



**HAMAMATSU**  
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**2<sup>nd</sup> FDSS Application Workshop Cor.4U  
& Neuron, June 10<sup>th</sup>, 2015**

## **In vitro predictive toxicity of GPCR modulators in human iPS-derived Cor.4U cardiomyocytes by Ca<sup>2+</sup> transients detection**



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# Pre-clinical drug discovery process

- **Target screening, Hits identification and validation**

Binding assay, functional assays

- **Selection of lead compounds, SAR and drug candidate**

***In vitro***

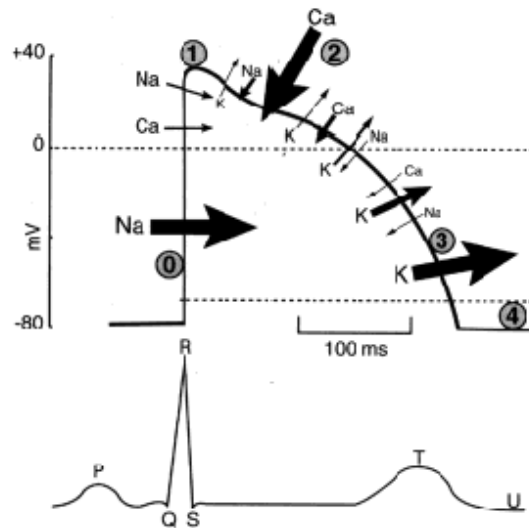
- Biochemical and functional characteristics
- Selectivity assays

***In vivo***

- PK studies
- Pre-clinical animal models (proof of concept)
- Toxicity assays (CNS, CV, Respiratory, genotoxicity)
- Safety pharmacology

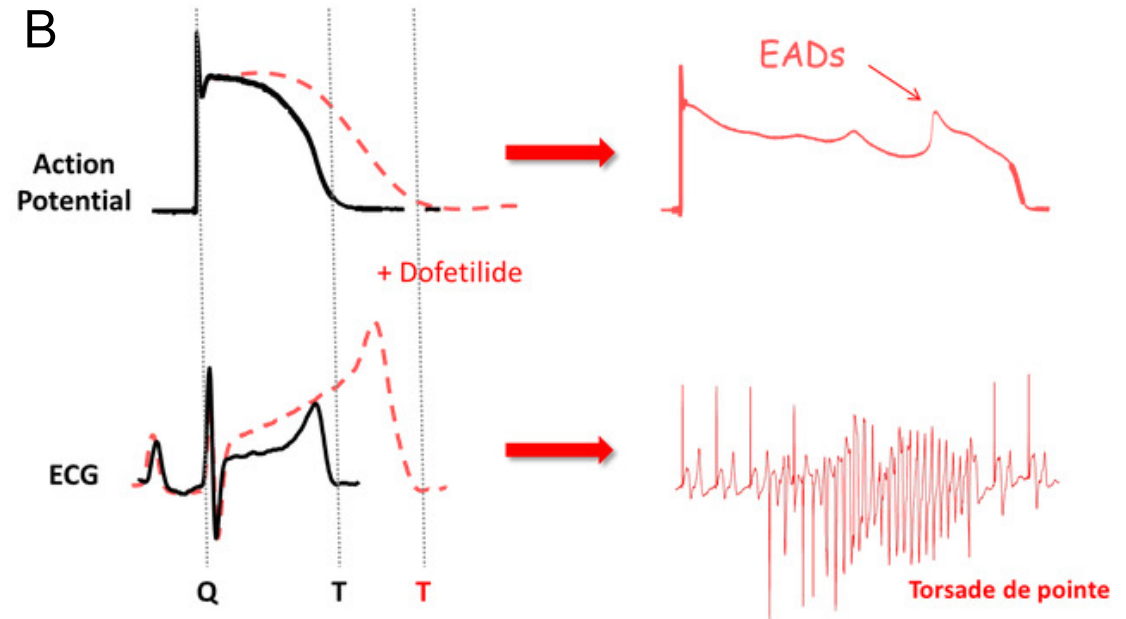
# Cardiovascular toxicity

A



Involvement of the different ionic channels in the cardiac action potential (AP) with corresponding ECG measure

B



Direct correlation between arrhythmia (EAD type) on the AP and torsades de pointes (TdP) on the ECG

# Cardiotoxicity prediction of GPCR modulators

## *In vitro*

- Dofetilide binding (hERG)
- Patch clamp (native or recombinant ion channels)
- hERG trafficking

## *In vivo*

- Cardiovascular safety package
- Rabbit Purkinje fibers
- Telemetry in vigil animals

