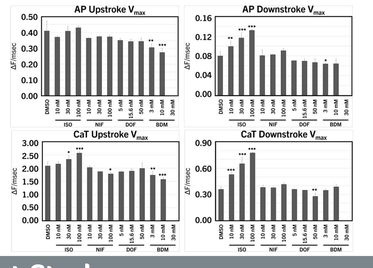
Contractility



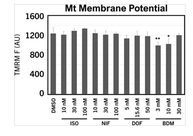
Amplitude



Maximum Velocity



Mito Membrane Potential



- Traditional live-cell fluorescence microscopy (single image, non-kinetic analysis)

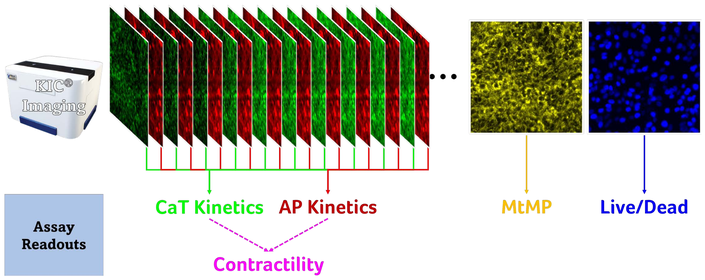
Statistical Analysis

- **Bar plots**: bars and error lines represent mean±S.D. (this section only)
- **Significance testing**: Ordinary one-way ANOVA followed by Dunnett's post-hoc multiple comparisons test (*p<0.05, **p<0.01, ***p<0.001) for N=3 (generally) using GraphPad Prism 9

CyteSeer® automatically computes >200 measurements per optical channel!

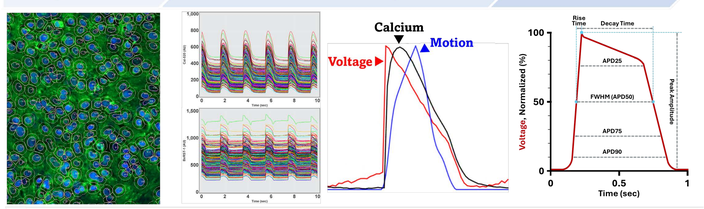
Methods: KIC® Cardiotoxicity Assay

Cell Culture	iPSC Differentiation → hiPSC-CM Maturation[12]
Multiplexed Staining	Cal-520 AM + BeRST-1 + TMRM + Hoechst 33342 (AAT Bioquest) (Lumencor) (Thermo Fisher Scientific)
Test Articles	**Drug Classes:** 6 SR, 10 plasma membrane/ion channel, 8 mitochondrial/myosin, 7 negative controls **Assay Format:** 7 test concentrations + vehicle control in triplicate/quadruplicate (acute exposure)
Assay Readouts	CaT Kinetics, AP Kinetics, MtMP, Live/Dead → Contractility

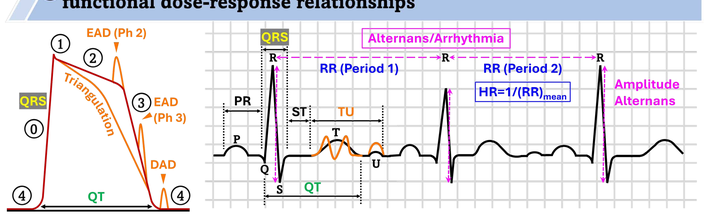


Methods: CyteSeer® Analysis

① Image segmentation → **② Single-cell waveform extraction and analysis** → **③ Transient measurements**



④ Waveform classification and predicted ECG correlations from functional dose-response relationships



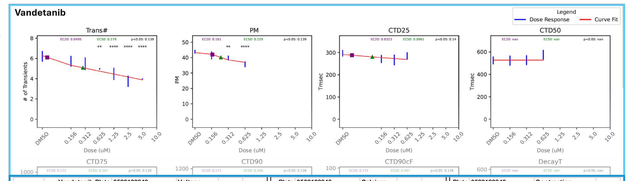
Results Summary: 31-Compound Pilot Study

