



# Human-induced pluripotent stem cell-derived cardiomyocytes have limited $I_{Ks}$ for repolarization reserve as revealed by specific KCNQ1/KCNE1 blocker

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## Abstract

**Objective:** We investigated if there is  $I_{Ks}$ , and if there is repolarization reserve by  $I_{Ks}$  in human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs).

**Design:** We used a specific KCNQ1/KCNE1 channel blocker, L-000768673, with an  $IC_{50}$  of 9 nM, and four hERG-specific blockers, astemizole, cisapride, dofetilide, and E-4031 to investigate the issue.

**Results:** L-000768673 concentration-dependently prolonged feature point duration (FPD)-a surrogate signal of action potential duration-from 1 to 30 nM without pacing or paced at 1.2 Hz, resulting from  $I_{Ks}$  blockade in hiPSC-CMs. At higher concentrations, the effect of L-000768673 on  $I_{Ks}$  was mitigated by its effect on  $I_{Ca-L}$ , resulting in shortened FPD, reduced impedance amplitude, and increased beating rate at 1  $\mu$ M and above, recapitulating the self-limiting properties of L-000768673 on action potentials. All four hERG-specific blockers prolonged FPD as expected. Co-application of L-000768673 at sub-threshold (0.1 and 0.3 nM) and threshold (1 nM) concentrations failed to synergistically enhance the effects of hERG blockers on FPD prolongation, rather it showed additive effects, inconsistent with the repolarization reserve role of  $I_{Ks}$  in mature human myocytes that enhanced  $I_{Kr}$  response, implying a difference between hiPSC-CMs used in this study and mature human cardiomyocytes.

**Conclusion:** There was  $I_{Ks}$  current in hiPSC-CMs, and blockade of  $I_{Ks}$  current caused prolongation of action potential of hiPSC-CMs. However, we could not demonstrate any synergistic effects on action potential duration prolongation of hiPSC-CMs by blocking hERG current and  $I_{Ks}$  current simultaneously, implying little or no repolarization reserve by  $I_{Ks}$  current in hiPSC-CMs used in this study.

## Keywords

Stem cell, cardiomyocyte

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## Introduction

In recent years, human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have been shown to have unique predictability in terms of cardiac safety of pharmaceutical reference agents and candidates.<sup>1–5</sup> One key application is to detect and predict QT prolongation and, more importantly and relevantly, *Torsades de Pointes* (TdP) liability of drug candidates, which could result from enhanced depolarizing  $Na^+$  or  $Ca^{2+}$  currents, or reduced repolarizing  $K^+$  currents (mainly  $I_{Kr}$  and  $I_{Ks}$  currents).

Effects of many hERG channel (or  $I_{Kr}$  current) blockers on QT prolongation of hiPSC-CMs have been widely reported.<sup>6–8</sup>

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$I_{Ks}$  current, conducted by KCNQ1 channel subunits associated with KCNE1 or min-K subunits, is deemed a “repolarization reserve”<sup>9</sup>  $K^+$  current during repolarization of cardiac action potential (see Jost et al.<sup>10</sup> for review). In theory, a KCNQ1/KCNE1 blocker should cause QT prolongation, and, sequentially, TdP in vivo. However, JNJ303 has been the only KCNQ1 blocker, reported to cause TdP in a dog model.<sup>11</sup> Although KCNQ1 and KCNE1 proteins are expressed in hiPSC-CMs,<sup>12</sup> the effect of  $I_{Ks}$  blockade on iCell hiPSC-CMs has not been thoroughly studied and reported, to our best knowledge, due to a lack of selective KCNQ1/KCNE1 blockers. JNJ303 caused small prolongation of action potential<sup>13</sup> or showed little effects (unpublished observation, and communications). Therefore, we selected to use a specific in-house developed KCNQ1/KCNE1 blocker-L-000768673 (L-673)-to investigate if there is  $I_{Ks}$ , and if there is repolarization reserve by  $I_{Ks}$  in iCell hiPSC-CMs.

## Experimental procedures

### Test compound preparation

L-673 was synthesized in house. Astemizole, E-4031, cisapride and dofetilide were from Sigma-Aldrich (St. Louis, MO, USA). Test agent stock solutions were prepared in 100% DMSO, and the final dilution was 1:1000 with culture media.

### hiPSC-CM assay

The hiPSC-CMs (iCells<sup>®</sup>) were purchased from Cellular Dynamics International (CDI, Madison, WI, USA). They were placed onto 48-well Cardio ECR E-Plates<sup>®</sup> (ACEA Biosciences Inc., San Diego, CA, USA) and cultured for 14 days before use as described previously.<sup>14</sup> Briefly, E-plates were pre-coated with 10  $\mu$ g/mL fibronectin (Sigma Aldrich, Catalog # F1141), and then cells were seeded at a density of 30,000 cells per well, and maintained at 37°C with 5% CO<sub>2</sub> and 95% O<sub>2</sub>, and fresh culture media every two to three days per manufacturer’s recommendations. Fresh culture media exchange was performed ~3 h prior to data acquisition using an xCELLigence<sup>®</sup> RTCA CardioECR instrument (ACEA Biosciences Inc., San Diego, CA, USA) with the following setting: Sweeps 1; Interval 1; Unit minute; Block duration 0 s; Cardio Speed 12 mS; Cardio point/blk 1; ECR speed 1 mS; ECR point/blk 1. Pre-reads of 1-min were recorded sequentially as baseline with no pacing, and then with pacing at 1.2 Hz using built-in square pulses of 500 mV voltage and 1 mS duration.<sup>15</sup> The empirical voltage and duration were optimized to minimize any potential damage to cells, and to avoid baseline shift. The test agent stock solutions (prepared