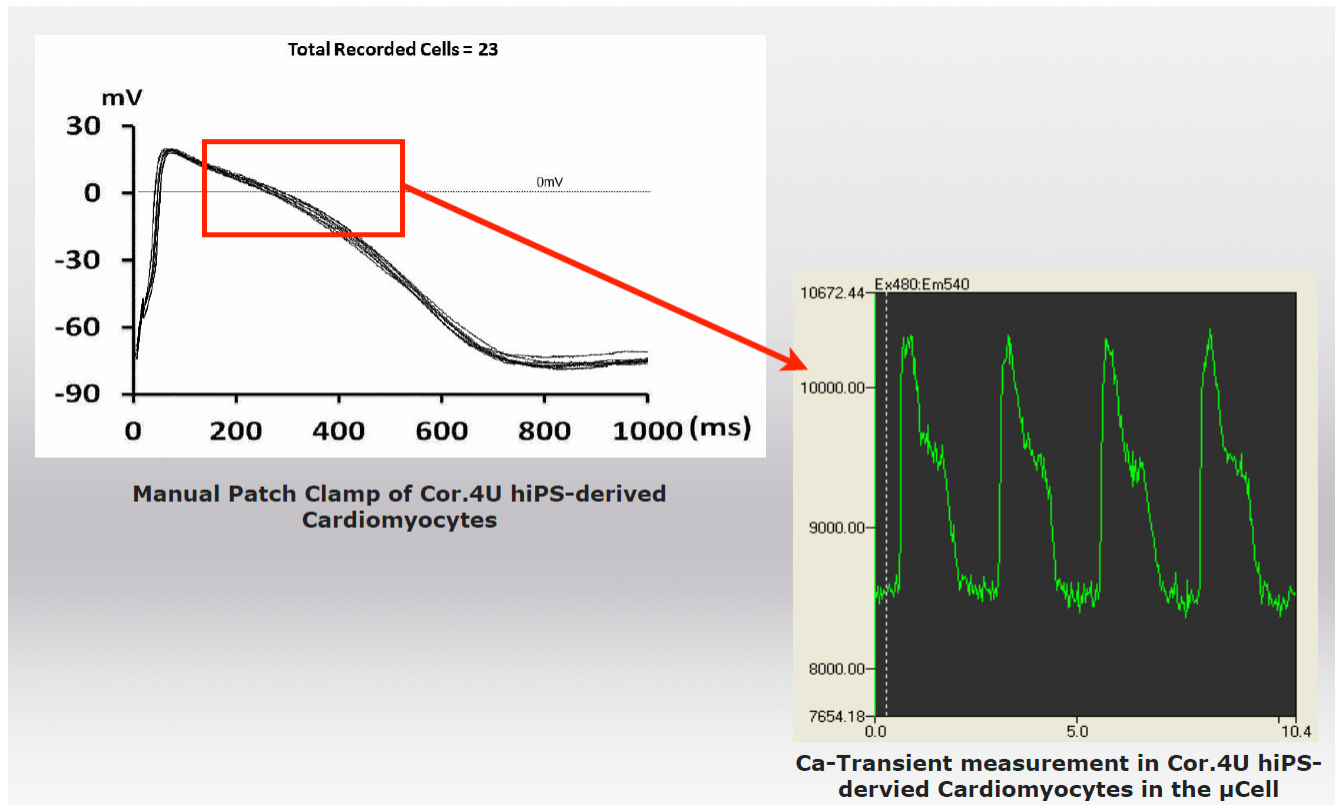
**Cor.4U cardiomyocytes ?**

## Cor.4U cardiomyocytes

### Cytosolic Ca<sup>2+</sup> transients detection on FDSS $\mu$ cell (Hamamatsu)

- Basic configuration
- Basic + 37 °C heater
- Electric Field Stimulation (EFS): synchronized beating

### Excitation-contraction coupling in heart: Ventricular PA vs. Ca<sup>++</sup> transients



# Protocol

## Cardiomyocytes plating

- Fibronectin coating (3h at 37 °C, PBS + Ca<sup>2+</sup>, Mg<sup>2+</sup>)
- Plating cryopreserved Cor.4U cardiomyocytes into 1/2 wells
- **15 000 cells per 1/2 well**
- Two media changes per day
- **Spontaneously beating cells at ~20 beats / min**

# Protocol

## Ca<sup>2+</sup> dye loading

- **0.5  $\mu$ M Fluo-4 (30 min, 37°C) into Cor.4U culture medium (BMCC medium or HBSS buffer not used)**
- **1.25 mM probenecid, 0.05% pluronic F27**
- **DMSO final concentration: 0.6%**
- **No quencher**
- **Washing with Cor.4U culture medium**

# Protocol

## FDSS $\mu$ Cell settings

- Pipetting: 25 $\mu$ l (5X concentrated, 125 $\mu$ l final volume)
- **Injection: 40 $\mu$ l/sec, 3.5 mm deep at 3 min post starting reading**
- Exposition 100 ms, sensitivity 2, binning 2x2
- Reading: 40 minutes



# Tested toxic reference drugs

## Positive chronotropic and inotropic:

- **Isoproterenol**

$\beta$  adrenergic receptor agonist

Treatment of bradycardia, heart block

## Arrhythmia:

- **Dofetilide**

Class III antiarrhythmic agent (delayed rectifier outward potassium current IKr)

Maintenance of sinus rhythm (US), serious side effect (torsades de pointes)

hERG/ I<sub>Kr</sub> target

- **Astemizole**

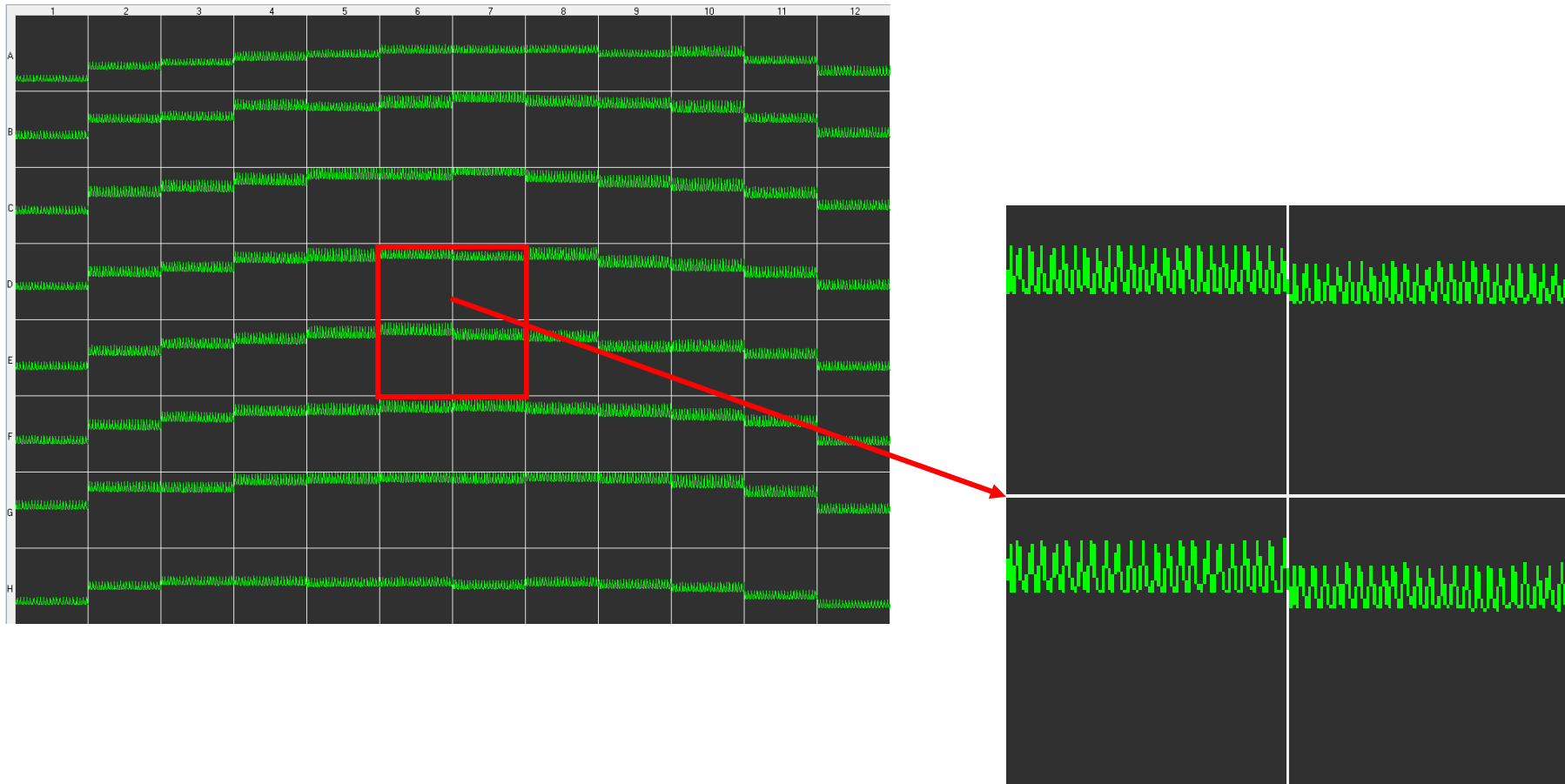
H1 receptor antagonist (withdrawn from the market: long QT)

