Patient-Specific Drug Screening Using a Human Induced Pluripotent Stem Cell Model of Catecholaminergic Polymorphic Ventricular Tachycardia Type 2

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Background—Catecholaminergic polymorphic ventricular tachycardia type 2 (CPVT2) results from autosomal recessive CASQ2 mutations, causing abnormal Ca²⁺-handling and malignant ventricular arrhythmias. We aimed to establish a patient-specific human induced pluripotent stem cell (hiPSC) model of CPVT2 and to use the generated hiPSC-derived cardiomyocytes to gain insights into patient-specific disease mechanism and pharmacotherapy.

Methods and Results—hiPSC cardiomyocytes were derived from a CPVT2 patient (D307H-CASQ2 mutation) and from healthy controls. Laser-confocal Ca²⁺ and voltage imaging showed significant Ca²⁺-transient irregularities, marked arrhythmogenicity manifested by early afterdepolarizations and triggered arrhythmias, and reduced threshold for store overload–induced Ca²⁺-release events in the CPVT2-hiPSC cardiomyocytes when compared with healthy control cells. Pharmacological studies revealed the prevention of adrenergic-induced arrhythmias by β-blockers (propranolol and carvedilol), flecainide, and the neuronal sodium-channel blocker riluzole; a direct antiarrhythmic action of carvedilol (independent of its α/β-adrenergic blocking activity), flecainide, and riluzole; and suppression of abnormal Ca²⁺ cycling by the ryanodine stabilizer JTV-519 and carvedilol. Mechanistic insights were gained on the different antiarrhythmic actions of the aforementioned drugs, with carvedilol and JTV-519 (but not flecainide or riluzole) acting primarily through sarcoplasmic reticulum stabilization. Finally, comparable outcomes were found between flecainide and labetalol antiarrhythmic effects in vitro and the clinical results in the same patient.

Conclusions—These results demonstrate the ability of hiPSCs cardiomyocytes to recapitulate CPVT2 disease phenotype and drug response in the culture dish, to provide novel insights into disease and drug therapy mechanisms, and potentially to tailor patient-specific drug therapy. (Circ Arrhythm Electrophysiol. 2017;10:e004725. DOI: 10.1161/CIRCEP.116.004725.)

Key Words: drug therapy ■ induced pluripotent stem cells ■ myocytes, cardiac ■ stem cell ■ tachycardia, ventricular

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmogenic disorder characterized by abnormal Ca²⁺ handling leading to catecholamine-induced Ca²⁺ leak, ventricular arrhythmias, and sudden cardiac death in young and otherwise healthy individuals. Most CPVT cases (CPVT1) are associated with dominant mutations in the cardiac ryanodine receptor gene (RyR2). The minority of cases results from autosomal recessive mutations in the cardiac calsequestrin gene (CASQ2), termed collectively CPVT2. The study of CPVT-related mutations provided invaluable insights on normal Ca²⁺-handling in cardiomyocytes and the arrhythmogenic mechanisms of this disorder. Nevertheless, the study of this disorder has been hampered by the lack of appropriate human cardiomyocyte models and by the inability to study this disease in a patient-specific manner.

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The introduction of the human induced pluripotent stem cells (hiPSCs) technology offers a unique opportunity to overcome these obstacles. This approach allows reprogramming of patient-specific somatic cells into pluripotent stem cells that can be coaxed to differentiate into several cell types including cardiomyocytes. In the cardiac field, hiPSCs were established from healthy individuals and from patients inflicted with acquired and several types of inherited cardiac disorders. Patient-specific hiPSC-derived cardiomyocyte (hiPSC-CM) models of different inherited arrhythmogenic syndromes (including the long-QT, Brugada, and sodium channel overlap syndromes; arrhythmogenic right ventricular cardiomyopathy; and both types of CPVT) were
WHAT IS KNOWN
- Catecholaminergic polymorphic ventricular tachycardia type 2 (CPVT2) results from autosomal-recessive CASQ2 mutations, causing abnormal Ca\(^{2+}\) handling and malignant ventricular arrhythmias.
- Patient/disease-specific human induced pluripotent stem cell derived cardiomyocytes hiPSC-CMs can recapitulate the abnormal clinical phenotype of a variety of inherited cardiac disorders in the culture-dish including of CPVT2.

WHAT THE STUDY ADDS
- The CASQ2 mutation reduced the threshold for development of SOICR in the CPVT2-hiPSC-CMs supporting a physiological role of CASQ2 in luminal Ca\(^{2+}\) sensing and in RyR2 stabilization.
- The patient/disease-specific hiPSC-CMs model allowed screening of potential therapies and provided novel insights into mechanism of action of several pharmacological agents (propranolol, labetalol, JTV519, carvedilol, flecainide and riluzole) in CPVT2.
- A positive correlation was noted between the in vitro effects of some of the drugs studied in the patient-specific hiPSC-CMs model and the clinical exercise-test results in the same patient, supporting the role of this technology in the field of precision medicine.

Methods
Detailed information on various methods used in this study is available in the Data Supplement.

Generation and Cardiomyocyte Differentiation of the CPVT2-hiPSC-CMs
We used previously well-established healthy control and CPVT2 hiPSC lines,28 the latter derived from a 19-year-old male patient with CPVT2 because of the D307H-CASQ2 homozygous mutation) can recapitulate the CPVT phenotype in vitro, to provide novel mechanisms into the disease process, and offer a unique experimental platform for patient-specific drug testing. To test the latter hypothesis, we evaluated the potential antiarrhythmic effects of several pharmacological agents using the generated CPVT2-hiPSC-CMs model, investigated, and provided novel insights into the mechanisms of action of these drugs and correlated the in vitro pharmacological responses of several agents with their in vivo clinical response in the same patient.