in 100% DMSO) were diluted into media and quickly added to the plate at 1:1000 ratio, so the final DMSO concentration in each well was 0.1%. After 1-h incubation in the instrument that was hosted in a cell culture incubator at 37°C with 5% CO<sub>2</sub> and 95% O<sub>2</sub>, plates were read again using identical procedure as pre-reads. All data were analyzed with built-in analysis software and normalized using Microsoft EXCEL<sup>®</sup>. The experimental system, CardioECR, used in this study simultaneously acquires electrical activities (via field potential measurement), and motion activities (via impedance change) of hiPSC-CM syncytia. Field potential signal and impedance signal are not two aspects of one signal, but two separate signals for electrical and motion activities respectively, coupled temporally. Therefore, the feature point duration (FPD, the time duration from the start of a beat to its inflectionpoint in the impedance signal, as detailed in our previous study<sup>15</sup>) matches exactly the field potential duration (i.e. the time interval from Na<sup>+</sup> spike to “T” wave in field potential signal), as shown in Figure 1 of our recent publication.<sup>15</sup> We found that external stimulation suppressed field potential signals but had little impact on impedance signal. Therefore, we used FPD from impedance signal measurement throughout this study.

### Ion channel assay

Whole-cell currents were measured from cells expressing cardiac ion channels using the QPatch HTX automated patch clamp system (Sophion Bioscience, DM) at room temperature. Resistances of the planar patch plate chambers (holes) were 1–3 M $\Omega$ .

CHO-hERG cells<sup>16</sup> or HEK-KCNQ1/KCNE1 cells<sup>17</sup> were washed with PBS once, treated with 0.05% Trypsin/EDTA for 6 min at 37°C, re-suspended in PBS, spun down 1300 r/min for 1.15 min, and then re-suspended in 450  $\mu$ L external solution before putting them into QPatch.

hERG external solution contained (in mM): 132 NaCl, 4 KCl, 3 CaCl<sub>2</sub>, 0.5 MgCl<sub>2</sub>, 10 HEPES, 11.1 glucose, pH = 7.35  $\pm$  0.1, adjusted with NaOH. hERG internal solution contained (in mM): 70 KF, 60 KCl, 15 NaCl, 5 HEPES, 5 EGTA, pH 7.25  $\pm$  0.1, adjusted with NaOH.

KCNQ1/KCNE1 external solution contained (in mM): 137 NaCl, 1 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 11 Dextrose, pH = 7.25  $\pm$  0.1, adjusted with NaOH. KCNQ1/KCNE1 internal solution contained (in mM): 90 KCl, 50 KF, 1.6 MgCl<sub>2</sub>, 2.5 K<sub>2</sub>ATP, 10 HEPES, 10 EGTA, pH 7.3  $\pm$  0.1, adjusted with KOH.

hERG current was elicited with the following voltage – Step protocol: from a holding potential ( $V_h$ ) of –70 mV, first a brief 20-mS depolarizing pre-pulse to –40 mV was applied to obtain a baseline current

measurement with a return to  $V_h$  for 80 mS, followed by a 4-S depolarizing step to a test potential ( $V_t$ ) of 30 mV and finally a 1-S repolarizing step ( $V_{tail}$ ) to  $-40$  mV. The inter-pulse interval is 20-S. The hERG current amplitude was measured as the peak deactivating tail current amplitude during  $V_{tail}$  relative to the baseline current at  $-40$  mV.

KCNQ1/KCNE1 current was elicited with a voltage – Step protocol from  $V_h$  of  $-50$  mV with 3-S depolarizing steps to  $V_t$  of 50 mV followed by repolarization to  $-50$  mV. The inter-pulse interval is 20-S. The KCNQ1/KCNE1 current amplitude was measured as the peak tail current amplitude.

After forming whole cell configuration and 8-min waiting time, external solution (containing 0.3% DMSO as vehicle control) was applied, and currents were monitored for stability during vehicle control for 5 min before addition of test compounds. To achieve steady state, test compound was applied three times at 0-S, 90-S, and 180-S time point during the 5-min assay time at each test concentration. Test compound response of each cell was averaged from the last three data points at each concentration, and then normalized to its vehicle control for % inhibition.  $IC_{50}$  values were fit with a Hill equation:

$$\begin{aligned} \% \text{ inhibition} \\ &= 100 / (1 + (IC_{50} / [\text{test compound concentration}])^h) \end{aligned}$$

where  $h$  = Hill coefficient.