hERG/ I<sub>Kr</sub> target

## Cardiotoxicity properties of BP tested compounds

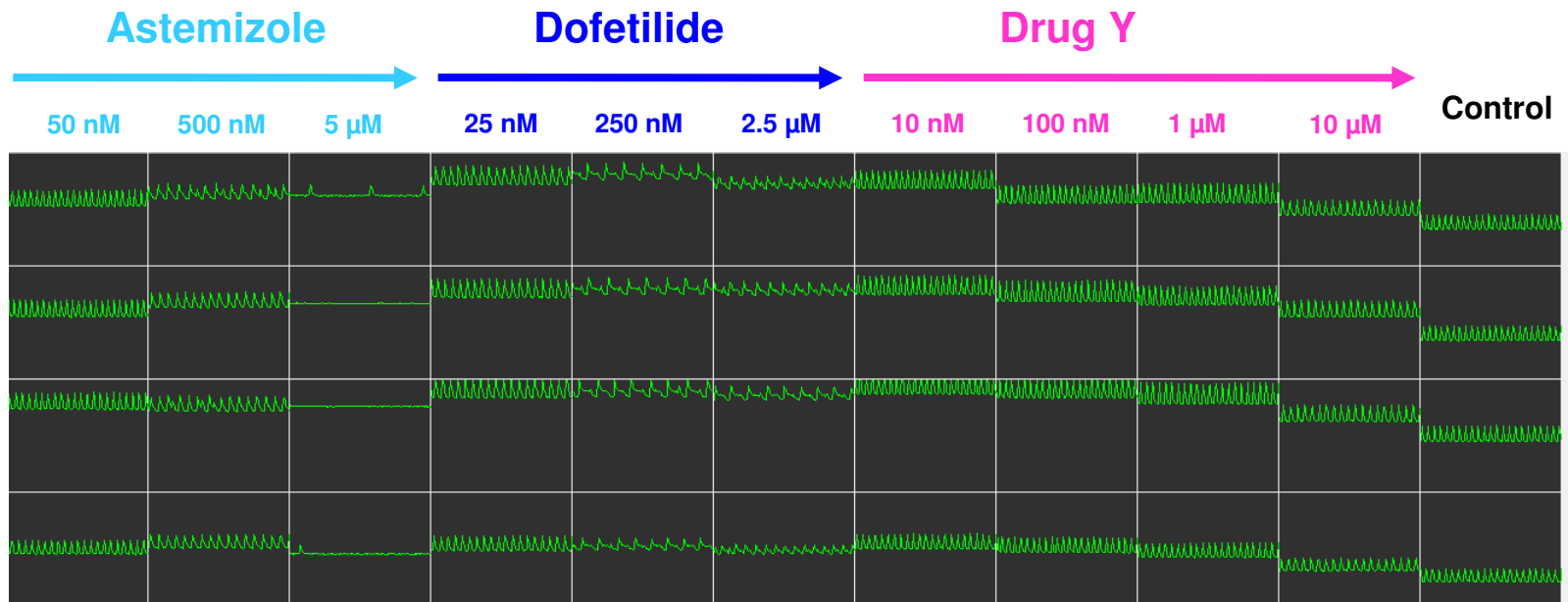
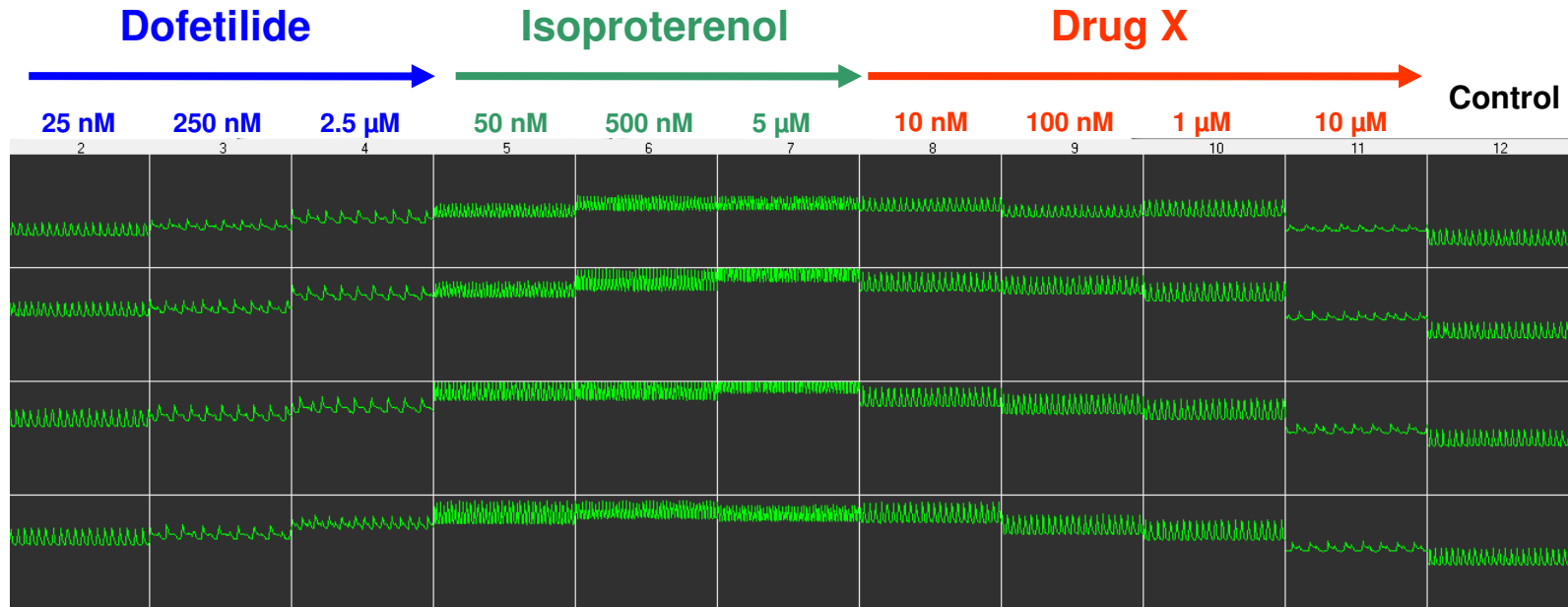
- **X compound: hERG and calcium channel inhibitor**
- **X metabolite: No observed effect**
  