Supplement minus insulin (Life Technologies), 1% penicillin/streptomycin, and 6 µmol/L CHIR99021 (Stemgent) for 2 days. Medium was changed to RPMI/B27 (without CHIR) supplemented with 5 µmol/L IWR-1 (days 3 and 4). Beating monolayers were collected at 30 to 60 days, enzymatically dissociated into single cardiomyocytes (using TrypLE), and plated on Matrigel-coated optical culture dishes.

Laser Confocal Ca\(^{2+}\) and Voltage Imaging
For Ca\(^{2+}\) imaging, the studied hiPSC-CMs were loaded with 5 µmol/L Fluo-4 (Molecular Probes) to allow whole-cell [Ca\(^{2+}\)] transients recording. Dispersed hiPSC-CMs were incubated with 5 µmol/L Fluo4 containing culture medium at 37°C for 30 minutes. Subsequently, cells were washed and incubated with dye-free Tyrode solution for additional 30 minutes to allow complete de-esterification of intracellular acetoxyethyl esters.

For voltage imaging, hiPSC-CMs were loaded with the voltage-sensitive indicator FluoVolt (Molecular Probes), according to the manufacturer’s protocol. Experiments were performed at 35°C in Tyrode solution containing (in mmol/L): NaCl, 140; KCl, 5.4; CaCl\(_2\), 1.8; MgCl\(_2\), 1; HEPES, 10; and glucose, 10. Optical Ca\(^{2+}\) transients and action potentials were recorded from spontaneously beating dispersed CMs (single cells or small cell clusters) using the line-scan mode on a Zeiss LSM-710 confocal system. Ca\(^{2+}\) imaging data were analyzed using Matlab-based custom-written software.27 Optical action potentials were analyzed using Clampfit software. For simultaneous Ca\(^{2+}\) and voltage imaging, hiPSC-CMs were loaded with the Ca\(^{2+}\)-sensitive dye Rhod-3-AM (Molecular Probes) and FlouVolt consecutively, according to the manufacturer’s protocols. Fluorescence was then recorded after simultaneous excitation of both dyes.

Store overload-induced Ca\(^{2+}\) release (SOICR) was evaluated using a modification of the method described by Jiang et al.26 In brief, hiPSC-CMs were treated with tetrodotoxin (10 µmol/L), lidocaine (50 µmol/L), and CsCl (5 mmol/L) to eliminate spontaneous automaticity and action potential–dependent Ca\(^{2+}\) releases. Subsequently, cells were exposed to increasing extracellular [Ca\(^{2+}\)] concentrations (0.1–4 mmol/L), and the development of SOICR events was identified using the line-scan mode. Recordings were performed for at least 60 seconds to allow identification of infrequent Ca\(^{2+}\) oscillations.

In experiments including pacing, relatively quiescent cells at baseline were identified and paced at 1 Hz using field stimulation.

Pharmacological Studies
hiPSC-CMs were treated with isoproterenol (10 µmol/L), forskolin (10 µmol/L), carvedilol (0.3–1 µmol/L), propranolol (1 µmol/L), flecainide (1–6 µmol/L), riluzole (10 µmol/L), JTV519 (1 µmol/L), and labetalol (2 µmol/L), all from Sigma. Preparations were incubated with the tested drugs for >10 minutes except for carvedilol (0.3 µmol/L, 2 hours). Forskolin, carvedilol, riluzole, and JTV519 were dissolved in dimethyl sulfoxide.

In all experiments quantifying arrhythmogenicity, a single ectopic Ca\(^{2+}\) release (Figure 1C) was regarded as the minimal finding defining Ca\(^{2+}\) cycling abnormality, similarly to previous reports.22 Early and delayed afterdepolarization (EADs, DADs, respectively) were defined as low-amplitude depolarizations occurring during phase 3 or phase 4 of the action potential, respectively. Arrhythmogenicity suppression was regarded as the complete resolution of any ectopic Ca\(^{2+}\) release events after drug application, whereas proarrhythmic effects were documented when new Ca\(^{2+}\)-cycling abnormalities occurred in previously normal cells. Similarly, SOICR suppression was regarded as the complete cessation of Ca\(^{2+}\) oscillations after drug application.

Clinical Correlation
Because of suboptimal medical response to maximal ß-blocker therapy additional pharmacological solutions were sought and the patient’s response to flecainide and labetalol was evaluated. An exercise test was performed after 2 days of treatment with labetalol alone or flecainide in addition to ß-blockers. The patient gave his written informed consent for the study, and the trial was approved by the institution’s ethics committees. Both investigators performing the
Figure 1. CPVT2-hiPSC-CMs are arrhythmogenic. A, Identifying the D307H-CASQ2 homozygous mutation (*G/C substitution) in the CPVT2-hiPSC-CMs (top). Positive immunostaining of the CPVT2-hiPSC-CMs for cTnI and α-actinin (bottom). B–F, Line-scan tracings depicting changes in intracellular Ca\(^{2+}\) in fluo4-loaded CPVT2-hiPSC-CMs. The CPVT2-hiPSC-CMs demonstrated a variety of Ca\(^{2+}\) abnormalities including local Ca\(^{2+}\)-release events (black arrows, B), broad double-humped transients (red arrows) appearing consistently (top) or intermittently (bottom). C, Multiple consecutive Ca\(^{2+}\)-release events (red arrows) occurring while diastolic Ca\(^{2+}\) levels remain elevated (D), and sustained high-frequency oscillating Ca\(^{2+}\)-release events (red arrows; E and F). G and H, Line scan Ca\(^{2+}\) (Continued)
exercise test and the in vitro drug testing were blinded to results of the other study.