### CaV1.2 influx assay

HEK-293 cells stably expressing human Cav 1.2 L-type  $Ca^{2+}$  channel (HEK-CaV1.2 E74 cell line)<sup>18</sup> were plated to a 96-well Greiner Plate at 60,000 cells/well on the day before the experiment. PPB solution contains (in mM): 127 NaCl, 25 KCl, 0.005  $CaCl_2$ , 1.7  $MgCl_2$ , and 10 HEPES (pH = 7.2, adjusted with NaOH). CTB solution contains (in mM): 119 NaCl, 25 KCl, 4  $CaCl_2$ , 1.7  $MgCl_2$ , and 10 HEPES (pH = 7.2, adjusted with NaOH). The  $Ca^{2+}$ -sensitive fluorescence ACTOne<sup>®</sup> dye (Codex BioSolutions, Gaithersburg, MD, USA) was used as previously described.<sup>5</sup> On experiment day, cells were washed with 100  $\mu$ L of PPB per well twice, then incubated with 50  $\mu$ L of PPB + 45  $\mu$ L of dye solution at 37°C for 1 h (test plate); 5  $\mu$ L of test compound stock was then added to each well for another 30-min incubation (the final DMSO concentration was 0.1%). FDSS/ $\mu$ Cell (Hamamatsu Ltd, Hamamatsu, Japan) was used to acquire  $Ca^{2+}$  transient signals at a sampling rate of 16 Hz with 485 nm excitation and 530 nm emission. After 20-S baseline recording (i.e. to acquire

baseline signal of each well), 100  $\mu$ L of CTB solution, containing test compounds at the same corresponding concentrations, was added to each well using the built-in solution addition machinery to trigger  $Ca^{2+}$  influx without interrupting signal acquisition; 100 nM of isradipine and 0.1% DMSO were used as maximal (or 100% inhibition,  $n = 12$ ) and minimal (or 0% inhibition  $n = 12$ ) responses, respectively. Normalized test compound response (or % inhibition  $n = 4$ ) was calculated as follows:

$$\begin{aligned} \text{Inhibition}_{\text{MIN}} \\ &= \text{Average of (signals of 0.1\% DMSO} \\ &\quad \text{– signals of the corresponding baselines)} \end{aligned}$$

$$\begin{aligned} \text{Inhibition}_{\text{MAX}} \\ &= \text{Average of (signals of 100 nM isradipine} \\ &\quad \text{– signals of the corresponding baselines)} \end{aligned}$$

$$\begin{aligned} \text{Inhibition}_{\text{test}} \\ &= \text{signals of test compound} \\ &\quad \text{– signals of their corresponding baselines} \end{aligned}$$

$$\begin{aligned} \% \text{ inhibition} \\ &= (\text{Inhibition}_{\text{MIN}} - \text{Inhibition}_{\text{test}}) \\ &\quad / (\text{Inhibition}_{\text{MIN}} - \text{Inhibition}_{\text{MAX}}) * 100\% \end{aligned}$$

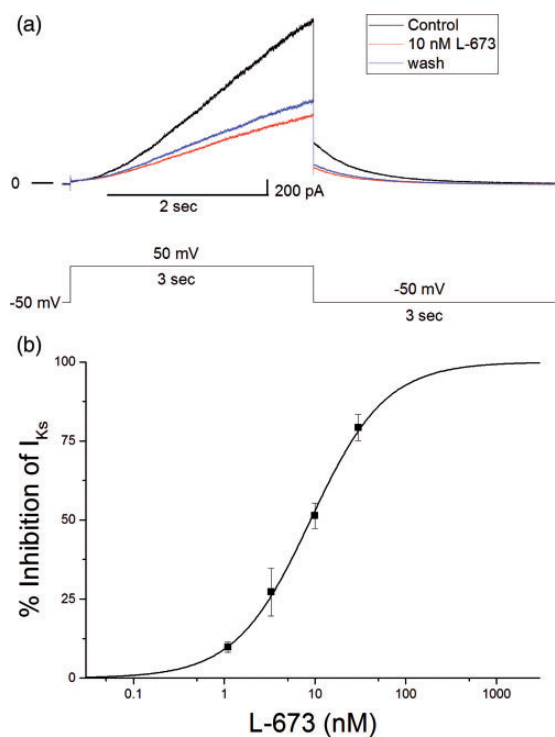
### Statistical analysis

All data were normalized to appropriate controls as described above and expressed as: Mean  $\pm$  SEM ( $n = 4-9$ , as indicated specifically in individual legend and table). Since all the statistical analyses were with vs. without type, one-time comparison of means between two groups with similar variances (as indicated in figure legends), Student's t-test was used for statistical comparison on normalized data, and  $P < 0.05$  was deemed statistically significant.