- **Y compound : Induces slight tachycardia**
  
- **Z compound : Induces bradycardia**

## Base Line reading (2-3 min), compound injection at 3 min



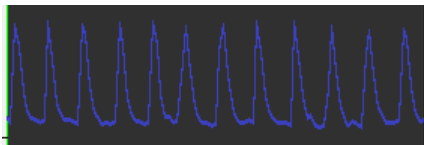
- All wells containing cardiomyocytes are « beating » at the same frequency and at equivalent amplitude
- Basal fluorescence level are different depending on the cell number per well

# Evaluation of different compounds (calcium measurement) on Cor.4U human cardiomyocytes (Readings 5-6 min)

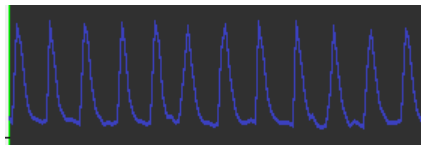


# Compounds evaluation by calcium flux on Cor4U human cardiomyocytes (Readings 5-6 min)

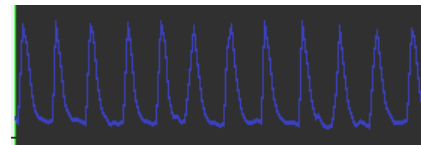
control



control

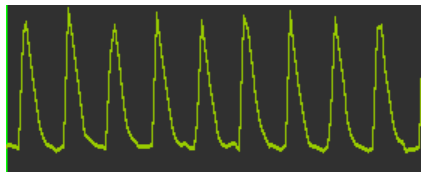


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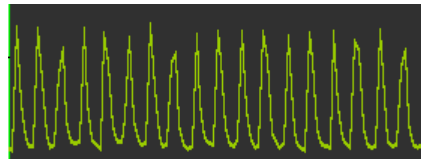
Dofetilide

25 nM



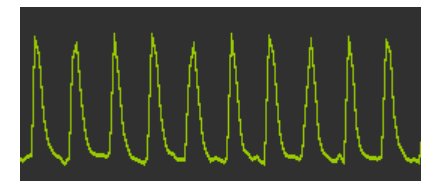
Isoproterenol

50 nM

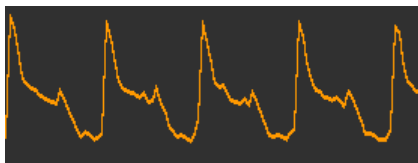


Astemizole

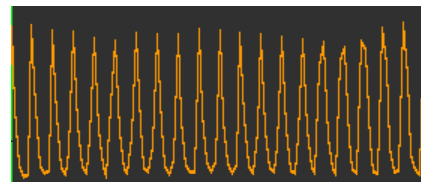
50 nM



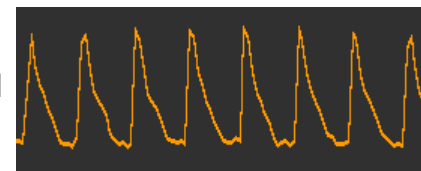
250 nM



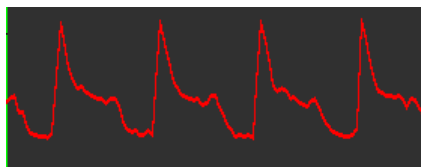
500 nM



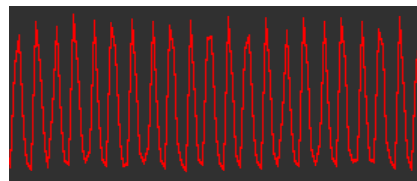
500 nM



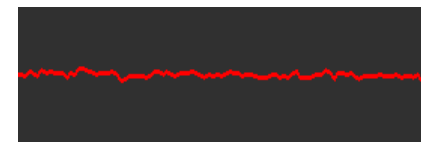
2.5 μM



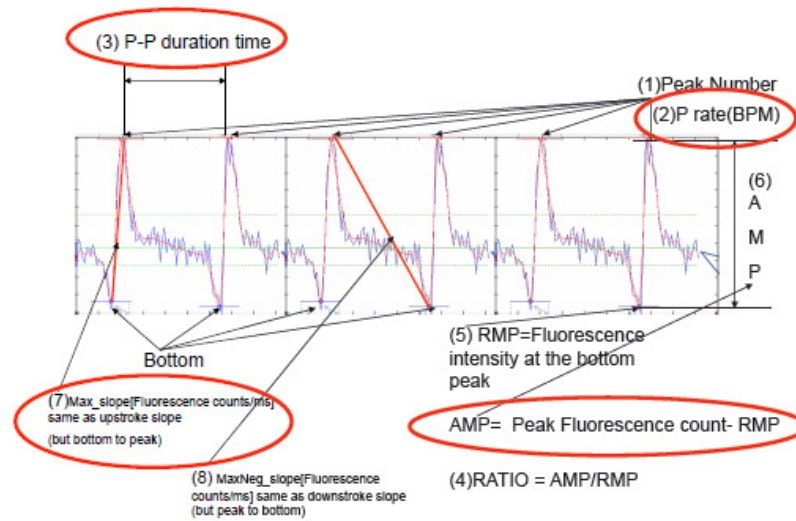
5 μM



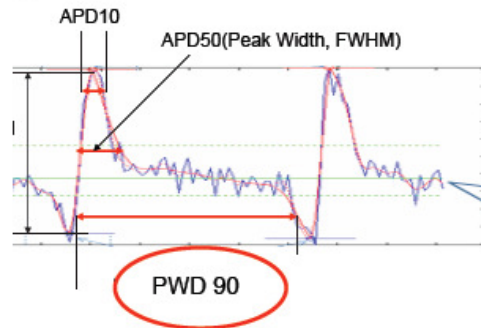
5 μM



## Fig. 2: Parameters calculated from raw data of Ca<sup>2+</sup> transients



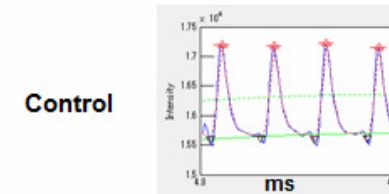
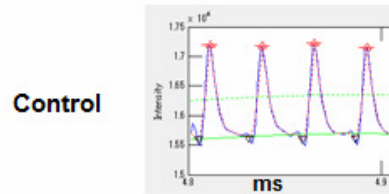
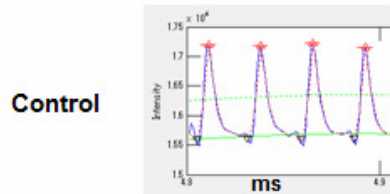
(9) APD 10 to 90