Statistical Analysis
Categorical variables were expressed as frequencies. The \( \chi^2 \) test was performed to compare such variables for unpaired groups. The McNemar test was used when paired nominal data were analyzed. \( P<0.05 \) was considered statistically significant.

Results

Characterization of Ca\(^{2+}\) Handling in the CPVT2-hiPSC-CMs

CPVT2-hiPSC-CMs Are Arrhythmogenic

The CPVT2-hiPSCs, displaying the D307H-CASQ2 mutation (Figure 1A), were coaxed to differentiate into cardiomyocytes (Figure 1A) using a modification of a directed monolayer differentiation system. Laser-confocal Ca\(^{2+}\) imaging of dispersed fluo4-loaded CPVT2-hiPSC-CMs revealed marked calcium-handling abnormalities in 69% of the cells (Figure 1B through 1F and 1J). Such abnormalities in the CPVT2-hiPSC-CMs were manifested as multiple diastolic Ca\(^{2+}\) release events (Figure 1B) or as broad transients with ectopic Ca\(^{2+}\) releases ranging in complexity from single (Figure 1C) to multiple high-frequency consecutive Ca\(^{2+}\) release events occurring while diastolic Ca\(^{2+}\) levels remain elevated (Figure 1D through 1F). In contrast, the majority (85%) of healthy control hiPSC-CMs displayed normal Ca\(^{2+}\) cycling (Figure 1G and 1J). The remaining 15% (Figure 1H and 1J) displayed some Ca\(^{2+}\) cycling abnormalities, which were not as complex as those observed in the CPVT2-hiPSC-CMs.

Laser-confocal action-potential recordings, using the voltage-sensitive fluorescent indicator FluoroVolt, confirmed that the aforementioned Ca\(^{2+}\) abnormalities led to the development of arrhythmias in the CPVT2-hiPSC-CMs. Thus, 63% of CPVT2-hiPSC-CMs displayed afterdepolarizations (mostly DADs but also EADs) and even triggered arrhythmias (Figure 1I and 1J) compared with only 13% of control hiPSC-CMs, which displayed only minor DADs (Figure 1I and 1J). To further elucidate the interplay between Ca\(^{2+}\)-handling abnormalities and membrane potential changes, we also performed simultaneous voltage- and Ca\(^{2+}\)-imaging studies. Local Ca\(^{2+}\) release events could result in membrane depolarizations causing either the development of action potentials or DADs (Figure 1K, left). A complex interplay between Ca\(^{2+}\) and voltage abnormalities could also be documented with ectopic Ca\(^{2+}\)-release events triggering membrane depolarizations and vice versa (Figure 1K, right).

Adrenergic Stimulation

Because arrhythmias in CPVT are usually triggered by exercise or emotional stress, we evaluated the effect of adrenergic pathway activation in the hiPSC-CMs. Isoproterenol (10 µmol/L) application was highly arrhythmogenic in the CPVT2-hiPSC-CMs (Figure 2A) but not in healthy control hiPSC-CMs (Figure 2B). Thus, focusing on normally beating cells at baseline, we observed the development of new Ca\(^{2+}\)-cycling abnormalities in 77% of isoproterenol-treated CPVT2-hiPSC-CMs. In contrast, no new arrhythmias were observed in the healthy control hiPSC-CMs after isoproterenol administration.

It was recently reported that \( \alpha \)-adrenoceptor stimulation might cause ventricular arrhythmias in the CASQ2\(^{Δ\beta/\gamma}\) mouse model. We, therefore, evaluated the effect of \( \alpha \)-adrenergic activation in the CPVT2-hiPSC-CMs through the application of phenylephrine (10 µmol/L). This resulted in development of new Ca\(^{2+}\)-cycling abnormalities in 41% of the phenylephrine-treated CPVT2-hiPSC-CMs (Figure 2C). Interestingly, both the incidence and complexity of such events (Figure 2C) were less severe than that after \( \beta \)-adrenergic activation (Figure 2A).

Store Overload–Induced Ca\(^{2+}\) Release

It was previously suggested that Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) may occur not only after Ca\(^{2+}\) entry via the cell membrane but also in the absence of membrane depolarization in a process termed SOICR. It was recently reported that some RyR2 mutations might decrease SOICR event threshold. We have previously described the presence of abnormal SOICR activity in CPVT1-hiPSC-CMs. It is not clear, however, whether such a phenomenon could also be modeled in the CPVT2-hiPSC-CMs.

To evaluate for SOICR, quiescent hiPSC-CMs (after suppression of automaticity) were subjected to increasing extracellular Ca\(^{2+}\) (\([\text{Ca}^{2+}]_o\)) concentrations (0.1–4 mmol/L) and analyzed by repeated line-scans to assess for spontaneous Ca\(^{2+}\) oscillations. As demonstrated in Figure 2D and 2E, SOICR events were noted in both CPVT2 and healthy control hiPSC-CMs, and their occurrence increased with elevated \([\text{Ca}^{2+}]_o\) concentration. Importantly, SOICR threshold was significantly reduced in the CPVT2-hiPSC-CMs, and the percentage of oscillating CPVT2-hiPSC-CMs was significantly higher than that of control hiPSC-CMs at each \([\text{Ca}^{2+}]_o\) concentration (Figure 2E).