### Results

L-673 is an in-house developed, specific KCNQ1/KCNE1 blocker.<sup>19,20</sup> The batch of L-673 used in this study had an  $IC_{50}$  of 9 nM on KCNQ1/KCNE1 channels overexpressed in HEK293 cells, measured with the QPatch platform (Figure 1). Its effects on overexpressed hERG and Cav1.2 channels are listed in Table 1. Due to the relative advantage in simultaneous measurement of impedance signal (a surrogate of contraction), and field potential signal (local ensemble of hiPSC-CM membrane potential), we used the ACEA

CardioECR platform to measure the response of hiPSC-CM to L-673 without external stimulation or paced at 1.2 Hz (or 72 beats/min [a close mimic of normal human heart rate]). Representative impedance traces before and after L-673 addition are shown in Figure 2. As shown in Figure 3(a), L-673 had no effects



**Figure 1.** Effect of L-673 on KCNQ1/KCNE1 channels over-expressed in HEK293 cells. Example traces of whole-cell  $I_{Ks}$  currents (a), elicited with a voltage step, are superimposed during control condition and after addition of 10 nM L-673. The voltage-clamp protocol is shown above the current traces. The concentration dependence of KCNQ1/KCNE1 inhibition by L-673 (b) is quantified as the peak tail current amplitude measured at steady state for each concentration of L-673, normalized to the control tail current amplitude for each cell, expressed as % inhibition, and fitted with a Hill equation (solid line) to the data ( $n = 6-9$ ).

on beating rate from 0.3 to 300 nM, but concentration-dependently increased beating rate starting at 1000 nM. Similarly, it only started to concentration-dependently reduce impedance amplitude at 1000 nM. On the other hand, L-673 concentration-dependently increased FPD with Frederica correction (FPDcF) from 1 nM to 30 nM. This effect was then reduced with increasing concentrations of L-673, and it was converted to a concentration-dependent decrease starting at 1000 nM (Figure 3(a)). When paced at 1.2 Hz (Figure 3(b)), the effect of L-673 on beating rate was masked until 3000 nM (at which concentration, cells had a faster spontaneous beating rate than 1.2 Hz), but effects on impedance amplitude and FPD (no correction, as rate was a constant under pacing condition, Figure 3(b)) were similar to those without external stimulation.

Because L-673 had shown effects on FPDcF beginning at 1 nM concentration, we co-applied L-673 (0.1, 0.3 and 1 nM) with the specific hERG blocker cisapride at 3, 10 and 30 nM concentrations that do not cause early afterdepolarizations (EADs) alone in hiPSC-CMs to avoid measurement interference. As shown in Figure 4, cisapride at 3, 10 and 30 nM had little effect on beating rate and impedance amplitude, and co-application of L-673 up to 1 nM had no impact on the effect of cisapride on beating rate and impedance amplitude. However, addition of L-673 did slightly enhance the effect on FPDcF (without pacing) and FPD (paced at 1.2 Hz) prolongations by cisapride, and the enhancement effects were statistically significant only in some combinations (Figure 4(e) and (f)).

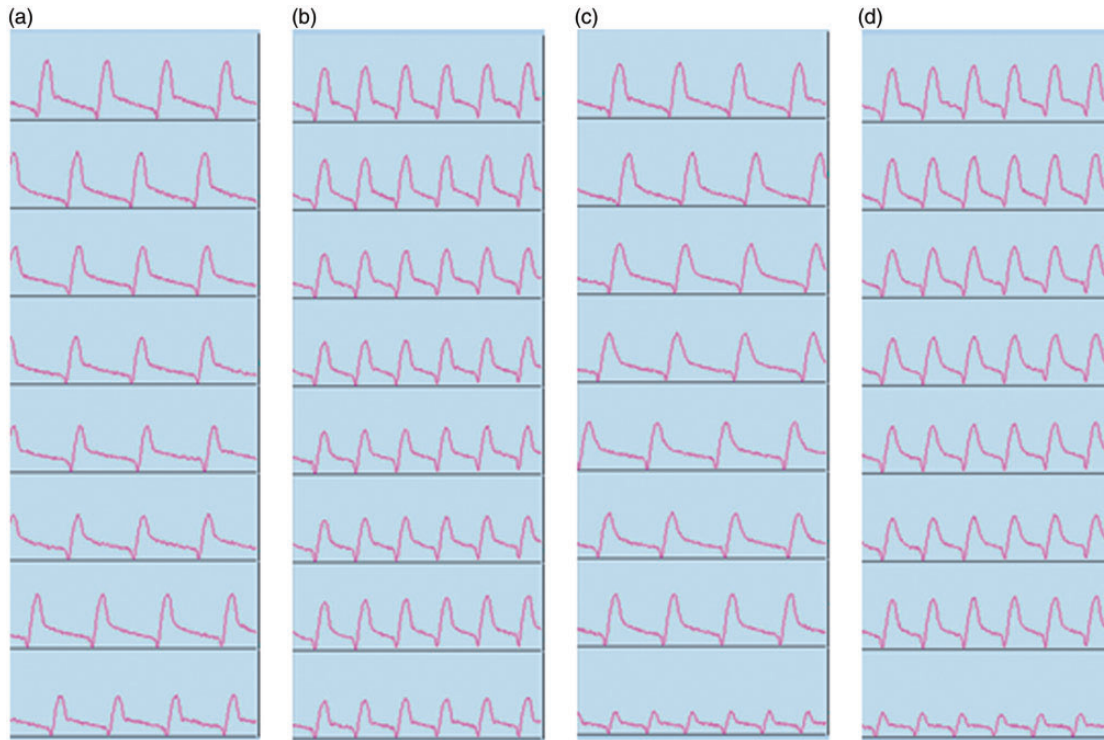
Next, we repeated the study with different, yet specific hERG blockers, astemizole, dofetilide, and E-4031 (Table 1). The highest test concentrations of each reference hERG blocker were set to half-log lower than their respective EAD-caused concentration in this study (data not shown). Just like cisapride, all three agents at tested concentrations had little effect on beating rate and impedance amplitude, and co-application of L-673 up to 1 nM did not change these effects (data