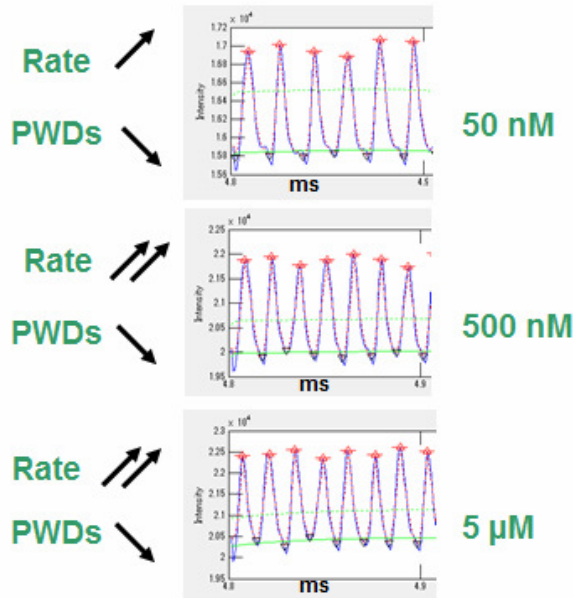
(1)	Peak number (total, BPM)
(2)	P-P time [ms] (Ave, Std, Max, Min)
(3)	Ratio (Ave, Std) Ratio = (AMP+RMP)/RMP
(4)	AMP (Ave, Std)
(5)	RMP (Ave, Std)
(6)	Slope (Ave, Std) Rising Slope: Slope from bottom to peak Falling Slope: Slope from peak to bottom 0% - 10%, 10% - 90%, 20% - 80%, 30% - 70%
(7)	Integration (Ave, Std)
(8) - (16)	PWD (10% - 90%) [ms] (Ave, Std)

**PWD: PULSE WIDTH DURATION → ~ APD**

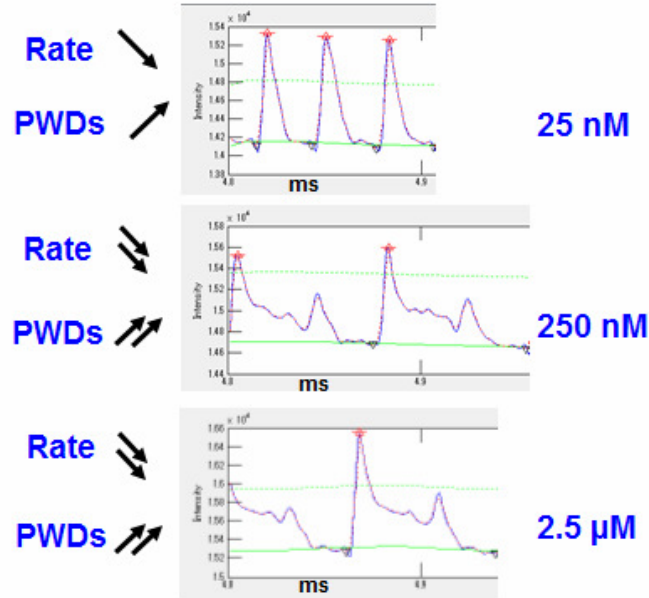
# Evaluation of different compounds (calcium measurement) on Cor4U human cardiomyocytes (Readings 5-6 min)



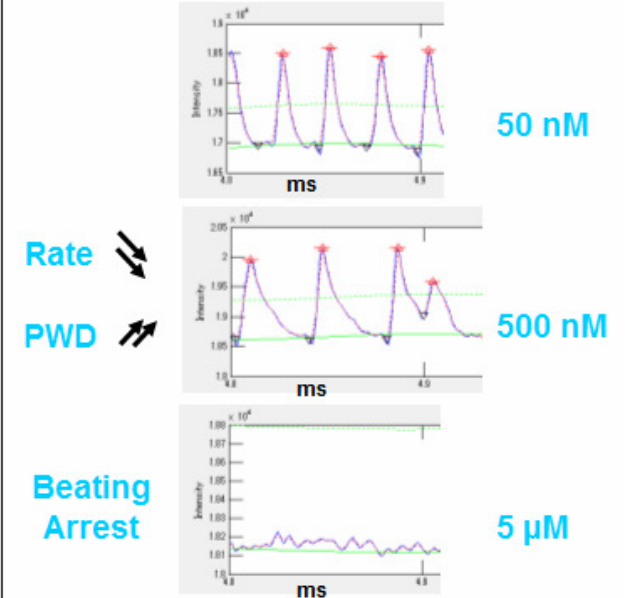
## Isoproterenol



## Dofetilide

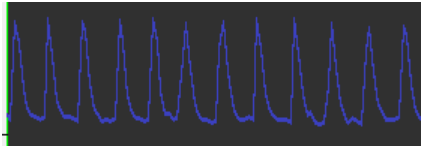


## Astemizole



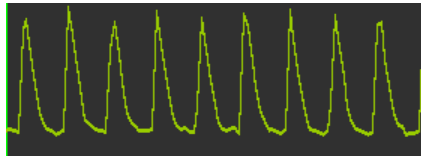
PWDs = PWD10, PWD50 and PWD90

**control**

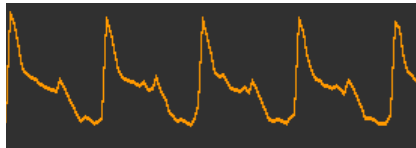


**Dofetilide**

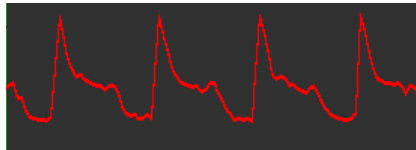
25 nM



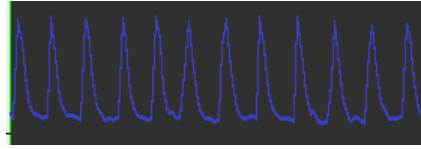
250 nM



2.5 μM

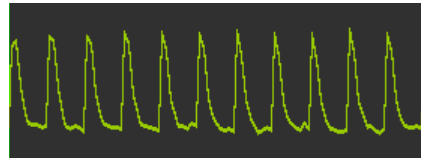


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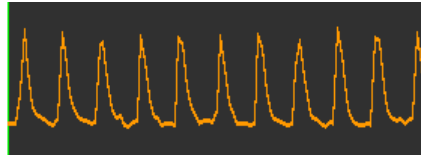