Drug Screening Using the CPVT2-hiPSC-CMs

We next aimed at using the CPVT2-hiPSC-CMs model to evaluate the potential therapeutic effects and mechanism of action of several pharmacological agents, which could
theoretically be beneficial in treating CPVT2. Both the suppression of arrhythmias and SOICR were evaluated (Figures 3 and 4). Suppression of arrhythmias was regarded as the complete resolution of any ectopic Ca\(^{2+}\) release events after drug application (Figure 3A, 3B, 3D, and 3E, in contrast to Figure 3C and 3F). A proarrhythmic effect was defined when new Ca\(^{2+}\)-cycling abnormalities occurred after drug application in previously normal cells.

**Figure 2.** Adrenergic stimulation and store overload–induced Ca\(^{2+}\) release (SOICR) in CPVT2 and control human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs). **A** and **B**, Ca\(^{2+}\)-cycling abnormalities (red arrows) were induced in the CPVT2-hiPSC-CMs by isoproterenol (10 µmol/L; **A**) but not in control hiPSC-CMs (**B**). **C**, Induction of Ca\(^{2+}\)-cycling abnormalities (red arrows) in the CPVT2-hiPSC-CMs by phenylephrine (10 µmol/L). **D**, Line-scan images showing the development of SOICR in the CPVT2- (**left**) and healthy control (**right**) hiPSC-CMs when increasing extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{o}\)) concentrations. All tracings were recorded from the same cell, respectively. The incidence and complexity of the SOICR events were significantly greater in the CPVT2-hiPSC-CMs at each [Ca\(^{2+}\)]\(_{o}\) concentration. **E**, Summary of the percentage of oscillating CPVT2- (red) and control- (blue) hiPSC-CMs at increasing [Ca\(^{2+}\)]\(_{o}\) concentrations. Note that the proportion of oscillating cells was significantly higher in the CPVT2-hiPSC-CMs at each [Ca\(^{2+}\)]\(_{o}\) concentration (n=27, 27, 27, 222, 27; P=0.03, 0.01, 0.03, 0.03, 0.01, 0.002, 0.001, respectively) when compared with healthy control cells (n=24 in all [Ca\(^{2+}\)]\(_{o}\) levels). *Statistically significant differences.

**JTV-519**

We first evaluated the effect of JTV-519, a potent RyR2 stabilizer. Application of JTV-519 (1 µmol/L) resulted in significant suppression of the CPVT2-hiPSC-CMs arrhythmic behavior, even when Ca\(^{2+}\)-cycling abnormalities were complex at baseline (Figure 3A). Overall, JTV-519 eliminated Ca\(^{2+}\)-cycling abnormalities in 72% of arrhythmic CPVT2-hiPSC-CMs (Figure 4A).
β-Blockers and Combined α/β-Adrenergic Blockade

Because β-blockers are the mainstay treatment for both CPVT types, we next evaluated their therapeutic potential in the CPVT2-hiPSC-CMs model. Administration of propranolol (1 µmol/L) or carvedilol (1 µmol/L) before adrenergic stimulation with isoproterenol (10 µmol/L) completely prevented the development of Ca²⁺-cycling abnormalities (Figure 5A and 5B). At lower (and more clinically relevant) concentrations of these β-blockers, some isoproterenol-induced Ca²⁺-cycling abnormalities were still noted, albeit at a much lower frequency than compared with isoproterenol alone (Figure 5B).

It was suggested recently that carvedilol, but not other β-blockers (such as propranolol), may possess direct antiarrhythmic properties in CPVT by stabilizing RyR2.36,37

![Figure 3. Antiarrhythmic drug screening. A–F. Line scan Ca²⁺ recordings from CPVT2-hiPSC-CMs at baseline (top tracings, red arrows indicate abnormal Ca²⁺ cycling) and after application of different pharmacological agents (bottom tracings). Note the antiarrhythmic actions of JTV-519 (A), carvedilol (B), flecainide (D), and riluzole (E). In contrast, propranolol and labetalol (C and F) did not suppress baseline Ca²⁺-cycling abnormalities in CPVT2-hiPSC-CMs.](http://circep.ahajournals.org/doi/abs/10.1161/CIRCEP.116.007784)
We also noted a direct antiarrhythmic action of carvedilol, significantly reducing the incidence of Ca\textsuperscript{2+}-cycling abnormalities in the CPVT2-hiPSC-CMs in the absence of isoproterenol (Figures 3B and 4A) and using clinically relevant doses (0.3 µmol/L). In contrast, propranolol effectively prevented isoproterenol-induced arrhythmogenicity (Figure 5A and 5B), it had no effect on eliminating baseline Ca\textsuperscript{2+}-cycling abnormalities (Figures 3C and 4A). Overall, carvedilol (0.3 and 1 µmol/L) eliminated such abnormalities in \approx50\% of previously abnormal CPVT2-hiPSC-CMs (Figure 4A). In agreement with previous studies,\textsuperscript{6} a longer incubation period was required to achieve a therapeutic effect with the lower dose. In contrast, propranolol demonstrated no significant reduction in baseline Ca\textsuperscript{2+}-cycling abnormalities (Figures 3C and 4A).

Labetalol (a mixed α/β-adrenergic blocker) was recently reported to prevent arrhythmias in the CPVT2 mouse model.\textsuperscript{32} Because phenylephrine was somewhat arrhythmogenic in

### Table 1: Summary of Pharmacological Effects

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration</th>
<th>% Change in Ca\textsuperscript{2+}-cycling Abnormalities</th>
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<tr>
<td>JTV-519</td>
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<tr>
<td>JTV-519</td>
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<td>-50%</td>
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<tr>
<td>Carvedilol</td>
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<td>Propranolol</td>
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<td>Riluzole</td>
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<td>-44%</td>
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<tr>
<td>Forskolin</td>
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<td>-15%</td>
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*Statistically significant differences as compared with baseline conditions before drug application.*

### Figure 4

**Pharmacological effects on arrhythmogenicity and store overload–induced Ca\textsuperscript{2+} release (SOICR).**

**A.** Summary of the relative reduction in the incidence of Ca\textsuperscript{2+}-cycling abnormalities by the different pharmacological agents. Note the significant antiarrhythmic action of JTV-519 (1 µmol/L; n=36; P=2.98E\textsuperscript{-8}); carvedilol (0.3 µmol/L; n=34; P=0.00001), 1 µmol/L (n=31; P=0.0004), flecainide (1 µmol/L; n=34; P=0.02), 6 µmol/L (n=90; P=8.55E\textsuperscript{-14}), and riluzole (1 µmol/L; n=35; P=0.003). In contrast, propranolol (1 µmol/L; n=9; P=1) and labetalol (2 µmol/L; n=18; P=0.5) did not possess a direct antiarrhythmic effect.