Drug #	Drug Name	Clinical Cardiac Effect*	Mechanism of Action/Cardiac Target Class	Cardiac Target(s)	Target (AG)onist or (ANT)agonist	Measurement(s) of Cardiac Effect† [Dose (µM)] AP	SR	CON	MT	Predicted Human Outcome?
1	Milrinone	HF− (HF+, Arr+)	SR	PDE3	ANT	[50] ▲APD75, ▲Ampl, ▲BPM, ▲UVel, ▲DVel	[50] ▲DVel	[50] ▲DVel	*ns*	Yes
2	Flecainide	Arr−	SR	$Na_V1.5$ (I_{Na}), RyR2	ANT, ANT	[9.49] Q	[30] ▼Ampl, ▼UVel, ▼DVel	[0.095] ▼MTD75; [9.49] ▼Ampl, Q	*ns*	Yes
3	Istaroxime	HF−	SR	SERCA2, Na^+/K^+ ATPase	AG, ANT	[0.0125] ▼APD75; [0.025] ▲Tri	[0.0125] ▼CTD75	[0.025] ▼MTD75; [0.05] ▲UVel, ▲DVel; [0.1] ▲Ampl	*ns*	Yes
4	Cisapride	Arr+	AP	hERG (I_{Kr})	ANT	[0.125] ▲APD75	[0.125] ▼DVel	[0.25] ▲MTD75	*ns*	Yes
5	Diltiazem	Arr−	AP	$Ca_V1.2$ ($I_{Ca,L}$/$_{Ca,N}$)	ANT	[0.625], ▼BPM, ▼APD75	[1.25] ▼Ampl, ▼CTD75	[1.25] ▼MTD75	[2.5] ▼MtMP	Yes
6	Propranolol (racemic mixture)	Arr−	AP	*S(−)*: β-AR, *R(+)*: $Na_V1.5$ (I_{Na})	ANT, ANT	[3.125] ▼BPM	[3.125] ▼UVel, ▼DVel	[12.5] ▼DVel	[25] ▼MtMP	Yes
7	Mexiletine	Arr−	AP	$Na_V1.5$ ($I_{Na,L}$), hERG (I_{Kr})	ANT	[0.32] ▼UVel	[3.125] ▼DVel	[6.25] ▲MTD75	[100] ▼MtMP	Yes
8	Sotalol	Arr+	AP	hERG (I_{Kr})	ANT	[3.25] ▼BPM	[6.25] ▼DVel	[3.16] ▼MTD75	*ns*	Yes
9	Ivabradine	HF-	AP	HCN (I_f), hERG (I_{Kr})	ANT, ANT	[1.25] ▲Tri	[0.625] ▼DVel	[1.25] ▼Peak#	[10] ▼MtMP	Yes
10	Ibutilide	Arr− / Arr+	AP	hERG (I_{Kr})	ANT	[0.0156] ▲APD75	[0.0156] ▲CTD75	[0.0156] ▼Peak#	*ns*	Yes
11	Quinidine	Arr−	AP	hERG (I_{Kr})	ANT	[0.156] ▼DVel	[0.312] ▼DVel, ▼BPM	[0.312] ▼DVel	*ns*	Yes
12	Dofetilide	Arr+ / Arr−	AP	hERG (I_{Kr})	ANT	[0.0156] ▲Tri	[0.0156] ▲CTD75	[0.0156] ▲MTD75	[0.5] ▼MtMP	Yes
13	Isoproterenol	Arr−	AP	β-AR, $K_V7.1$ (I_{Ks})	AG, AG	[0.01] ▼Tri, ▲DVel	[0.01] ▲Ampl, ▲DVel	[0.03] ▲DVel	[10] ▼MtMP	Yes
14	Trimetazidine	HF−	CON / MT	Mt LC 3-KAT	ANT	[50] ▲Tri	[50] ▼DVel	*ns*	*ns*	No
15	Levosimendan	HF−	CON	Ca^{2+}→TnC	AG	[50] ▲APD75	[25] ▲Ampl, ▲CTD75	[100] ▲Ampl	[100] ▲MtMP	Yes
16	Omecamtiv	HF− (failed trial)	CON	Myosin	AG	[2.5] ▼Tri, ▲APD75	*ns*	[0.625] ▼UVel, ▼DVel; [10] Q	*ns*	Yes
17	Mavacamten	HF−	CON	Myosin	ANT	[0.095] ▲APD75	[5] ▼CTD75	[0.095] ▼Peak#	*ns*	Yes
18	Vandetanib	Arr+, HF+	All	hERG (I_{Kr})	ANT	[1.25] ▲APD75	[1.25] ▼BPM	[0.625] ▲MTD75	*ns*	Yes
19	Sorafenib	HF+	All	SERCA2 / cytotoxic	ANT	[0.00032] ▼UVel	[0.0316] ▼Ampl	[0.00316] ▼Ampl	*ns*	Yes
20	Regorafenib	HF+	All	$I_{Na,L}$	AG	[31.6] ▼BPM	[31.6] ▼UVel	[50] ▼Peak#	*ns*	Yes
21	Carboplatin	HF+	All	$I_{Ca,L}$	AG	[12.5] ▲APD75	[12.5] ▲CTD75	[6.25] ▲MTD75	*ns*	Yes
22	Ranolazine	Arr−	SR / AP	RyR2 / I_{Na}	ANT	[25] ▲APD75, ▲Tri	[6.25] ▼UVel	[25] ▲MTD75	*ns*	Yes
23	Veratridine	Arr+ / Q?	AP	$Na_V1.5$, $Na_V1.8$ (I_{Na})	ANT	[0.312] ▲APD75	[0.312] ▲CTD75	[0.156] ▼Peak#	*ns*	Yes
24	Thapsigargin	Q	SR	SERCA	ANT	[0.625] ▼APD75	[0.312] ▼Ampl	[0.625] ▼Ampl, ▼MTD75	*ns*	Yes
25	Caffeine	Neg	Neg	–	–	*ns*	*ns*	*ns*	*ns*	Yes
26	Loratadine	Neg	Neg	–	–	[0.2] ▲APD75	[0.2] ▲CTD75	ns	*ns*	Yes
27	Acetaminophen	Neg	Neg	–	–	*ns*	*ns*	*ns*	*ns*	Yes
28	Doxycycline	Neg	Neg	–	–	*ns*	*ns*	*ns*	*ns*	Yes
29	Ciprofloxacin	Neg	Neg	–	–	*ns*	*ns*	*ns*	*ns*	Yes
30	Oseltamivir	Neg	Neg	–	–	*ns*	*ns*	*ns*	*ns*	Yes
31	Aspirin	Neg	Neg	–	–	*ns*	*ns*	*ns*	*ns*	Yes