**Drug X**

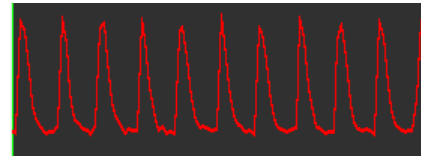
10 nM



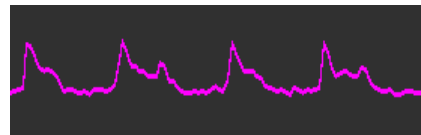
100 nM



1 μM



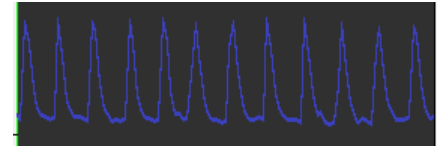
10 μM



30 μM

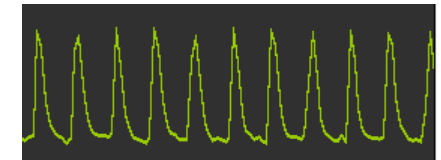


**control**

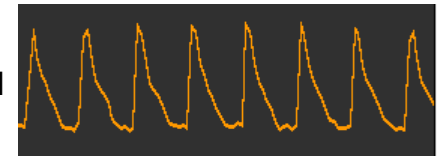


**Astemizole**

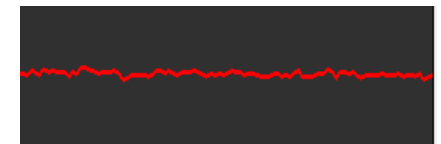
50 nM



500 nM

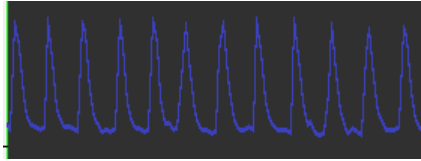


5 μM



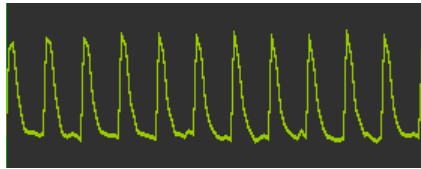


**control**

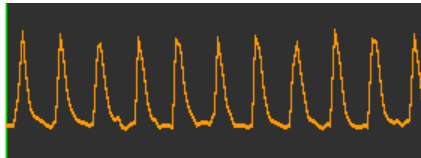


**Drug X**

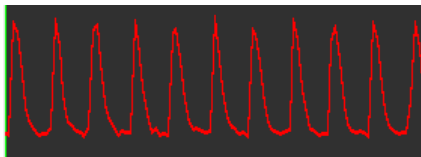
**10 nM**



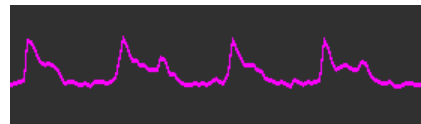
**100 nM**



**1 μM**



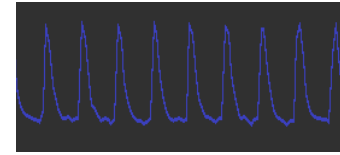
**10 μM**



**30 μM**

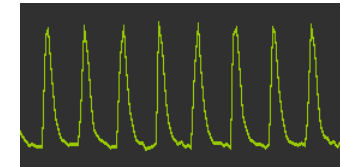


**control**

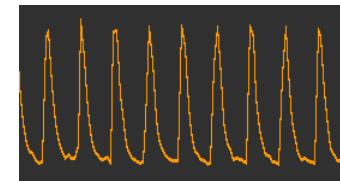


**X metabolite**

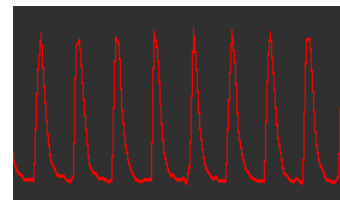
**1 μM**



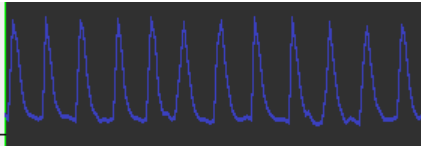
**10 μM**



**30 μM**

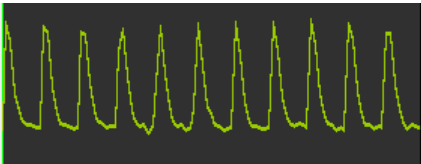


**control**

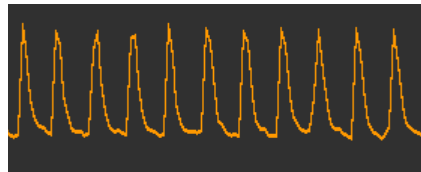


**Drug Y**

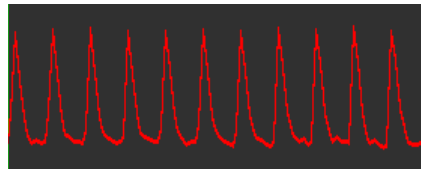
10 nM



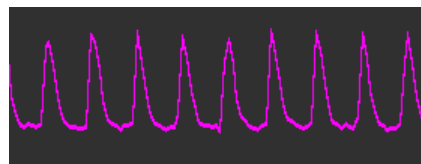
100 nM



1  $\mu$ M



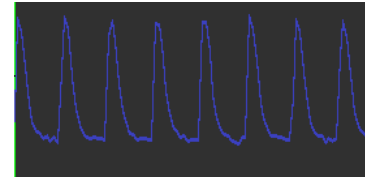
10  $\mu$ M



PWDs ↗

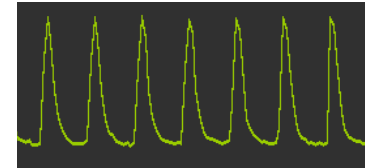
Rate ↘

**control**

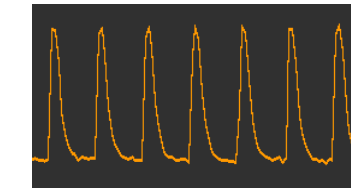


**Drug Z**

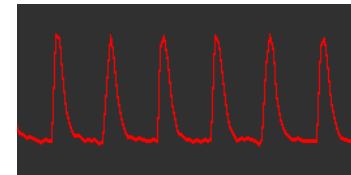
10 nM



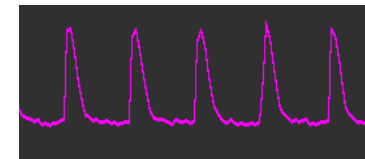
100 nM



1  $\mu$ M



10  $\mu$ M



PWDs ↗

Rate ↘

# SUMMARY

	Rate	PWD10	PWD50	PWD90	Beating arrest	Rising slope	Falling slope	Integration	Basal Fluorescence	Max Fluorescence
Astemizole	Red	Green	Green	Green	Green	Orange	Red	Green	Yellow	Pink
Dofetilide	Orange	Green	Green	Green	Green	Orange	Red	Green	Yellow	Pink