**Table 1.** Effect on cardiac ion channels.

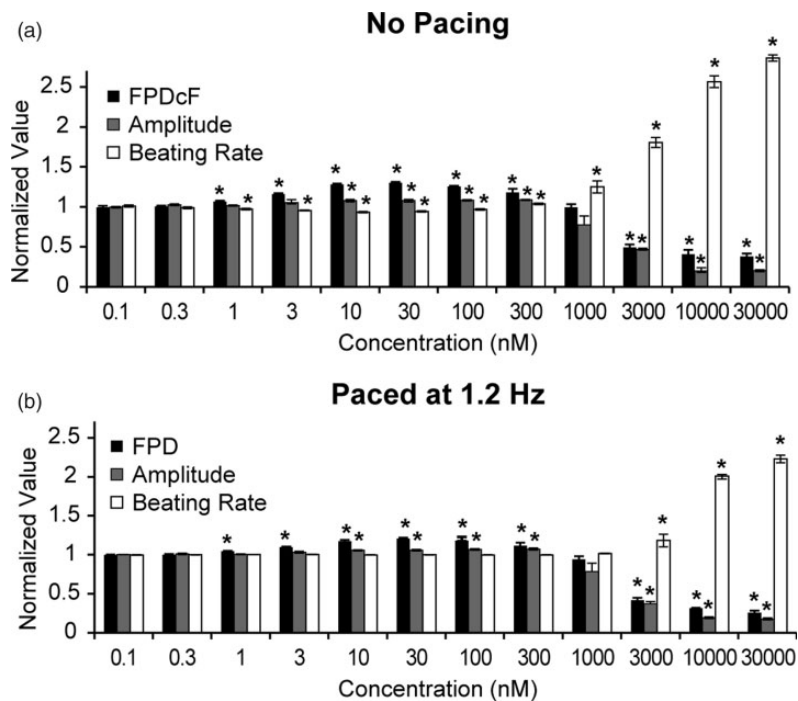
Test compound	IC <sub>50</sub> or % inhibition (nM) [N]		
	KCNQ1/KCNE1	hERG	CaV1.2
L-673	9 [6-9]	27 ± 3%@30,000 [4]	1500 [4]
Astemizole	25,000 [6]	3 [7]	n.d.
Cisapride	18 ± 5%@30,000 [7]	18 [5]	n.d.
Dofetilide	3 ± 6%@30,000 [6]	23 [8-9]	n.d.
E-4031	9 ± 3%@30,000 [6]	40 [6]	n.d.

Note: % Inhibition = percentile inhibition (Mean ± SEM) between 0 and 100% at the highest test concentration of 30,000 nM. IC<sub>50</sub> value was not determined when the inhibition at the highest test concentration was less than 50%.

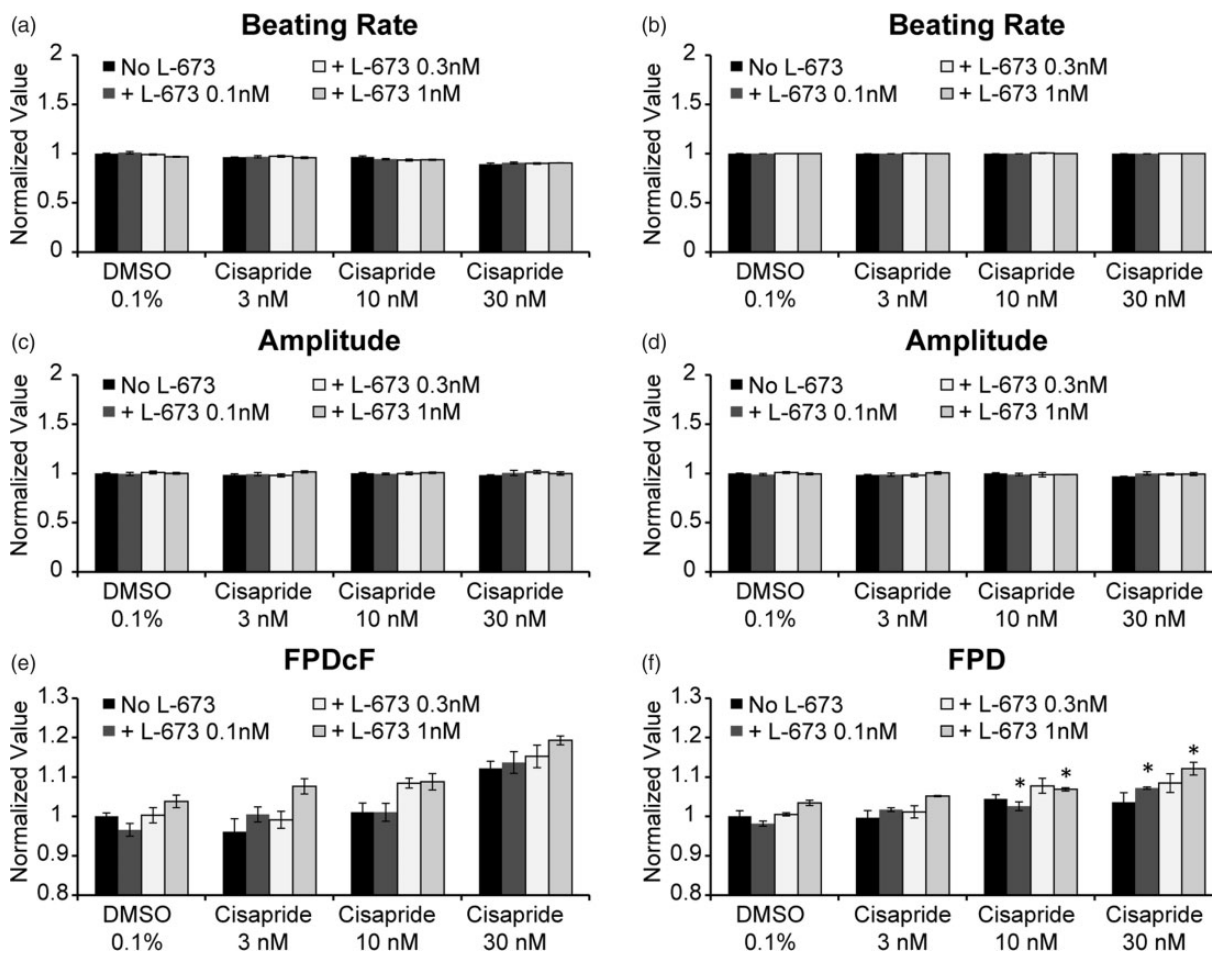
[N] = individual n-number; n.d. = not determined.