*** Clinical Cardiac Effect:** Arr=arrhythmia (+, causes; −, treats); HF=heart failure (+, causes; −, treats); Q=arrest (quiescence); Neg=negative control/no clinical cardiac effect
† Measurement(s) of Cardiac Effect: [X]=lowest dose at which significant changes were observed, in µM; ▲=increase/▼=decrease; AP=action potential/membrane voltage; SR=sarcoplasmic reticulum/Ca^{2+} transient; CON=contraction (APD/CTD/MTD75=Action Potential/Ca^{2+} Transient/Motion [contractile] Transient Duration, 75% down from peak; U.Vel/D.Vel=max rate of peak upstroke/downstroke; Ampl=peak amplitude; Tri=triangulation; BPM=beats/min; Peak#=number of contractile peaks); MT=mitochondria (MtMP = mitochondrial membrane potential); *ns*=no significant changes observed

prophECG: "Predicting" the Safety of Vandetanib

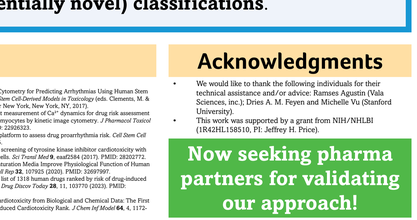
1. **Functional dose-responses & curve-fitting**
2. **ECG classifications from *in vitro* measurements**
3. **Example prophECG summary data report**