**B.** Changes in the percentage of SOICR-oscillating CPVT2-hiPSC-CMs (at [Ca\textsuperscript{2+}]\textsubscript{o}, 1.8 mmol/L) after drug applications. Note the significant SOICR suppression by JTV-519 (1 µmol/L; n=13; P=0.03) and carvedilol (0.3 µmol/L; n=36; P=0.01 and 1 µmol/L; n=18; P=0.008); the neutral effect of propranolol (1 µmol/L; n=5; P=1), labetalol (2 µmol/L; n=16; P=1), and riluzole (10 µmol/L; n=7; P=1); and the increase in SOICR by forskolin (10 µmol/L; n=13; P=0.03). Flecainide at 1 µmol/L (n=9; P=1) did not alter SOICR frequency, whereas at 6 µmol/L, it modestly (yet insignificantly) decreased its incidence by 15% (n=28; P=0.28). *Statistically significant differences as compared with baseline conditions before drug application.*

[Figure 4](http://circep.ahajournals.org/)

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Figure 5. Drug effects on adrenergic-induced arrhythmogenicity, postpacing triggered activity, and diastolic Ca$^{2+}$ release (DCR). A, Line-scan recordings from normally beating CPVT2-hiPSC-CMs pretreated with different pharmacological agents, before (left) and after (right) isoproterenol (upper tracings) or phenylephrine (lower tracing) treatments. Note the protective action of carvedilol, propranolol, flecainide, and riluzole against isoproterenol-induced arrhythmias. Labetalol, in contrast, failed to completely prevent phenylephrine-induced arrhythmias (arrows). B, Summary of the isoproterenol- (upper) and phenylephrine- (lower) induced arrhythmogenicity in CPVT2-hiPSC-CMs. Isoproterenol application induced novel arrhythmias in 77% of the cells (10 μmol/L; n=13; P=0.002). Application of (Continued)
the CPVT2-hiPSC-CMs, we next analyzed the arrhythmia-malpotic potential of labelatal. Similar to the minimal arrhythmia-suppressing effect in the absence of adrennergic stimulation by propranolol, labetalol (2 µmol/L) application demonstrated no significant reduction in baseline Ca²⁺-cycling abnormalities (Figures 3F and 4A). However, in contrast to the robust β-blocker effects in preventing isoproterenol-induced arrhythmias, labetalol (2 µmol/L) had no protection against isoproterenol-induced arrhythmogenicity (Figure 5B) while exhibiting only a modest decrease in the development of phenylephrine-induced arrhythmias in CPVT2-hiPSC-CMs (Figure 5A and 5B). Importantly, carvedilol but not propranolol was also protective against phenylephrine-induced arrhythmogencity (Figure 5B).

Flecainide
Flecainide, a class Ia antiarrhythmic agent, was suggested to play an important therapeutic role in CPVT.21,38-44 This was also manifested in our CPVT2 model (Figure 3D), as application of flecainide significantly suppressed baseline arrhythmogenic activity in the CPVT2-hiPSC-CMs. Overall, flecainide (1 and 6 µmol/L) abolished Ca²⁺-cycling abnormalities in 26% and 57% of previously arrhythmic CPVT2-hiPSC-CMs, respectively (Figures 3D and 4A). To further test flecainide’s clinical relevance, we evaluated its ability to prevent isoproterenol- and phenylephrine-induced arrhythmias and noted a significant decrease in the incidence of new Ca²⁺-cycling abnormalities when administrated before isoproterenol or phenylephrine as compared with isoproterenol or phenylephrine alone (Figure 5A and 5B).

Riluzole
A recent study45 described a novel arrhythmogenic action of the neuronal Na⁺-channel blocker riluzole in an animal model of CPVT. This arrhythmogenic effect was suggested to stem from a novel mechanism involving Na⁺/Ca²⁺-exchanger reverse-mode activity and RyR2 priming.45 To test the relevance of these findings in a human model of CPVT, we evaluated the arrhythmogenic properties of riluzole in the CPVT2-hiPSC-CMs. Riluzole (10 µmol/L) abolished Ca²⁺-cycling abnormalities in 31% of previously abnormal CPVT2-hiPSC-CMs (Figures 3E and 4A). Moreover, riluzole was found to protect the CPVT2-hiPSC-CMs from catecholamine-induced arrhythmias, with a significant reduction in the incidence of new Ca²⁺-cycling abnormalities when administrated before isoproterenol or phenylephrine as compared with isoproterenol or phenylephrine alone (Figure 5A and 5B).