**Figure 2.** Representative impedance traces before and after L-673 addition. Panel (a) shows the pre-reads without external stimulation. Panel (b) is the pre-reads when paced at 1.2 Hz. Panel (c) shows the impedance traces 1 h after L-673 addition (from top to bottom: 0.1% DMSO, 1, 3, 10, 30, 100, 300, and 1000 nM L-673) without pacing. Panel (d) is the immediately following recordings but with external 1.2 Hz stimulation. Five second traces are shown in all panels.



**Figure 3.** Effects of L-673 alone on hiPSC-CMs. Normalized changes on impedance amplitude, beating rate, and FPDcF or FPD of hiPSC-CMs at the presence of L-673 at different concentrations from 0.1 to 30000 nM as labeled in figure without pacing (a) or paced at 1.2 Hz (B). All data ( $n = 6$ ) were normalized to 0.1% DMSO control at pre-read.  $*P < 0.05$ , compared to time-matched 0.1% DMSO control (detailed in Experimental procedures).



**Figure 4.** Impact of L-673 on the effects of cisapride. L-673 at 0.1, 0.3, or 1 nM was co-applied with cisapride at different concentrations (3, 10, or 30 nM) to hiPSC-CM for effects on impedance amplitude (a, no pacing; b, paced at 1.2 Hz), beating rate (c, no pacing; d, paced at 1.2 Hz), and FPDcF (e, no pacing) or FPD (f, paced at 1.2 Hz). All data ( $n=6$ ) were normalized to 0.1% DMSO control at pre-read. \* $P<0.05$ , compared to time-matched 0.1% DMSO control; # $P<0.05$ , compared to time-matched, same concentration without L-673 (detailed in Experimental procedures).