		Vandetanib Plate 0522122848 Voltage							Plate 0522122848 Calcium							Plate 0522122848 Contraction						
		Dunnett's Least p<0.05	Dose-Response Equation Fit-Derived						Dunnett's Least p<0.05	Dose-Response Equation Fit-Derived						Dunnett's Least p<0.05	Dose-Response Equation Fit-Derived					
ECG Class	Feature	p <<<	R^2	Trend		ZD [µM] p=0.05	EC_{50} [µM]	EC_{25} [µM]	p <<<	R^2	Trend		ZD [µM] p=0.05	EC_{50} [µM]	EC_{25} [µM]	p <<<	R^2	Trend		ZD [µM] p=0.05	EC_{50} [µM]	EC_{25} [µM]
HR	Trans#	0.16	0.0003	0.858	T	-	0.139	0.139	0.395	0.156	0.0033	0.863	T	-	0.139	0.139	0.395	0.31	0.0033	0.906	T	-	0.140	0.158	0.917
HR	BPM	0.16	0.0392	0.880	T	-	0.139	0.217	0.775	0.156	0.0287	0.862	T	-	0.139	0.318	0.807	0.31	0.0287	0.927	T	-	0.139	0.238	1.237
QT	CTD25	0.625	0.0074	0.586	T	+	4.837	4.849	6.484	N/A	N/A	N/A	F	NS	NaN	NaN	NaN	N/A	N/A	0.327	F	NS	NaN	NaN	NaN
QT	CTD50	N/A	N/A	0.178	F	+	NaN	NaN	NaN	N/A	N/A	0.228	F	+	NaN	NaN	NaN	0.31	0.0426	0.654	T	NS	NaN	0.049	0.279
QT	CTD75	N/A	N/A	0.231	F	+	NaN	NaN	NaN	N/A	N/A	0.472	F	+	NaN	NaN	NaN	0.31	0.0413	0.944	T	+	0.140	0.347	1.486
QT	CTD90	0.312	0.0398	0.572	T	+	0.140	0.409	0.858	0.312	0.0392	0.601	T	+	0.139	0.687	1.350	0.31	0.0382	0.957	T	+	0.140	0.648	1.584
QT	CTD90cF	0.625	0.0009	0.547	T	+	0.279	0.483	0.897	0.625	0.0010	0.504	T	+	0.291	0.812	1.513	0.63	0.0010	0.934	T	+	0.229	0.783	2.230
QT	DecayT	N/A	N/A	0.170	F	NS	NaN	NaN	NaN	N/A	N/A	0.186	F	+	NaN	NaN	NaN	0.31	0.0175	0.675	T	+	0.139	0.139	0.279
QT	TDT6-25	N/A	N/A	0.425	F	+	NaN	NaN	NaN	N/A	N/A	0.310	F	+	NaN	NaN	NaN	1.25	0.0187	0.949	T	+	0.149	0.782	2.228
QT	DnSVel	0.625	0.0001	0.945	T	-	0.140	0.330	0.886	1.250	0.0001	0.905	T	-	0.300	0.574	1.031	0.63	0.0000	0.766	T	-	0.540	0.540	0.540
QT	DnST	N/A	N/A	0.150	F	NS	NaN	NaN	NaN	N/A	N/A	0.066	F	+	NaN	NaN	NaN	N/A	N/A	0.000	F	+	NaN	NaN	NaN
QRS	UpSVel	N/A	N/A	0.485	F	-	NaN	NaN	NaN	N/A	N/A	0.205	F	NS	NaN	NaN	NaN	N/A	N/A	0.021	F	NS	NaN	NaN	NaN
QRS	UpST	N/A	N/A	0.374	F	NS	NaN	NaN	NaN	N/A	N/A	0.006	F	NS	NaN	NaN	NaN	N/A	N/A	0.160	F	+	NaN	NaN	NaN

prophECG

		HR [µM] MoS	HR [µM] EC_{25}	QT [µM] MoS	QT [µM] EC_{25}	QRS [µM] MoS	QRS [µM] EC_{25}	TU [µM] MoS	TU [µM] EC_{25}	Rhy [µM] MoS	Rhy [µM] EC_{25}	Arr [µM] MoS	Arr [µM] EC_{25}
Ciprofloxacin	Voltage	0.22	2.00	1.09	9.87	4.44	40.1	-	-	-	-	-	-
	Calcium	0.17	1.51	0.16	1.41	-	-	0.16	1.47	1.75	15.8	-	-
	Contraction	0.27	2.49	1.42	12.9	-	-	-	-	-	-	-	-
Chlorpromazine	Voltage	-	-	10.83	0.37	28.2	0.97	29.0	1.00	-	-	-	-
	Calcium	0.01	0.0002	0.02	0.00	0.79	0.03	2.02	0.07	1.28	0.04	57.9	2.00
	Contraction	-	-	21.0	0.72	-	-	30.4	1.05	34.1	1.18	-	-
Ranolazine	Voltage	0.72	1.40	4.59	8.94	-	-	7.27	14.2	8.14	15.8	-	-
	Calcium	0.90	1.76	0.97	1.89	2.68	5.22	1.02	1.99	8.64	16.8	44.3	86.3
	Contraction	2.69	5.24	5.43	10.6	-	-	7.19	14.0	-	-	-	-
▶ Vandetanib	Voltage	0.72	0.22	1.20	0.36	1.09	0.33	2.04	0.61	4.28	1.28	15.1	4.54
	Calcium	0.66	0.20	1.27	0.38	5.49	1.65	2.01	0.60	4.02	1.21	16.1	4.83
	Contraction	2.00	0.60	3.25	0.97	0.37	0.11	5.06	1.52	-	-	18.6	5.59



- KIC® of hiPSC-CMs provides **sensitive, data-rich readouts** of **multiplexed voltage, calcium, contraction, and mitochondrial membrane potential**.
- *In vitro* KIC® measurements **correspond to *in vivo* clinical readouts** (*e.g.*, ECG); large KIC® data sets can be used to **predict compound effects** in humans, thus **accelerating early-stage drug discovery**.
- In the next year, we **will screen 300+ drugs** with known cardiotoxicity risk profiles (*e.g.*, from databases like DICTrank[13,14]), as well as re-analyze existing KIC® data (*e.g.*, from Pfeiffer *et al.*[7]) with these newer methods.
- We continue to develop and refine computational strategies (including **classical AI/ML**) to provide further insight into the complex interplay among all the different KIC® measurements in these data for **improved, explainable predictivity** and **more clinically-relevant (and potentially novel) classifications**.

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