Mechanisms of Antiarrhythmic Drug Activities in the CPVT2-hiPSC-CMs
Traditional therapeutic approaches in CPVT focused on either blocking the adrenergic signaling pathway (β-blockers) or on stabilizing RyR2. Newly suggested drugs presented new or unexpected mechanisms. For instance, carvedilol was suggested to stabilize RyR2 beyond its β-blocking action.36,37 Similarly, many recent in vivo and clinical studies suggested a potential role for flecainide in CPVT. Two main mechanisms were suggested (1) flecainide may increase triggered-activity threshold by directly blocking Na⁺ channels39,44,46 or (2) flecainide may stabilize SR thereby decreasing diastolic Ca²⁺ leak.38,40,42-44

To provide mechanistic insights into the antiarrhythmic action of the tested pharmacological agents, we initially evaluated their ability to alter SOICR incidence in the CPVT2-hiPSC-CMs as a surrogate for SR stabilization. To provide additional information on their capacity to alter excitability or stabilize the SR, we also evaluated their effects on triggered activity and diastolic Ca²⁺ release events after pacing.

SOICR Suppression
JT519 and Forskolin
To validate our model, we first evaluated JT519 and forskolin, based on the known RyR2-stabilizing property of the former and the direct adrenergic activation by the latter. As expected, application of JT519 (1 µmol/L) resulted in suppression of SOICR events in 46% of oscillating CPVT2-hiPSC-CMs (at [Ca²⁺] of 1.8 mmol/L; Figure 4B), whereas forskolin (10 µmol/L) induced Ca²⁺ oscillations in 55% of previously quiescent CPVT2-hiPSC-CMs (Figure 4B).

Carvedilol, Propranolol, and Labetalol
In agreement with recent reports suggesting RyR2-stabilizing properties of carvedilol,36 we found that carvedilol suppressed SOICR in the CPVT2-hiPSC-CMs model, decreasing the percentage of oscillating cells by 37% (0.3 µmol/L) and 44% (1 µmol/L; Figure 4B). Carvedilol was also hypothesized to
Figure 6. Clinical correlation of flecainide and labetalol effects. ECG tracings obtained during exercise testing of the CPVT2 index patient after pretreatment with either flecainide (A) or labetalol (B). Flecainide displayed a significant antiarrhythmic effect, eliminating almost all ventricular ectopy (with only a few remaining isolated premature ventricular complexes, red arrows, appearing at a heart rate of 115 beats per minute during Bruce protocol level 6 workload). Labetalol, in contrast, did not prevent arrhythmias, with development of multiple episodes of polymorphic ventricular tachycardia (red arrow) at a heart rate of 69 beats per minute soon after stress test initiation.
stabilize RyR2 independently of its β/α adrenergic blocking properties. In support of this hypothesis, we found that neither propranolol nor labetalol affected SOICR in the CPVT2-hiPSC-CMs (Figure 4B).

**Flecainide**

To test whether flecainide may harbor RyR2-stabilizing properties, we exposed oscillating CPVT2-hiPSC-CMs to flecainide at 1 and 6 µmol/L. As presented in Figure 4B, flecainide did not alter SOICR at 1 µmol/L, whereas at 6 µmol/L flecainide suppressed SOICR in 15% of the cells (not reaching, however, statistical significance).

**Riluzole**

Radwański et al. showed that riluzole’s antiarrhythmic action in the CPVT mouse model was not associated with RyR2 blocking. Supporting this observation, we found that riluzole (10 µmol/L) application did not alter SOICR events in the CPVT2-hiPSC-CMs (Figure 4B).

**Postspacing Triggered Activity and Diastolic Ca2+ Release Events**

It was shown that rapid pacing of CPVT cardiomyocytes causes SR Ca2+ overload, which may translate into diastolic Ca2+ release (DCR) events and triggered activity at the postspacing interval, especially during adrenergic stimulation. To simulate this scenario, we studied forskolin-treated CPVT2-hiPSC-CMs after pacing. As demonstrated in Figure 5, the CPVT2-hiPSC-CMs developed marked DCR events and triggered activity during the postspacing period (Figure 5C through 5E). Carvedilol (1 µmol/L) completely suppressed triggered activity, concomitantly with marked suppression of DCR events (Figure 5D and 5F). Flecainide (1 µmol/L) also significantly reduced the incidence of triggered activity in the postspacing interval (Figure 5C and 5F). However, its effect on reducing DCR was only modest (not reaching statistical significance, Figure 5C and 5F). This differential effect was even more pronounced with flecainide 6 µmol/L, almost completely abolishing triggered activity, whereas only modestly reducing DCR (Figure 5F). Similarly, riluzole (10 µmol/L) completely suppressed triggered activity with minor (and statistically nonsignificant) reduction of DCR (Figure 5E and 5F).

**Clinical Correlation**

Finally, we aimed to evaluate the correlation between our in vitro CPVT2-hiPSC-CMs findings and the clinical setting. To this end, we compared the drug-testing results from the patient-specific CPVT2-hiPSC-CMs model with those from the exercise-test studies performed in exactly the same patient. The patient studied had a history of multiple exercise-induced arrhythmias, which were only partially relieved by high-dose β-blocker (320 mg/d propranolol) treatment.

Pretreatment with flecainide (200 mg/d for 2 days), as shown previously in CPVT2 patients, resulted in a significant antiarrhythmic effect in our patient. Thus, although a few premature ventricular complexes were still noted during the exercise test (at a heart rate of 115 beats per minute, Bruce protocol level 6), no ventricular tachycardia events were documented (Figure 6A). In contrast, labetalol treatment (800 mg/d for 2 days) failed protecting against such ventricular arrhythmias, and short runs of polymorphic ventricular tachycardias were frequently recorded during the stress test, initiating already at a heart rate of 69 beats per minute shortly after stress-test initiation (Figure 6B).