X drug 30 $\mu$ M	Red	Green	Green	Green	Green	Orange	Red	Green	Yellow	Pink
X drug 10 $\mu$ M	Red	Green	Green	Green	Teal	Orange	Red	Green	Yellow	Pink
X drug 1 $\mu$ M	Pink	Light Green	Light Green	Light Green	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
X drug 100 nM	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
X drug 10 nM	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
X metabolite 30 $\mu$ M	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Z drug 10 $\mu$ M	Orange	Light Green	Light Green	Light Green	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Z drug 1 $\mu$ M	Pink	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Y drug 10 $\mu$ M	Yellow	Light Green	Light Green	Light Green	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Isoproterenol	Green	Orange	Orange	Orange	Yellow	Teal	Green	Yellow	Yellow	Yellow

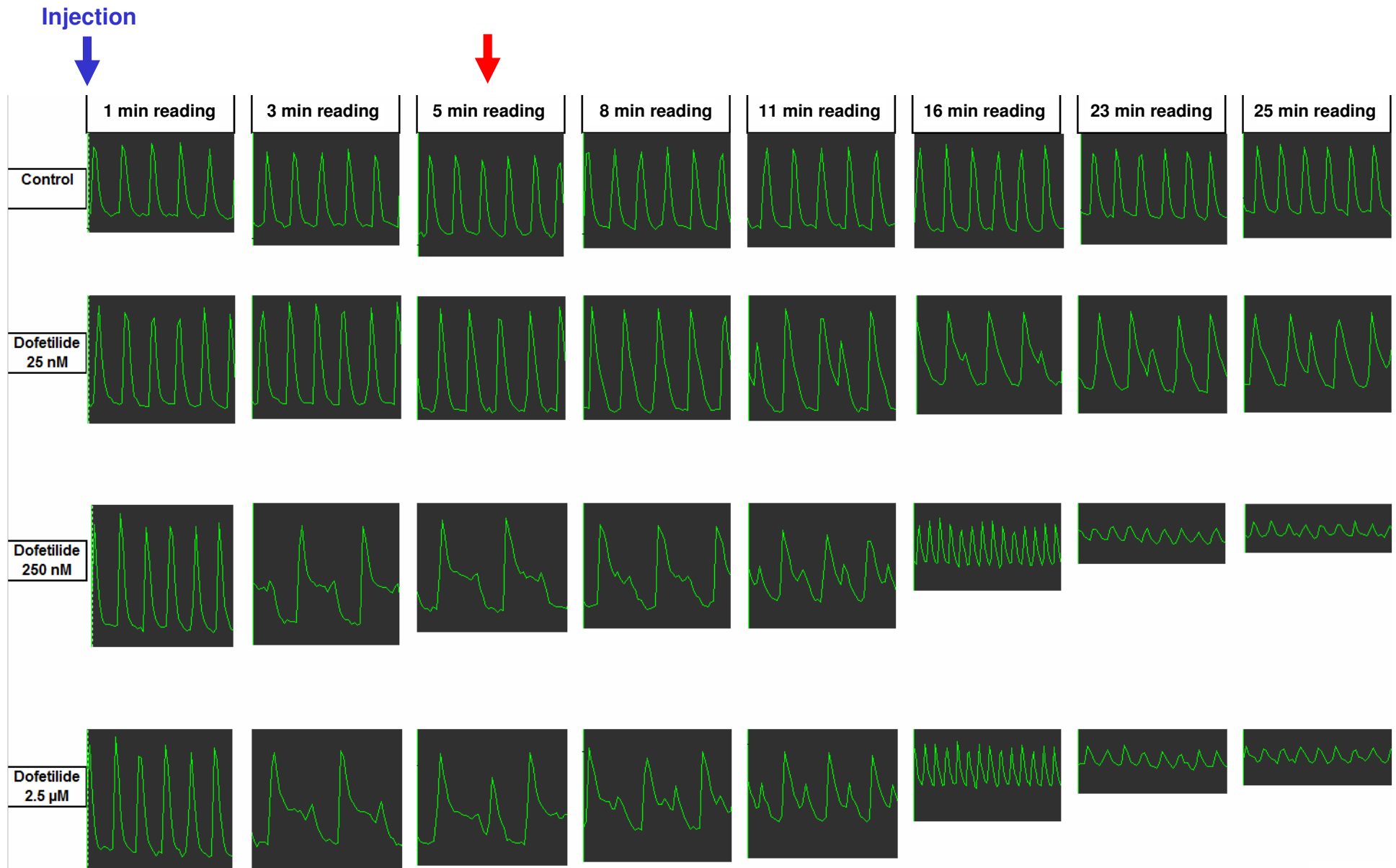


# Correlation

## Cardiovascular data and Cor.4U transient Ca<sup>2+</sup>

	Recombinant hERG (HEK293 cells)		<i>In vivo</i> Cardiovascular	Cardiomyocytes Patch clamp	Cor.4U Cardiomyocytes	
	3H Dofetilide binding (K <sub>i</sub> )	Patch Clamp (IC <sub>50</sub> )			Calcium transients	NOAEL Dose
Drug X	1.9 μM	1.3 μM	QT Elongation NOAEL= 900 nM	Decrease APD at 3 and 10 μM APD20 (26% and 38%) Increase APD at 3 and 10 μM APD90 (18% and 11%)	Decreasing rate at ~3 μM Increasing PWDs at ~3 μM Induces Arrhythmia at ~3 μM Beating arrest at 30 μM	~ 1 μM
X metabolite	> 20 μM	> 20 μM	N.D	N.D	No effect at 30 μM	≥ 30 μM
Drug Y	> 20 μM	~ 30 μM	Slight tachycardia NOAEL= 1 μM	N.D	Rate decreasing Slightly at 10 μM Slight Increase PWDs at 10 μM	~ 3 μM
Drug Z	> 3 μM	> 3 μM	Bradycardia NOAEL= 1 μM	N.D	Rate Decreasing at 1 μM Slight Increase PWDs at 10 μM	1μM > ≥ 100 nM