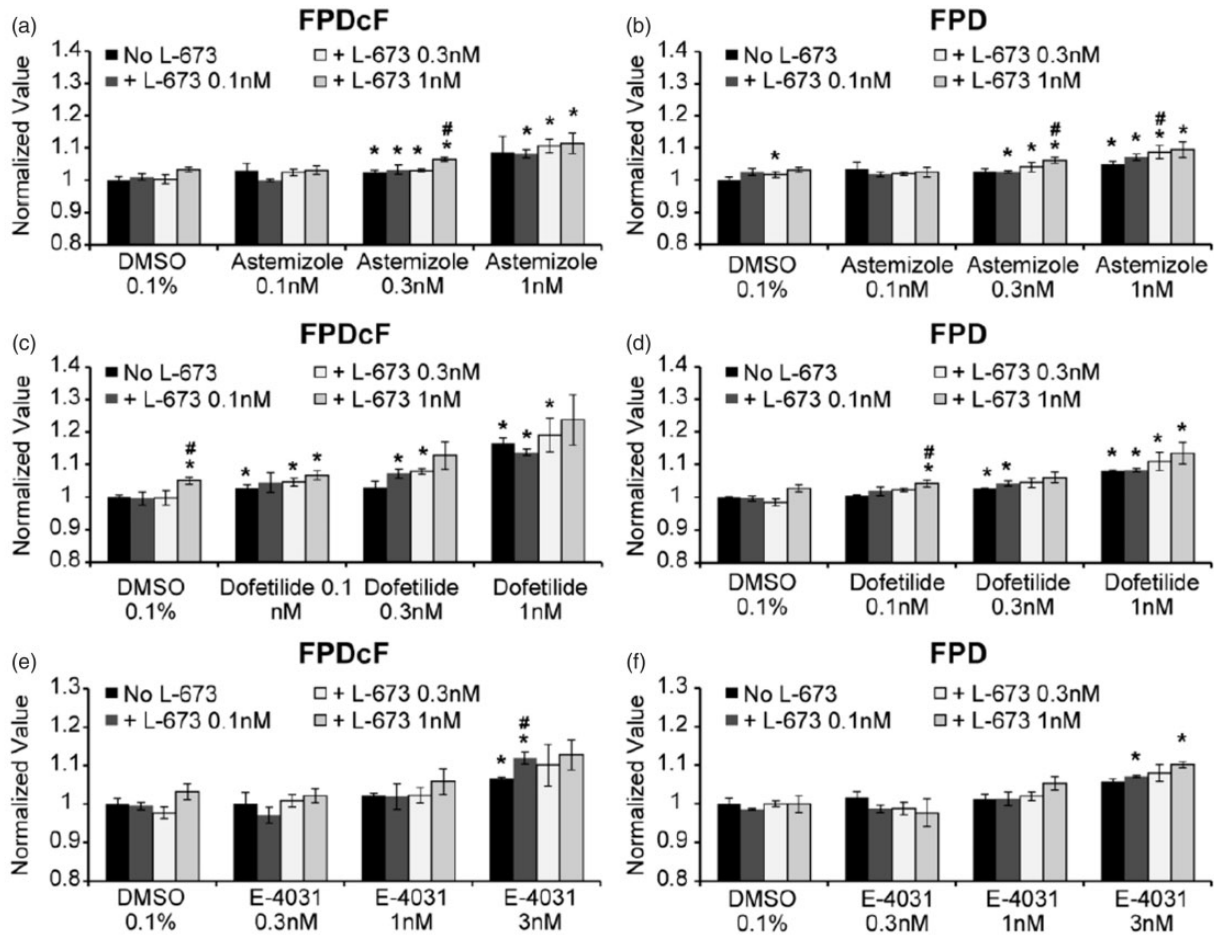
not shown). Similarly, all three agents increased FPDcF (without pacing) and FPD (paced at 1.2 Hz), and the effects were enhanced by co-application of L-673 in an additive manner (statistically significant only in some combinations), as shown in Figure 5.

## Discussion

To the best of our knowledge, impact of  $I_{Ks}$  blockade on behavior of iCell hiPSC-CMs was not thoroughly investigated, except that JNJ303 was used in CiPA phase one study, intended as a positive KCNQ1/KCNE1 blocker, but it showed minor or no effects on hiPSC-CMs<sup>13</sup> (and unpublished observation and communication). Therefore, we used a specific KCNQ1/KCNE1 blocker to investigate this issue. We confirmed  $I_{Ks}$  current in hiPSC-CMs, demonstrated the self-limiting property of L-673, but could not prove any

synergistic effects on FPD by blocking hERG and  $I_{Ks}$  currents simultaneously.

Since it was initially published in year 1997 by Selnick et al.,<sup>19</sup> L-673 has been widely accepted as a specific KCNQ1/KCNE1 blocker and used in many in vitro and in vivo studies. The L-673 used by Qu and Vargas<sup>21</sup> was purchased from Albany Molecular Research Inc. with an  $IC_{50}$  of 27 nM on  $I_{Ks}$ , and it failed to prolong field potential duration up to 300 nM in their study. We observed that L-673 could break down and the batch of L-673 used in our study was synthesized in house and verified for potency immediately prior to use in this study. This may explain the discrepancy between these two studies. We have tested JNJ303 in house and cannot get definitive results. In addition, as described by Towart et al.,<sup>11</sup> L-673 was 10 fold more potent than the other two well-known KCNQ1/KCNE1 blockers, JNJ303 or



**Figure 5.** Impact of L-673 on the FPD prolongation effects of hERG blockers. L-673 at 0.1, 0.3, or 1 nM was co-applied with astemizole (a, no pacing; b, paced at 1.2 Hz), E-4031 (c, no pacing; d, paced at 1.2 Hz), and dofetilide (e, no pacing; f, paced at 1.2 Hz) for its impact on the FPD prolongation effects of these hERG blockers at different concentration as labeled. All data ( $n = 6$ ) were normalized to 0.1% DMSO control at pre-read. \* $P < 0.05$ , compared to time-matched 0.1% DMSO control; # $P < 0.05$ , compared to time-matched, corresponding test compound without L-673 (detailed in Experimental procedures).

HMR-1556, on  $I_{Ks}$  current inhibition, and all three were highly selective over  $I_{Na}$ ,  $I_{Ca}$ ,  $I_{Kr}$ ,  $I_{To}$ , and  $I_{K1}$  currents involved in cardiac repolarization. Therefore, we conducted this thorough study using L-673 as a reliable, specific KCNQ1/KCNE1 blocker.