Our in vitro results correlated with these clinical findings, with propranolol at clinically relevant concentrations protecting (but not completely preventing) isoproterenol-induced arrhythmia in the CPVT2-hiPSC-CMs. Importantly, flecainide application was also found to be protective against the development of arrhythmias in the CPVT2-hiPSC-CMs, whereas labetalol was ineffective (Figure 5A and 5B).

**Discussion**

We used a patient-disease-specific hiPSCs-CMs model of the autosomal recessive form of CPVT (because of the D307H-CASQ2 mutation) to provide mechanistic insights into disease pathogenesis and treatment. Our results show that (1) CPVT2-hiPSC-CMs recapitulate the clinical CPVT phenotype in vitro by displaying significant Ca2+-handling abnormalities, diastolic Ca2+-leak, and arrhythmic activity that was aggravated by adrenergic stimulation; (2) the CASQ2 mutation resulted in reduced SOICR threshold in the CPVT2-hiPSC-CMs when compared with healthy control cells; (3) using this patient-specific disease-specific approach, we were able to screen several therapeutic agents (propranolol, labetalol, JTV519, carvedilol, flecainide, and riluzole) in clinically relevant concentrations and to provide mechanistic insights into their potential mode of action; and (4) the in vitro effects of some of these agents in the patient-specific hiPSC-CMs were concordant with the clinical exercise-test results in the same patient.

More than a decade has passed since RyR2 mutations were identified as responsible for CPVT1. Studies in mouse models of different CASQ2 mutations (including the D307H-CASQ2 mutation) suggested several possible mechanisms explaining CPVT2 arrhythmogenicity. It was proposed that CASQ2 mutations decrease CASQ2 protein levels and SR Ca2+ buffering or interfere with CASQ2-mediated regulation of RyR2 and luminal Ca2+-sensing thereby causing catecholamine-induced diastolic Ca2+ leak. Nevertheless, the clinical correlation between such animal-based models and human patients was suboptimal at times.

Using hiPSC-CMs to establish disease-in-a-dish models may allow the study of diseased human cardiomyocytes in a patient-specific manner. Subsequently, many hiPSC-CMs models of both CPVT types were shown to recapitulate the clinical phenotype in the culture-dish by displaying abnormal Ca2+ handling and arrhythmias. Here, we focused on CPVT2 and found, similar to previous reports, that most CPVT2-hiPSC-CMs display marked Ca2+-handling abnormalities including local Ca2+-release events, whole-cell Ca2+ irregularities, and arrhythmogenicity of varying complexity, which worsened after adrenergic stimulation. Using voltage-sensitive dyes to record optical action potentials, we noted that membrane potential manifestations of the aforementioned calcium abnormalities in the CPVT2-hiPSC-CMs were the development of DADs, EADs, and triggered arrhythmias. By the simultaneous recording of Ca2+ transients and Ca2+ transients and...
optical action potentials, we were able to document a complex interplay between Ca\(^{2+}\)-handling abnormalities and membrane potential changes, with ectopic Ca\(^{2+}\) releases triggering membrane depolarizations and vice versa. Such Ca\(^{2+}\)-triggered EADs could be the result of increased Na\(^{+}/Ca^{2+}\) exchanger activity because of elevated intracellular Ca\(^{2+}\) levels.

It is hypothesized that DAD formation is preceded by diastolic Ca\(^{2+}\) leak, occurring when SR Ca\(^{2+}\) content exceeds a certain threshold.\(^5,30\) Such SOICR events were implicated as a fundamental component associated with arrhythmia during Ca\(^{2+}\) overload. RyR2 mutations were shown to decrease SOICR threshold.\(^21,30\) We recently investigated this phenomenon in a CPVT1-hiPSC-CMs model demonstrating a reduced SOICR threshold in the affected cells.\(^21\) Here, we demonstrated the ability to model SOICR also in the CPVT2-hiPSC-CMs and revealed that the D307H-CASQ2 may also reduce SOICR threshold in the affected cardiomyocytes. These findings further elucidate the mechanistic nature of arrhythmogenicity in CPVT2, supporting the potential role of CASQ2 in luminal Ca\(^{2+}\) sensing and in RyR2 stabilization.\(^33,47,48\)

Subsequently, we performed drug-screening studies using the CPVT2-hiPSC-CMs model. Initially, we showed that JTV-519 suppressed Ca\(^{2+}\)-cycling abnormalities in CPVT2-hiPSC-CMs. This stresses the potential antiarrhythmic role of RyR2 stabilization not only in CPVT1 but also in CPVT2 and further suggests that CPVT2 arrhythmogenicity is mediated via RyR2 deregulation. We next showed, similarly to the mainstay clinical treatment of CPVT2, that \(\beta\)-blockers were protective against isoproterenol-induced arrhythmogenicity in the CPVT2-hiPSC-CMs. Interestingly, carvedilol at clinically relevant doses, but not propranolol or labetalol, exhibited a direct antiarrhythmic action in CPVT2-hiPSC-CMs, suppressing Ca\(^{2+}\)-cycling–related abnormalities even in the absence of adrenergic stimulation. This effect could not be attributed to its \(\beta\)- or \(\alpha\)-adrenergic blocking activity and supports the potential future use of carvedilol (or its recently described analogues with RyR2-stabilizing activity) for the treatment of CPVT2.

Flecainide was recently suggested as a potent antiarrhythmic agent in CPVT.\(^21,38-42\) Our results support this concept by showing that flecainide, at clinically relevant concentrations, could significantly suppress baseline Ca\(^{2+}\)-cycling abnormalities and protect against isoproterenol-induced arrhythmogenicity in the CPVT2-hiPSC-CMs. Finally, riluzole, a neuronal Na\(^{+}\) channel blocker used for treatment of amyotrophic lateral sclerosis, which was recently shown to be antiarrhythmic in a mouse model of CPVT,\(^41\) was also found beneficial in our CPVT2-hiPSC-CMs model.