# Dofetilide time course specific study



## Analysis of waveform multiparameters

Rate	1 min	1 min S.D	3 min	3 min S.D	5 min	5 min S.D	8 min	8 min S.D	11 min	11 min S.D	16 min	16 min S.D	23 min	23 min S.D	25 min	25 min S.D
Control	22.4	0.9	23.0	1.5	23.3	1.3	23.1	1.1	23.0	0.9	23.5	0.7	24.2	0.6	24.5	0.5
Dofetilide 25 nM	23.5	0.4	21.3	0.2	18.9	0.5	16.3	0.6	19.5	1.4	20.1	1.2	23.2	1.1	23.0	0.7
Dofetilide 250 nM	23.2	0.1	9.5	0.2	8.2	0.2	9.1	0.5	15.7	2.4	28.1	0.5	32.1	0.4	31.6	0.7
Dofetilide 2.5 µM	19.5	0.2	8.9	0.3	9.3	0.9	10.7	1.0	17.0	3.0	29.1	0.8	32.1	0.4	31.8	0.6

Rising slope	1 min	1 min S.D	3 min	3 min S.D	5 min	5 min S.D	8 min	8 min S.D	11 min	11 min S.D	16 min	16 min S.D	23 min	23 min S.D	25 min	25 min S.D
Control	2.3	0.1	2.5	0.1	2.5	0.1	2.6	0.1	2.6	0.1	2.7	0.1	2.8	0.1	2.8	0.1
Dofetilide 25 nM	2.8	0.2	2.9	0.2	2.9	0.2	2.8	0.1	2.3	0.2	1.9	0.1	2.0	0.2	1.9	0.2
Dofetilide 250 nM	2.9	0.2	2.7	0.2	2.5	0.2	2.0	0.1	1.5	0.1	1.0	0.1	0.6	0.1	0.5	0.1
Dofetilide 2.5 µM	2.9	0.1	2.6	0.1	2.1	0.1	1.8	0.1	1.3	0.05	0.7	0.1	0.5	0.0	0.4	0.1

# Analysis of waveform multiparameters

<b>Falling slope</b>	1 min	1 min S.D	3 min	3 min S.D	5 min	5 min S.D	8 min	8 min S.D	11 min	11 min S.D	16 min	16 min S.D	23 min	23 min S.D	25 min	25 min S.D
Control	1.2	0.0	1.2	0.1	1.3	0.1	1.3	0.05	1.3	0.04	1.3	0.04	1.4	0.04	1.4	0.03
Dofetilide 25 nM	1.5	0.1	1.3	0.1	1.1	0.04	0.7	0.04	0.7	0.03	0.7	0.03	0.7	0.04	0.7	0.03
Dofetilide 250 nM	1.4	0.1	0.3	0.0	0.2	0.02	0.2	0.01	0.4	0.04	0.4	0.03	0.3	0.02	0.3	0.02
Dofetilide 2.5 µM	1.1	0.0	0.3	0.0	0.3	0.01	0.3	0.03	0.4	0.02	0.4	0.02	0.3	0.01	0.2	0.02
<b>Integration</b>	1 min	1 min S.D	3 min	3 min S.D	5 min	5 min S.D	8 min	8 min S.D	11 min	11 min S.D	16 min	16 min S.D	23 min	23 min S.D	25 min	25 min S.D
Control	8960.5	300.1	9462.4	389.0	9464.6	400.0	9984.2	423.5	10182.0	400.8	10302.1	371.0	10604.9	376.0	10550.9	376.5
Dofetilide 25 nM	10196.6	577.3	12398.4	833.8	14794.2	1183.0	17719.0	1451.1	12678.7	1945.1	8830.5	1183.0	9397.6	1156.1	8903.5	975.7
Dofetilide 250 nM	10751.4	729.5	27752.7	2187.5	31008.5	2167.3	33186.4	5323.9	15726.1	4025.3	4184.1	413.4	2179.0	219.0	1953.8	167.9
Dofetilide 2.5 µM	13876.6	564.2	30101.7	2048.0	26477.0	1901.0	22676.8	2651.0	16528.1	4784.8	3178.5	413.8	1822.0	147.7	2644.4	931.1
<b>PWD50</b>	1 min	1 min S.D	3 min	1 min S.D	5 min	1 min S.D	8 min	1 min S.D	11 min	1 min S.D	16 min	1 min S.D	23 min	1 min S.D	25 min	1 min S.D
Control	611.8	15.1	623.4	28.0	601.1	12.3	613.0	11.6	613.2	9.3	614.7	8.0	610.5	7.6	609.8	7.9
Dofetilide 25 nM	580.6	6.6	686.7	8.2	790.1	17.4	849.1	13.8	734.5	42.8	685.0	35.2	733.2	30.9	736.2	26.2
Dofetilide 250 nM	607.9	6.4	931.5	15.7	1008.0	24.3	1136.4	33.2	884.2	43.9	687.7	10.7	630.9	8.4	662.1	22.5
Dofetilide 2.5 µM	712.7	8.2	1001.4	24.9	1389.7	322.1	1264.6	222.0	912.1	121.0	719.6	27.0	647.6	9.0	676.2	13.3

# Prediction of GPCR modulators cardiotoxicity using Cor.4U cardiomyocytes

## Calcium transient can be impacted by different parameters

- Time dependent drug effect : Cell penetration needed for intracellular target (hERG)
- Compound injection settings and diffusion properties
  - Depth and rate of compound injection
  - Nature and size of considered compound
  - Viscosity of the assay buffer
  - Volume and surface area of the assay well
- Waveform Analysis depends on multiple parameters (rate, slope, PWD ..)



**When evaluating cardiotoxicity with calcium  
transient for a tested compound on Cor.4U cells**

**we suggest to consider**

**NOAEL DOSE (No Observed Adverse Effect Level)**