Based on cardiac ion channel profile (Table 1), L-673 at low nM concentration range seemed a selective KCNQ1/KCNE1 blocker. It concentration-dependently prolonged FPDcF and FPD (paced at 1.2 Hz) from 1 to 30 nM, implying an effect resulting from enhanced depolarized currents (e.g.  $I_{Na}$  or  $I_{Ca-L}$ ) or reduced repolarized  $I_K$  currents ( $I_{Ks}$  or  $I_{Kr}$ ). As the effective concentration range only matched its  $IC_{50}$  value on KCNQ1/KCNE1 channel, but was much lower than that of hERG channel, and it did not show any enhancement effect on NaV1.5 or CaV1.2 channels (data not shown), rather blocked CaV1.2 channel with an  $IC_{50}$  of 1500 nM, we believed the effect of FPDcF prolongation was mediated via  $I_{Ks}$

blockade in hiPSC-CMs. This conclusion is consistent with *in vivo* dog studies by Lynch et al.<sup>22</sup> and Stump et al.,<sup>20</sup> and the available selectivity data by Towart et al.<sup>11</sup>

At concentrations starting from 100 nM, the effect on FPDcF and FPD (paced at 1.2 Hz) prolongation was progressively reduced by increased concentrations of L-673, and it was even converted to FPDcF and FPD (paced at 1.2 Hz) shortening, associated with increased beating rate and reduced impedance amplitude, a signature of CaV1.2 channel blockade,<sup>3,4,6,7,23</sup> starting from 1000 nM, demonstrating gradually mixed ion channel effects from both KCNQ1/KCNE1 and CaV1.2 channel blockade and dominated effect from CaV1.2 blockade at high concentrations. This observation indeed matched L-673's  $IC_{50}$ s of 9 nM on KCNQ1/KCNE1 channel and of 1500 nM on CaV1.2 channel (Table 1), and explains the self-limiting property of L-673 in causing only limited FPD prolongation

without any arrhythmic effect, consistent with *ex vivo* study using guinea pig myocytes.<sup>19</sup> These data also implied relatively smaller  $I_{K_s}$  current density than  $I_{Ca-L}$  current density in hiPSC-CMs, otherwise, the effect at 1  $\mu$ M and higher should not be dominated by effect from CaV1.2 channel blockade.

FPD measured in this study is a surrogate of action potential duration of hiPSC-CMs.<sup>15</sup> All four hERG-specific blockers caused FPD prolongation as expected (Figures 4 and 5).  $IC_{50}$  value from individual ion channel measurement may not match the potency in hiPSC-CMs. L-673 statistically significantly prolonged FPD by 7% at 1 nM, although its  $IC_{50}$  on KCNQ1/KCNE1 was 9 nM. We also observed that 3 nM dofetilide caused EADs, while its  $IC_{50}$  value on hERG channel was 29 nM (unpublished observation); 3–100 nM L-673 prolonged FPD by 20–30%, which was near the threshold for EAD generation (Figure 3 (a)). The effect of hERG channel blockers above 20–30% FPD prolongation usually caused EADs (unpublished observations in this study), which prevented further data analysis. Therefore, we limited L-673 test concentration at 1 nM in this study to investigate the issue of synergism. Co-application of L-673 at sub-threshold (0.1 and 0.3 nM) and threshold (1 nM) concentrations failed to synergistically enhance the effects of hERG blockers on FPD or FPDcF prolongation, but rather showed additive effects. These results (i.e. lack of synergistic responses) failed to demonstrate any “repolarization reserve function (i.e., reduced  $I_{K_s}$  enhances the effect of  $I_{K_r}$  blockade on action potential prolongation)” of  $I_{K_s}$  current in hiPSC-CMs. That is, the quantity of  $I_{K_s}$  current in hiPSC-CMs did not seem to be large enough to mitigate proarrhythmic effects when hERG channels were blocked, otherwise EADs would be observed. The finding was inconsistent with the repolarization reserve role of  $I_{K_s}$  in mature human myocytes.<sup>24,25</sup> This discrepancy also implied a difference between hiPSC-CMs used in this study and matured human cardiomyocytes, and could help to explain the higher sensitivity of hiPSC-CMs to hERG blockers. However, as cardiac repolarization is a net effect of several cardiac currents, such as  $I_{K_r}$ ,  $I_{K_s}$ ,  $I_{Ca-L}$ ,  $I_{Na}$ , and  $I_{NCX}$ , that all exist in the hiPSC-CMs we used,<sup>3,15</sup> our findings could not exclude the repolarization reserve capacity of the hiPSC-CMs contributed by other cardiac currents.

In conclusion, as revealed by this study with our specific KCNQ1/KCNE1 blocker L-673, there was  $I_{K_s}$  current in iCell hiPSC-CMs, and blockade of  $I_{K_s}$  current caused prolongation of action potential of hiPSC-CMs. L-673 also exemplifies the concept that QT prolongation does not equal TdP. However, we could not demonstrate any synergistic effects on action potential duration prolongation of hiPSC-CM

by blocking hERG current and  $I_{K_s}$  current simultaneously, implying limited contribution of  $I_{K_s}$  current in hiPSC-CMs used in this study, under the two experimental conditions (spontaneous beating and 1.2 Hz pacing) used.

### Declaration of conflicting interests

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### Ethical approval

None.

### Guarantor

None.

### Contributorship

HZ, JW, HC, and AL designed and conducted experiments. HZ and AL analyzed, interpreted and presented results for group discussions. AL and FS provided rationale, background, framework and feedback. HZ, JW, and HC provided methods, description of results, and figures for the manuscript. HZ wrote and organized the manuscript, with editorial input from AL.

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