We also documented a phenylephrine-induced (\(\alpha\)-adrenergic) proarrhythmic effect in CPVT2-hiPSC-CMs, consistent with recent findings in a mouse model of CPVT2. Importantly, carvedilol, flecainide, and riluzole, but not propranolol, were protective against such arrhythmia. Because propranolol presents no \(\alpha\)-blocking activity, its ineffectiveness in this setting was not surprising. Based on our findings, carvedilol could presumably suppress such arrhythmogenicity by both an \(\alpha\)-blocking activity and a direct antiarrhythmic effect, whereas flecainide and riluzole could do so merely via a direct antiarrhythmic action.

To further characterize the mechanistic nature of the antiarrhythmic actions exhibited by these agents, we evaluated their influence on SOICR in the CPVT2-hiPSC-CMs as a surrogate for potential SR stabilization.\(^36\) Interestingly, both JTV-519 and carvedilol displayed marked SOICR suppression. Other \(\beta\)-blockers (propranolol and labetalol) did not affect SOICR in the CPVT2-hiPSC-CMs, supporting the notion\(^26,37\) that the SR-stabilizing property may be unique to carvedilol. Moreover, flecainide (1 \(\mu\)mol/L) or riluzole, despite possessing important antiarrhythmic actions, failed to significantly suppress SOICR, suggesting that their main antiarrhythmic action may not be related to SR stabilization. Interestingly, at 6 \(\mu\)mol/L, flecainide demonstrated a trend for decreasing SOICR, not reaching, however, statistical significance. To complement these findings, we compared the effects of carvedilol, flecainide, and riluzole during the postspacing period of forskolin-treated CPVT2-hiPSC-CMs. Although carvedilol significantly suppressed both triggered activity and DCR events at the postspacing period, flecainide, and riluzole markedly suppressed triggered activity but had only a mild effect on DCR.

Taken together, our findings indicate that the antiarrhythmic action of carvedilol in CPVT2 also involves RyR2 stabilization. In contrast, although flecainide may possess some SR-stabilizing properties at higher concentrations (as evident from the trends in the SOICR and postspacing experiments), at lower concentrations, flecainide’s direct suppression of triggered activity in CPVT2 is probably the more important mechanism.

Finally, to explore the potential of hiPSC-based models in predicting pharmacological clinical responses, we compared our in vitro drug-testing findings in the CPVT2-hiPSC-CMs model with the exercise test results performed in exactly the same patient. Similar to our in vitro observations, the clinical data and stress test results advocated that propranolol may not be entirely protective against catecholamine-induced arrhythmia in our patient and that flecainide treatment may contribute an additional antiarrhythmic effect while preventing exercise-induced arrhythmias. In contrast, although labetalol was recently suggested to exhibit beneficial antiarrhythmic activity in a CPVT2 mouse model,\(^23\) it failed to achieve similar effects in both the in vitro CPVT2-hiPSC-CMs model and in the corresponding clinical study. These findings may suggest that hiPSC-based models could potentially aid in predicting patient-specific clinical response, as was also evident from a recent report comparing the flecainide clinical response with in vitro findings using hiPSC-CMs from a CPVT1 patient.\(^28\)

Although of significant potential, hiPSC-CM–based disease models of arrhythmogenic syndromes are not flawless. One limitation of such models, including our current work, is that cells are usually obtained from only a small number of individuals (because of the rare incidence of such syndromes and to the large amount of work associated with creation and detailed characterization of the generated cell lines), and hence, the results of these studies cannot necessarily be generalized to differences between patient populations. This is also an obvious limitation for correlating in vitro results with the clinical setting, necessitating many more patients to prove significant trends. Indeed, extensive further research is warranted to test the prediction ability of drug screening studies using hiPSC-based models with the clinical setup.
Finally, a major limitation in the field is the degree of maturity of the hiPSC-CMs, which do not reach the full adult phenotype, therefore potentially affecting the clinical relevance of at least some hiPSC-based disease models. In this regard, the high frequency of baseline arrhythmia noted in the current study and in previous CPVT-hiPSC-CM-based models may be at least partially attributed to the early-stage phenotype exhibited by hiPSC-CMs and to the experimental setting using single cells rather than multicellular tissues. Such augmented arrhythmogenicity may on the one hand facilitate the screening for antiarrhythmic agents but on the other hand may not accurately reflect the actual clinical setup.

Nevertheless, despite the aforementioned limitations, our findings stress the unique potential of hiPSC-CMs for modeling arrhythmogenic syndromes in general and CPVT in particular. This approach allowed recapitulating the disease phenotype in the patient-specific CPVT2-hiPSC-CMs, provided important insights into the mechanistic nature of arrhythmias and drug response in this syndrome, and highlighted the potential of hiPSC-CMs to serve as a platform for drug screening and potentially for individualizing drug therapy.

Acknowledgments

We thank Dr Doron Aronson for his statistical advice, Dr Ilanit Itzhaki for the custom-written analysis software, and Dr Edith Suss-Toby for her help in imaging.

Sources of Funding

This study was funded in part by the European Research Council (ERC) Ideas-Program [ERC-2010-StG-260830-Cardio-iPS].

Disclosures

None.

References

Patient-Specific Drug Screening Using a Human Induced Pluripotent Stem Cell Model of Catecholaminergic Polymorphic Ventricular Tachycardia Type 2
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_Circ Arrhythm Electrophysiol._ 2017;10:
doi: 10.1161/CIRCEP.116.004725

_Circulation: Arrhythmia and Electrophysiology_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-3149. Online ISSN: 1941-3084

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